The Quorum-Sensing Regulon of *Vibrio fischeri*:
Novel Components of the Autoinducer/LuxR Regulatory Circuit

by

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ABSTRACT

In the marine bacterium *Vibrio fischeri* two intercellular homoserine-lactone signal molecules (*luxI*-dependent $3OC_6$-HSL and the *ainS*-dependent C_8-HSL) and the transcriptional activator LuxR regulate the luminescence system in a cell-density dependent manner by a process termed quorum sensing. In this study, five additional proteins whose production is regulated by quorum sensing are described, and the genes encoding four of the five proteins, denoted as QsrP, RibB, QsrV, and AcfA, are analyzed. Each protein is positively regulated by $3OC_6$-HSL and LuxR and negatively regulated at low population density by C_8-HSL. Probable LuxR/autoinducer binding sites are found in the promoter region of each. QsrP and RibB are encoded monocistronically, whereas AcfA and QsrV appear to be encoded by a two-gene operon. On the basis of sequence similarity to proteins of known function from other organisms, RibB is believed to be an enzyme that catalyzes the transformation of ribulose 5-phosphate to 3,4-dihydroxy-2-butanone 4-phosphate, a precursor for the xylene ring of riboflavin; AcfA is believed to be a pilus subunit; and the functions of QsrP and QsrV are unknown at this time. A *qsrP* mutant was reduced in its ability to colonize its symbiotic partner, *Euprymna scolopes* when placed in competition with the parent strain. On the other hand, a mutant strain of *V. fischeri* containing an insertion in *acfA*, which is believed to be polar with respect to *qsrV*, displayed enhanced colonization competence in a competition assay. A *ribB* mutant grew well on media not supplemented with additional riboflavin and displayed normal induction of luminescence. Both phenotypes suggest that the lack of a functional *ribB* gene is complemented by another gene of similar function in the mutant. Oriented divergently from *acfA* are open reading frames that code for two putative proteins that are similar in sequence to members of the LysR family of transcriptional regulators. Organization of the two divergent sets of genes and the shared promoter region suggests that transcription of *acfA* and *qsrV* may be regulated by one or both of these divergently transcribed proteins. This work defines a quorum-sensing regulon in *V. fischeri*. A model describing its regulation is presented.

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Table of abbreviations

$3OC_6$-HSL	*N*-3-oxohexanoyl-L-homoserine lactone
AI-1	*N*-3-oxohexanoyl-L-homoserine lactone
AI-2	*N*-octonoyl-L-HSL
C_8-HSL	*N*-octonoyl-L-HSL
CHAPS	Cloromidopropyldimethylammonio 1-propane sulfate
CNP	3':5'-Cyclic nucleotide phophodiesterase
EDTA	Ethylenediaminetetraacetic acid
FMN	Flavin mononucleotide
GFP	Green fluorescent protein
HPLC	High performance liquid chromatography
HSL	Homoserine lactone
ICPES	Inductively coupled plasma emission spectroscopy
LTTR	LysR-type transcriptional regulator
MES	4-Morpholinepropanesulfonic acid
NCBI	National Center for Biotechnology Information
2-D PAGE	Two-dimensional polyacrylamde gel electrophoresis
PAI	Pathogenicity island
PCR	Polymerase chain reaction
pI	Isoelctric point
PVDF	Polyvinylidiene difluoride
QSR	Quorum-sensing regulated
SDS	Sodium dodecyl sulfate
TIGR	The Institute for Genomic Research
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
X-gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside

Chapter 1.　Introductory remarks

In bacteria many processes are coordinated by cell-cell communication that is mediated by signal molecules generated by the bacteria. Information transfer between cells is used to determine population density, to synchronize behavior, and to develop spatial patterns during morphogenesis and differentiation. The signals known to be used by bacteria are diverse in structure, but most appear to serve the same short term function; as with environmental cues, the intercellular signal affects the cell's program of gene transcription through a regulatory circuit capable of interpreting it and effecting the appropriate response.

Two distinct conceptual models of bacterial communication have emerged from different views of bacterial populations. The first perceives bacterial cells as individuals that employ pheromones to coordinate activity of the population. Bacteria that release diffusable compounds to coordinate cell-density dependent behaviors are often regarded in this manner [1-3]. The second model considers assemblages of certain species as a single multicellular organism that uses signals in the course of morphogenesis and cellular differentiation in the same way that higher eukaryotes develop from a single fertilized embryo [4]. Both models are useful, and their coexistence highlights the variety of processes that are governed by cell-cell communication. Both models can often be applied effectively to the same developmental process due to the crossover between signaling systems and the processes they regulate. What follows is a survey of cell-cell signalling systems used by bacteria. It is meant to highlight the properties of each system and, by comparison, to give perspective on the acyl-homoserine lactone (acyl-HSL) system used by *Vibrio fischeri*.

Cell-cell signaling systems. Recently, the term quorum sensing has been used to describe mechanisms that both Gram-negative [2] and Gram-positive bacteria [1] use to monitor population density and regulate gene transcription. In Gram-negative bacteria the most well studied signal compounds are small *N*-acyl-L-homoserine lactone molecules called autoinducers that combine with a transcriptional activator protein of the LuxR family. The homoserine lactone (HSL) moiety of autoinducers is conserved, and the length and degree of oxidation of the *N*-acyl side chain, which is thought to be derived from the fatty acid biosynthesis pathway [5], provide the structural diversity and specificity of the signal. At low cell density, autoinducer is produced at a basal level and diffuses into the medium where it is diluted. Conversely, at high population density or in an enclosed environment levels of autoinducer increase with the population density. Transduction of the signal across the bacterial envelope in some cases is by simple diffusion [6], and in others active efflux from the cell has been proposed [7]. At a certain threshold concentration, autoinducer binds to the LuxR-type protein and the complex enhances transcription from genes that have specific target DNA sequences in the promoter region. The increase in transcription of some autoinducer-regulated genes can be in excess of 1000-fold. Processes regulated by autoinducers include bioluminescence, biofilm formation, production of virulence determinants, and synthesis of antibiotics, amongst others (see below).

Vibrio harveyi also uses acyl-HSL as signal molecules, but in a manner different from that presented in the model above. Instead of interacting directly with a LuxR-type transcriptional activator , the two autoinducers of *V. harveyi* interact with separate two-component sensor kinases on the surface of the cell, at high cell density, which results in the inactivation of a repressor of transcription via a phosphorelay system [8-10]. With the repressor inactivated, a transcriptional regulator activates transcription of target

genes. The transcriptional activator in this system is not similar in sequence to the LuxR family of proteins. Nonetheless, the protein was also designated LuxR [11].

Several species of *Streptomyces* also use butyrolactone molecules as autoregulatory signals to regulate production of antibiotics and to guide the morphological differentiation of aerial mycelia [12]. In this case, the secondary amine of the homoserine lactone autoinducers seen in Gram-negative bacteria is replaced by a carbonyl or an alcohol. As with the Gram-negative autoinducers, variability in the side-chain confers specificity. The receptor protein for A-factor, as the signal is called, occurs as a dimer in the cytoplasm and appears to also be a transcriptional regulator [13]. Despite the similarity between the two systems, BarA, the receptor/transcriptional activator, is not similar in sequence to LuxR-type proteins.

In addition to the acyl side chains of the lactone signal molecules, another fatty acid-derived signal molecule is 3-hydroxypalmitic acid methyl ester from the plant pathogen *Ralstonia solanacearum* [14]. Virulence gene expression is regulated by the long chain fatty acid methyl ester via a two-component regulatory system [15]. As a signal molecule, this compound is unique in its ability to act over long distances in the vapor phase [16].

In Gram-positive bacteria the intercellular signals that have received the most attention are small extracellular peptides that are derived from a larger precursor peptide by posttranslational processing and secreted by an ATP-binding-cassette exporter [1]. Unlike the lactone signals, these peptides cannot efficiently diffuse through the cell membrane. Instead, they either interact with a two component sensor-regulator system that transduces the signal across the cytoplasmic membrane as in the case of competence induction in *Streptococcus pneumoniae* [17], or the peptide is transported across the membrane and acts on an intracellular target as in the development of competence in

Enterococcus faecalis [18]. The signaling system in *E. faecalis* is noteworthy in two

respects. First, a protein has been identified that prevents self induction of cells [19].

The plasmid encoded inhibitor insures that only prospective recipients respond to the

signal. And second, evidence exists that, in addition to affecting transcription of target

genes, one of the targets of the cCF10 pheromone may be the translational machinery

[20]. The lone Gram-negative organism known to use a small peptide as an intercellular

signal is the cyanobacterium *Anabaena* 7120. The developmental pattern of heterocyst

differentiation requires PatS, a small unmodified peptide that has been proposed to travel

through the contiguous periplasm of a multi-celled filament to maintain a minimum

number of vegetative cells between heterocysts by inhibiting differentiation [21].

Myxococcus xanthus is a Gram-negative gliding bacterium that uses at least five

distinct signals, denoted as factors A-E, in the course of aggregation and sporulation [22,

23]. In response to nutrient limitation, several thousand rod-shaped cells swarm and

aggregate to form a macroscopic mound, or fruiting body, prior to cellular differentiation

to produce heat and desiccation resistant spores within the fruiting body [24]. Internal

patterning of cells in coherent arrays can be seen with light microscopy [25]. Genetic

analysis has been used to isolate mutants that are defective in fruiting body

morphogenesis. These mutants fall into five extracellular complementation groups that

were defined by mixing mutant cells with wild-type cells and with other mutants [22, 23].

The varied timing of growth arrest seen in the different mutant classes implies that a

series of sequential extracellular controls govern development [26]. Three of the factors

used as signals have been identified. A-factor is a group of fifteen common amino acids

that are generated, at least in part, by two proteases from a variety of exported peptides

[27, 28]. A-factor mutants exhibit a developmental defect prior to the onset of

aggregation. The extracellular amino acids are believed to specify the minimum cell

density required for fruiting body formation [26] in much the same way that other bacteria use homoserine lactone and peptide pheromones in the process of quorum sensing.

C-factor and E-factor are exceptional among the group of bacterial intercellular signals that have been mentioned in that they require contact between two cells for transmission, and therefore can be categorized as part of morphogenetic paracrine signaling systems [29, 30]. The other systems mentioned are presumed to be autocrine in nature. CsgA is a cell surface protein that produces C-factor, which possesses both an activity that allows coordinated cell movement into the center of aggregation and an activity that allows rod-shaped cells to differentiate into ovoid spores [29]. In addition to direct contact between cells, correct alignment of cells is necessary for C-factor function [25]. The CsgA protein is similar in sequence to members of the short-chain alcohol dehydrogenase family [31]. E-factor is believed to be branched-chain fatty acids, partly because the genetic locus that was mutated to define the E-complementation group encodes a gene that is necessary for their production [32]. The model infers that long branched-chain fatty acids that have been incorporated into membrane phospholipid are liberated, as a result of increased phospholipase activity during development, and are passed between adjacent cells [30].

Another myxobacterium that also forms spore-filled fruiting bodies in response to nutrient limitation is *Stigmatella aurantiaca*. Recently, a twelve carbon hydroxy ketone which the authors call stigmalone was shown to accelerate aggregation of starving cells in this bacterium [33]. Whether or not this compound is involved in fruiting body formation by other myxobacteria is unknown.

Acyl homoserine lactone signaling in *Vibrio fischeri*. In the early 1970's it became evident that the production of light by *V. fischeri* is regulated by a small

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diffusable extracellular signal that accumulated in the medium when cells reached high population density [34, 35]. Culture media that had been "conditioned" by the growth of *V. fischeri* to early stationary phase could be used to stimulate the luminescence of early exponential phase cultures, which provided an assay for the signal molecule. Autoinducer, as the signal was termed, was later identified as *N*-3-oxohexanoyl-L-homoserine lactone ($3OC_6$-HSL after Pearson, et al. [7]; formerly VAI-1) [36]. The molecule diffuses rapidly across the bacterial envelope in both directions indicating that binding of $3OC_6$-HSL is reversible [6]. A concentration of 200 nM elicits a maximal response, and concentrations of 10 nM, which reportedly corresponds to 1-2 molecules per cell, are sufficient for induction of luminescence [6].

Genetic analysis of bioluminescence and signaling by $3OC_6$-HSL was made possible by cloning of the *lux* operon into *Escherichia coli* in 1983 [37]. The *lux* locus consists of seven genes, *luxR luxICDABEG*; *luxR* and the *lux* operon are transcribed divergently [38, 39]. *luxR* encodes the receptor for $3OC_6$-HSL and the transcriptional activator of the *lux* operon, *luxI* encodes the autoinducer synthase, *luxC, D,* and *E* encode subunits of a fatty acid reductase complex involved in the generation of light, *luxA* and *B* encode the subunits of luciferase [39], and *luxG* is believed to encode a flavin reductase [40]. Fortunately, all of the functions necessary for light production in *E. coli* are encoded on the single 16 kb fragment, which facilitated its isolation by screening a heterologous chromosomal library for luminescence [37]. Cell density dependent light production in *E. coli* also suggests that the precursor molecules for $3OC_6$-HSL synthesis and the luminescent reactions are available in *E. coli*. Synthesis of $3OC_6$-HSL in a cell free system using purified LuxI demonstrated that the substrates for synthesis are S-adenosylmethionine and an acylated acyl carrier protein thought to come from the fatty acid biosynthesis pathway [5].

The transcriptional activator LuxR is a 250-amino acid protein that consists of

two domains. The C-terminus appears to bind DNA in the *lux* promoter and activate

gene transcription. By itself, the C-terminus can activate transcription to 200% that of

the wild-type in an autoinducer-independent fashion, and it has been proposed that the

N-terminal domain inhibits the activator function of the C-terminal domain in the absence

of autoinducer [41]. The N-terminal two-thirds of LuxR is involved in autoinducer

binding which may relieve its inhibition of C-terminus DNA-binding [41, 42]. Because

the kinetics of luminescence appear to be non-linear, it has been proposed that LuxR may

act as a multimer with cooperative binding of autoinducer [43]. Genetic dominance

experiments in *E. coli* containing wild-type and mutant alleles of LuxR also support the

multimerization of LuxR [44].

Situated between *luxR* and the *lux* operon is 218 bp of promoter DNA that

provides for the elegant regulation of luminescence [45]. Centered 40 nucleotides

upstream of the transcription start site of the *lux* operon is a 20 bp region with dyad

symmetry that has become known as the *lux* box and is believed to be the target binding

site of LuxR [46]. Its location relative to a putative -10 Pribnow box suggests that it

occurs in place of a typical σ^{70} -35 promoter element, and DNAseI protection assays that

demonstrated the synergistic binding of RNA polymerase and LuxR to this region

support the idea [47]. In addition to LuxR and autoinducer, cAMP and CRP are also

necessary for luminescence [48]. Between the *lux* box and the -35 polymerase

recognition site for LuxR is a consensus CRP binding site [45]. In addition to binding the

lux box region, LuxR also binds DNA upstream that includes the CRP binding site [47].

This finding supports the previous genetic analysis that led to the current model of

transcriptional regulation at the luminescence locus; LuxR/3OC$_6$-HSL enhances

transcription from the *lux* operon and down-regulates divergent transcription from *luxR*,

while cAMP/CRP enhances *luxR* transcription and inhibits transcription of *lux* [45, 46, 49, 50]. The positive feedback loop of autoinducer production, which accounts for the exponential increase in transcription of the *lux* operon once a critical concentration of autoinducer is reached, is balanced by the simultaneous steric hindrance of CRP binding by LuxR, an example of feedback inhibition of LuxR that may prevent runaway induction of luminescence.

The regulation of $3OC_6$-HSL synthesis varies between strains of *V. fischeri*. The fish symbiont MJ-1 produces large amounts of $3OC_6$-HSL in culture that result in high levels of light. In contrast, light production and luciferase levels are depressed in cultures of ES114, but they can be induced in culture to levels that approximate those in the light organ by the exogenous addition of $3OC_6$-HSL [51]. The symbiosis-dependent production of $3OC_6$-HSL by ES114 suggests that conditions in the squid light organ regulate $3OC_6$-HSL production via an undescribed bacterial mechanism. Alternatively, autoinducer could be supplied by the squid.

In addition to $3OC_6$-HSL, *V. fischeri* releases two other autoinducer molecules into the growth medium that induce luminescence to a limited extent [52]. AI-3, *N*-hexanoyl-L-HSL, is dependent on *luxI* for its synthesis like $3OC_6$-HSL. C_8-HSL, *N*-octonoyl-L-HSL, on the other hand, is dependent on a distinct gene, *ainS*, for its synthesis [52, 53]. It has been shown to antagonize the activity of $3OC_6$-HSL by lowering light levels and preventing premature induction [54]. Downstream of *ainS*, a putative receptor called *ainR* is encoded, which is similar in sequence to *luxN* of *V. harveyi*, another marine, luminous bacterium [53]. In *V. harveyi*, LuxN and autoinducer combine to inactivate a repressor of *lux* operon transcription [10]. LuxN is part of a two-component signaling system, and it contains both sensor kinase and response regulator domains [9]. The presence of a LuxN homolog in *V. fischeri* suggests that C_8-HSL and/or $3OC_6$-HSL

may also have functions that are mediated through a two-component regulatory system that includes AinR.

The use of acyl homoserine lactone signals by other bacteria. In 1981 Eberhard et al. determined the structure of the autoinducer from *V. fischeri*, and it wasn't until 1993, twelve years later, that acyl-HSL molecules were shown to be used to regulate processes other than luminescence in marine bacteria. In that year it was reported that both *Pseudomonas aeruginosa*, an opportunistic pathogen in humans that is also found in soil and water, and the plant pathogen *Agrobacterium tumefaciens* use autoinducer molecules in combination with LuxR-type proteins to regulate virulence gene expression [55-57]. Since that time, more than 25 species of bacteria have been found to use acyl homoserine lactones and homologs of LuxR to coordinate gene expression with population density [2, 58]. The search for additional bacteria that use autoinducers as signal molecules has been accelerated by the use of biological assays that allow the user to test the cell-free supernatant of "conditioned" media for their presence. These systems take advantage of luminescence or reporter constructs and the low substrate specificity of some LuxR homologs [57, 59, 60].

The preponderance of bacterial species that use LuxR-type quorum sensing have a host associated state in their lifestyle [58]. The discovery of acyl-HSL mediated quorum sensing systems in such high profile pathogens and symbionts as *E. coli* [61], *Salmonella typhimurium* [62], *Vibrio cholerae* [59], and *Rhizobium leguminosarum* [63] has led to speculation about the relationship between quorum sensing and host association. Delaying the production of virulence and symbiosis factors until they can be produced at levels high enough to overcome host defenses or elicit an appropriate host response, respectively, may confer an advantage to the bacterial population [64]. It has also been proposed that acyl-HSL molecules may be used as signals between bacterium and host

[2]. One such example of host involvement in bacterial quorum sensing is the interference of autoinducer mediated swarming motility in a *Serratia liquifaciens* population by a macroalga that produces two furanones that may also inhibit surface colonization by the bacterium [65]. Similarly, evidence exists of interspecies communication between bacteria [66].

The symbiosis between *V. fischeri* and the Hawaiian squid *Euprymna scolopes*. *V. fischeri* is found as free-living cells in the ocean's plankton and as a symbiont of monocentrid fish and certain cephalopods of the family Sepiolidae. The light produced by *V. fischeri* is used by its squid and fish hosts in bioluminescent displays to find mates, search for food, and avoid predators [67]. The small Hawaiian squid *E. scolopes*, while feeding at night in the water column, is believed to match levels of moonlight with its own ventral counterillumination to avoid creating a silhouette that would otherwise be visible to predators below [68]. In return, the bacteria are provided with an environment that supports the rapid growth of a monoculture of *V. fischeri*.

E. scolopes is a small (mantle length of 25 mm for mature adults), nocturnal squid indigenous to the Hawaiian archipelago. It buries itself in the sand during daylight and emerges at night to feed and lay eggs [69]. Between 100 and 300 eggs, each about 4 mm in diameter, are deposited per clutch on a solid surface, and juveniles hatch after twenty days at 24° C [69, 70]. Juveniles, which undergo direct development, are planktonic for about two days before adopting a diurnal cycle of daytime burial in the sand and nighttime foraging in the water column for food. They reach sexual maturity after a period of four months [69].

In the adult host, the colonized light organ is a conspicuous bilobed structure in the center of the mantle cavity. Within the light organ, about 10^7 bacteria are housed extracellularly as a pure culture in a pair of three highly branched crypts that are lined

with epithelial cells [71]. The tissue that houses the bacteria is surrounded by a dorsal reflector and a ventral lens, both of which modify the bacterial luminescence and are embedded in the ink sac [72].

The uncolonized light organ differs in structure from the adult organ: it is heart shaped instead of bilobed; a pair of ciliated epithelial appendages, which are believed to aid in colonization, are prominent in the nascent light organ but regress within 4 days after colonization; and the crypts that house the bacteria are simple sacculate structures devoid of the diverticulation in the adult organ. The differentiation and morphogenesis of the post-embryonic light organ is partially dependent on colonization by the bacteria [71]. Induction of apoptosis by a population of *V. fischeri* is believed to be involved in the remodeling of the host light organ tissues [73].

Delineation of the boundary between morphological events that are induced by the bacteria and those that are "hard wired" into the host's developmental program was made possible by the recent culturing of *E. scolopes* through the life cycle [74, 75]. It appears that the overall growth and development of the animal is not dependent on colonization; aposymbiotic animals grow and reach sexual maturity at the same rate as colonized animals under laboratory conditions. In addition, development of the light organ accessory tissues (lens, reflector and ink sac) are not influenced by colonization. In contrast, growth of the light organ crypts and the histology of the ciliated ducts that connect the crypts is markedly different in aposymbiotic and colonized animals [75, 76].

V. fischeri cells also differentiate when they enter the symbiotic state [77]. Competent squid symbiont strains grown in culture have a tuft of polar, sheathed flagella, are motile, have a doubling time of 30 min, and produce a very low level of light. In contrast, cells in the light organ are aflagellate, have a generation time between 10 and 18 h, and produce a level of light that is 1000 - 10,000 fold that of cells in culture. In

addition, cells in the symbiosis are significantly smaller than those grown in culture. Within three hours of release from the light organ, the bacteria revert to growth typical of that in complete media [77]. The spontaneous isolation of a small, visibly luminous, slow growing variant of a symbiosis competent strain that is reduced in flagellation suggests that the dimorphic nature of *V. fischeri* growth may be globally regulated [78].

The symbiosis can be thought to consist of three stages: initiation, colonization, and maintenance. During the initiation phase, as few as 1-10 *V. fischeri* cells enter the undifferentiated juvenile light organ [77] via a mucous plugged, ciliated pore [72]. Rapid growth of the bacteria to about 10^5 cells in 12 hrs [77] and development of the light organ define the colonization stage. Following colonization, a slow growing monoculture of *V. fischeri* is maintained in the light organ under conditions that fully induce the luminescence system. The events of each stage of the symbiosis raise several unanswered questions: what are the mechanisms of recognition and communication between the symbionts that lead to their respective differentiation; what are the conditions in the light organ that foster the rapid proliferation of *V. fischeri* at the exclusion of other bacteria; and, what events distinguish this mutualism from a pathogenic interaction?

Conditions within the host light organ have not been well characterized, but speculative correlations have been made between growth in laboratory culture conditions that result in high luminescence and growth in various fish light organs. In particular, nutrient limitation, oxygen limitation, iron limitation, and osmolarity are some of the factors that can be adjusted to yield slow growth and high levels of luminescence by certain fish symbionts [79]. *V. fischeri* is subject to catabolite repression, and cAMP is necessary for induction of luminescence [48, 50], so nutrient limitation may play a role in *lux* gene induction in the squid. Several amino acids are abundant in the crypt matrix

fluid of the squid light organ, and colonization experiments with various amino acid auxotrophs indicate that some of these host derived amino acids can support growth of the bacterium in the initial stages of the symbiosis [80]. Under conditions of low oxygen [81] or low iron [82] the growth rate of *V. fischeri* is repressed, and luciferase activity per cell is increased. The luminescence experiments described above involved fish light organ isolates such as *V. fischeri* MJ1 (*Monocentris japonicus*), which is brightly luminescencent in culture. In contrast, isolates from *E. scolopes,* such as ES114 (*E. scolopes*), are 1000 - 10,000 fold less luminous in culture than in the symbiosis [51]. Iron limitation and addition of cAMP have been shown to enhance luminescence of ES114 grown in culture [78].

Experimental system: The *E. scolopes-V. fischeri* symbiosis has been developed as a model system to study cellular interactions between bacteria and their animal hosts [83]. The short generation time, large clutch size, ease of laboratory maintenance, recent culturing [74, 75] and aposymbiotic hatching of the animal make it ideal for colonization experiments. The bacterium is easily cultured in a variety of complete and minimal media, has a short generation time, and can be manipulated genetically. Assays for colonization involve detection of light production by the prospective squid [71] and quantitation of the bacteria by platings of whole animal homogenates [84].

To date, two attributes of *V. fischeri* have been identified as symbiosis determinants. The first is motility. A collection of transposon-induced motility mutants were incapable of colonizing juvenile squid [84]. The second attribute, the production of a periplasmic catalase, was not necessary for colonization, but in competition with the wild-type strain, a catalase mutant colonized the squid less efficiently [85]. The results with the catalase mutant complement reports of peroxidase activity in the squid light organ [86, 87].

Cyclic nucleotide phosphodiesterase a possible symbiosis factor: In the course of studying the effects of cAMP and CRP on *lux* gene regulation, it was discovered that *V. fischeri* has the unusual ability to grow on cAMP as the sole carbon, nitrogen, phosphorus and energy source due to the location of a 3':5'-cyclic nucleotide phosphodiesterase in the periplasm, a novel location for an enzyme of this sort [88]. The gene encoding the enzyme was the first bacterial cyclic nucleotide phosphodiesterase (CNP) to be sequenced and characterized [89]. Because the ability to grow on cAMP is rare, the enzyme has been proposed to permit *V. fischeri*, exclusively, to utilize host provided cAMP as a source of nutrition. According to the hypothesis, *V. fischeri* in the animal light organ elicits the overproduction and release of host cAMP by secreting a toxin that, through an ADP-ribosylating activity, interferes with the regulation of host adenylate cyclase in a manner analogous to the secretion and activity of cholera enterotoxin by *V. cholera* in the human intestine [88]. The recent characterization of a secreted ADP-ribosyltransferase and the gene encoding it in *V. fischeri* is consistent with this hypothesis [90].

It was mentioned earlier that several species of bacteria use acyl-HSL signals and quorum sensing to regulate the production of virulence factors. Despite the obvious parallel between virulence factors and symbiosis factors, non-*lux* genetic loci that are transcriptionally regulated by autoinducer/luxR have not been investigated in *V. fischeri*. Since the luminescence system is fully induced at early stationary phase, the $3OC_6$-HSL/LuxR regulatory system is a good candidate for control of synthesis of proteins specific to the maintenance phase of the symbiosis. C_8-HSL would be a good candidate for control of synthesis of proteins that are specific to the colonization phase of the symbiosis since it is induced during exponential growth [54]. The recent discovery of an

autoinducer molecule [91] and receptor protein [92] that are produced by the symbiont *Rhizobium leguminosarum* in the course of pea root nodulation further suggests that autoinducers may play a role in symbiosis. The current study begins with the characterization of a suspected symbiosis determinant, the periplasmic CNP, and continues with the identification of proteins from *V. fischeri* that are distinct from those encoded by the *lux* operon and whose production is controlled by quorum sensing.

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Chapter 2. Purification and properties of periplasmic 3':5'-cyclic nucleotide phophodiesterase

Purification and Properties of Periplasmic 3':5'-Cyclic Nucleotide Phosphodiesterase

A NOVEL ZINC-CONTAINING ENZYME FROM THE MARINE SYMBIOTIC BACTERIUM *VIBRIO FISCHERI**

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The 3':5'-cyclic nucleotide phosphodiesterase (CNP) of *Vibrio fischeri*, due to its unusual location in the periplasm, allows this symbiotic bacterium to utilize extracellular 3':5'-cyclic nucleotides (*e.g.* cAMP) as sole sources of carbon and energy, nitrogen, and phosphorus for growth. The enzyme was purified to apparent homogeneity by a four-step procedure: chloroform shock, ammonium sulfate precipitation, and chromotography on DEAE-Sephacel and Cibacron Blue 3GA-agarose. The active enzyme consists of a single polypeptide with a mass of 34 kDa. At 25 °C, it has a pH optimum of 8.25, a K_m for cAMP of 73 μm, and a V_{max} of 3700 μmol of cAMP hydrolyzed/min/mg protein (turnover number of 1.24×10^5/min). The specific activity of the *V. fischeri* enzyme is approximately 20-fold greater than that of any previously characterized CNP when comparisons of activity are made at the same assay temperature. Activity increases with temperature up to 60 °C. The CNP contains 2 atoms of zinc/monomer, and zinc, copper, magnesium, and calcium can restore activity of the apoenzyme to varying degrees. The exceptional specific activity of the enzyme and its unusual location in the periplasm support proposals that the enzyme enables the bacterium to scavenge 3':5'-cyclic nucleotides in seawater and that the enzyme plays a role in cAMP-mediated host-symbiont interactions.

3':5'-Cyclic nucleotide phosphodiesterase (EC 3.1.4.17; CNP)[1] catalyzes the hydrolysis of 3':5'-cyclic nucleotides (*e.g.* cAMP and cGMP) to their corresponding 5'-nucleoside monophosphates. The enzyme typically is located in the cytoplasm of cells. In eukaryotic organisms, several isozymes function in a variety of signal-mediated processes by modulating cytoplasmic levels of cAMP and cGMP (1). Many prokaryotes also produce a cytoplasmic CNP. In bacteria, cAMP regulates gene transcription via interaction with a cAMP receptor protein. However, rather than CNP-mediated hydrolysis of cAMP or excretion from the cytoplasm, regulation of the synthesis of cAMP is thought to control cellular levels in bacteria (2, 3). A CNP with atypical locations in the cell has been described from the cellular slime mold *Dictyostelium discoideum*. That enzyme is unique in occurring as both extracellular and cell membrane-associated forms, which catalyze the hydrolysis of extracellular cAMP involved in morphogenetic and aggregational signaling during plasmodium formation (4, 5).

We recently described a second extra-cytoplasmic CNP. The enzyme occurs in the marine bacterium *Vibrio fischeri*, which establishes a luminescent mutualism with certain marine animals. Its periplasmic location and high activity confer on *V. fischeri* the novel ability to utilize extracellular 3':5'-cyclic nucleotides as sources of carbon and energy, nitrogen, and phosphorus for growth (6). The gene, *cpdP*, for this enzyme is the only bacterial CNP gene that has been cloned. The deduced amino acid sequence exhibits 34% identity with the extracellular CNP of *D. discoideum* and 30% identity with the low affinity CNP (PDE1) of the yeast *Saccharomyces cerevisiae* (7). The *V. fischeri* enzyme is specific for 3':5'-cyclic nucleotides (6) and, therefore, differs from previously described bacterial periplasmic phosphatases such as 2':3'-cyclic phosphodiesterase:3'-nucleotidase (EC 3.1.4.16) and 5'-nucleotidase (EC 3.1.3.5).

The *V. fischeri* enzyme has been proposed to play a central role in the luminescent (light organ) symbiosis of *V. fischeri* with sepiolid squids and monocentrid fish (6). Since periplasmic CNP activity is rare in bacteria (6), the enzyme may contribute to the specificity of the symbiosis by permitting *V. fischeri* cells to utilize putative host-released cAMP as a nutrient. Alternatively, the enzyme may function to degrade 3':5'-cyclic nucleotides released from organisms into the marine environment (6, 7).

In this report, we describe the purification and biochemical properties of the *V. fischeri* periplasmic CNP. A detailed understanding of the enzyme may lead to insights into the physiological and ecological roles of bacterial CNPs and the possibility of cAMP-mediated symbiotic interactions between *V. fischeri* and its hosts.

MATERIALS AND METHODS

Reagents—Myokinase (360 units/mg), pyruvate kinase (200 units/mg), lactate dehydrogenase (550 units/mg), and crystalline bovine serum albumin were purchased from Boehringer Mannheim. 5'-Nucleotidase (*Crotalus adamanteus* venom), 5'-ATP (disodium salt), NADH (disodium salt), 3':5'-cyclic nucleotides, Trizma grade tris, phosphenolpyruvate (tri-cyclohexylammonium salt), Cibacron Blue-agarose 3GA, DEAE-Sephacel, and Sephacryl HR200 were obtained from Sigma, and Coomassie R-250 was from Fisher Chemical Co. (Pittsburgh, PA).

Bacterial Strains and Culture Conditions—*V. fischeri* strain MJ-1, from the light organ of the monocentrid fish *Monocentris japonicus* (8), was maintained on LBS agar (6) at room temperature. Liquid cultures were grown at 26 °C with aeration (150 revolutions/min) in minimal medium containing 300 mM NaCl, 10 mM KCl, 50 mM MgSO₄, 10 mM

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[1] The abbreviations used are: CNP, 3':5'-cyclic nucleotide phosphodiesterase; ICPES, inductively coupled plasma emission spectroscopy; PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholinepropanesulfonic acid.

CaCl$_2$, 15 mM NH$_4$Cl, 0.3 mM α-glycerophosphate, 20 mg/liter ferric ammonium citrate, 10 mM glucose, and 100 mM HEPES, pH 7.5. Cultures for enzyme purification were initiated with a 1% inoculum that had been grown to mid-exponential phase in minimal medium. Cells were harvested by centrifugation approximately 3 h after cultures attained stationary phase, at which point they produced a high level of luminescence.

Cell Fractionation—Cells were separated into outer membrane, inner membrane, and cytoplasmic/periplasmic (*i.e.* soluble) fractions as described for *Vibrio cholerae* (9).

CNP Activity Assays—Three assays were used for measurement of CNP activity. In assay 1, the standard method used in this study, rates of hydrolysis of cAMP to AMP were measured by a modification of a coupled assay with adenylate kinase, pyruvate kinase, and lactate dehydrogenase (10). Specifically, identical reference and reaction mixtures consisted of 2 mM cAMP (except where otherwise indicated), 0.6 mM ATP, 0.17 mM NADH, 0.5 mM phosphoenolpyruvate, 4 units of adenylate kinase, 4 units of pyruvate kinase, 10 units of lactate dehydrogenase, 20 mM KCl, 5 mM MgCl$_2$, and 100 mM Tris-HCl, pH 8.25, in a volume of 2 ml. The reaction was initiated by addition of samples containing CNP, and the production of NAD$^+$ from NADH was monitored spectrophotometrically as the change in absorbance at 340 nm. Consistent with the coupled nature of the assay, the reaction exhibits a lag that is inversely proportional to the amount of CNP added. Following the lag, the reaction rate is linear up to a ΔA$_{340}$ of 0.8. CNP activity was calculated from the linear portion of the reaction using the extinction coefficient of 6.22/mM/cm (11) taking into account that 2 mol of NAD$^+$ are produced per mole of cAMP hydrolyzed. One unit of CNP activity is defined as the activity that catalyzes the hydrolysis of 1 μmol of cAMP in 1 min.

Control experiments demonstrated that the reaction rates obtained with assay 1 were linearly dependent on the amount of CNP in the reaction mixture over a 4-fold range (0.04–0.164 μmol of NADH oxidized/min), indicating that the observed CNP activity was not limited by any of the rate indicator components. This finding was confirmed at each temperature used in the determinations of K_m and V_{max}. Furthermore, since adenosine was found to be a competitive inhibitor of CNP (see "Results"), control experiments were conducted and verified that ATP (included in the assay at 0.6 mM as an adenylate kinase substrate) does not inhibit CNP activity.

Assay 2, a two-stage version of assay 1, was developed to examine the effects of pH, temperature, and metal ions on CNP activity without interference of these effects on the activities of the coupling enzymes. In the first stage, samples containing CNP were added to reaction mixtures containing 100 mM Tris-HCl, pH 8.25, and 6 mM cAMP in a volume of 1.0 ml; the reactions were initiated by the addition of the CNP and were terminated at various times by the addition of 53 μl of 60% (w/v) perchloric acid, which irreversibly inactivated the CNP. This mixture was placed on ice, and 142 μl of 3 M KOH was then added to raise the pH to approximately 6 and to precipitate the perchlorate anion. In the second stage, 20 μl of the perchlorate/KOH-treated reaction mixture was added to a mixture containing the components of Assay 1 except for the CNP and cAMP. The total ΔA$_{340}$ was used to calculate the amount of cAMP hydrolyzed to AMP during the first-stage reaction. Rates were linear with respect to the time of the first reaction. Rate measurements made with assay 2 agreed within 10% of those made with assay 1.

Assay 3, used for substrates other than cAMP, was a modification of a previously described method (6, 12) with the following additional changes: 100 mM Tris-HCl, pH 8.25 at 25 °C as the reaction buffer, without MnCl$_2$ which was not required for *V. fischeri* CNP activity, and with a 5′-nucleotidase reaction time of 30 min. Unless otherwise indicated, assays 1–3 were conducted at 25 °C. Protein concentrations were determined by the method of Bradford (13) using bovine serum albumin as the standard.

Purification—Periplasmic proteins were released from intact *V. fischeri* cells by a chloroform shock method (14). Cells from two 1-liter cultures were collected by centrifugation (10,000 × *g*, 10 min) and resuspended in 100 ml of 50 mM Tris-HCl, pH 8.0 (buffer 1) at 4 °C. 20 ml of chloroform was added to the cell suspension, which was inverted once to mix and then held at room temperature for 10 min. An additional 200 ml of buffer 1 was added, after which cells and chloroform were pelleted by centrifugation (16,000 × *g*, 20 min, 4 °C). Buffer 1 was added to the supernatant (periplasmic extract) to adjust its protein concentration to 1 mg/ml. All subsequent steps of the purification were conducted at 4 °C.

The periplasmic extract was fractionated with ammonium sulfate, and the protein that precipitated between 40 and 70% of saturation was

collected by centrifugation (16,000 × *g*, 10 min) and dissolved in 10 ml of 10 mM imidazole buffer, pH 7.5 (buffer 2). The protein solution was then dialyzed for 18 h against three 1-liter volumes of buffer 2, changed at 0, 6, and 12 h, to remove the residual ammonium sulfate.

The dialyzed ammonium sulfate fraction was applied to 2 ml of packed DEAE-Sephacel equilibrated with buffer 2 in a 10-ml Poly-Prep column (Bio-Rad). 10 ml of 50 mM NaCl in buffer 2 was run through the column and discarded. CNP was then eluted from the column with 10 ml of 100 mM NaCl in buffer 2. The entire eluate was immediately applied to 1 ml of Cibacron Blue-agarose equilibrated with 100 mM MgSO$_4$ in a second 10-ml Poly-Prep column. An initial wash with 10 ml of 1 M KCl, 50 mM Tris base (untitrated, pH 10.3; buffer 3) removed the majority of bound protein, and CNP was then eluted with 5 ml of buffer 3 containing 1 mM adenosine. At this point, aliquots were taken for SDS-PAGE analysis, activity assays, and protein determination. To preserve activity, crystalline bovine serum albumin was added to 5 mg/ml to the remaining sample. The adenosine was removed by 18 h of dialysis with three 1-liter changes of 1 M KCl and 50 mM Tris-HCl, pH 9.0. Without the addition of bovine serum albumin, CNP activity declined during storage at −80 °C. With bovine serum albumin, no loss of activity was detected after 12 months of storage at −80 °C with repeated thawing and freezing.

Molecular Weight Determinations—For SDS-PAGE, the method of Laemmli (15) was followed using 14% separating and 6% stacking gels. Proteins were stained with Coomassie Blue R-250 at 60 °C. For gel-filtration chromatography, a 1.5 × 50-cm column containing Sephacryl HR200 was used with 1 M KCl buffered with 100 mM Tris-HCl, pH 9.0, at 4 °C at a flow rate of 19 ml/h. Fractions were collected each minute and assayed for CNP activity by assay 1.

Inactivation of CNP by EDTA—A 300-μl aliquot of purified CNP solution containing 5 mg/ml bovine serum albumin was dialyzed for 140 h against three 100-ml volumes of 1 M KCl, 1 mM EDTA, and 50 mM Tris-HCl, pH 9.0, at 4 °C changed at 0, 48, and 96 h. After dialysis, 1% of the starting enzyme activity remained. The EDTA was then removed by size exclusion chromatography using a Bio-Spin 6 Column (Bio-Rad) prewashed with 1 mM EDTA and re-equilibrated with deionized water according to the manufacturer's instructions. Nine divalent metal ions (Specpure Analytical Standard salts (Johnson Matthey, Ward Hill, MA)) were added to reaction mixtures for the first stage of assay 2 at concentrations of 1, 10, and 100 μM to test for their ability to reactivate the apoenzyme. Those metals found to reactivate the apoenzyme were examined further to determine their optimal concentrations for reactivation. Percent reactivation of the apoenzyme was calculated as: [(activity of apoenzyme with metal ion addition) minus (activity without metal ion addition)]/[activity of control without EDTA] × 100.

Metal Content Determination—ICPES analysis of the purified CNP was performed by the Chemical Analysis Laboratory, University of Georgia (Athens, GA) using the Thermo Jarrell Ash 965 Inductively Coupled Argon Plasma with simultaneous 31 element capability. The CNP was tested for the presence of 31 elements including calcium, cadmium, cobalt, copper, iron, magnesium, manganese, nickel, and zinc which are most frequently found as components of metalloproteins. The metal content of CNP samples was corrected for metal content in control samples containing buffer alone.

RESULTS

Periplasmic Location—Previously, we demonstrated the CNP activity of *V. fischeri* to be soluble in the periplasm (6). That study, however, did not address the possibility that the enzyme also occurs in a cytoplasmic membrane- or outer membrane-associated form or that it is released into the extracellular environment. To test these possibilities, we examined membrane and soluble fractions of *V. fischeri* cells and the cell-free growth medium for CNP activity. Cells were separated into outer-membrane, cytoplasmic membrane, and cytoplasm/periplasm (soluble) fractions. Total cellular CNP activity before fractionation was 291 units, and the fractions were found to contain 0.5, 6, and 224 units, respectively, demonstrating that the enzyme is not associated with either the outer membrane or the cytoplasmic membrane. No CNP activity was observed in the cell-free growth medium following growth of *V. fischeri* cells to high population density or in protein precipitated from the cell-free growth medium by the addition of ammonium sulfate to 80% of saturation. The enzyme, therefore, does not appear to be released into the extracellular environment.

TABLE I
Purification of CNP

Assays were conducted at room temperature (21 °C) with assay 1. One unit of activity is defined as the amount of protein catalyzing the hydrolysis of 1 μmol of cAMP/min.

Step	Protein	Units	Specific activity	Recovery	Purification
	mg		*units/mg*	*%*	*-fold*
Intact cells	950	630	0.66	100	1
Periplasmic extract	350	620	1.8	98	2.7
40–70% $(NH_4)_2SO_4$	105	410	3.9	65	5.9
DEAE-Sephacel	4	400	100	63	152
Blue agarose	0.080	210	2625	33	3977

Purification—A four-step procedure was developed that resulted in purification of the enzyme by approximately 4000-fold (Table I) and to apparent homogeneity (Fig. 1). The first step, chloroform shock, served to disrupt the outer membrane and release the periplasmic contents from the cells, giving an approximately 3-fold increase in specific activity relative to whole cells. No cell lysis was detected by phase contrast microscopy when cells were mixed gently with the chloroform. Vigorous mixing of cells with the chloroform eliminated most of the CNP activity, as did freezing of the sample at any step in the purification procedure before it was complete.

The second step, fractionation of the periplasmic suspension with ammonium sulfate, gave a 2-fold increase in specific activity. It also served to concentrate the sample for the two subsequent chromatographic steps, in which most of the purification was achieved.

In the third step, the resolubilized ammonium sulfate fraction was bound to and eluted from DEAE-Sephacel, resulting in a 25-fold purification. Earlier purification attempts using buffer 2 in this anion-exchange step at a pH of 6.0, which is close to the protein's calculated pI of 5.5 (7), were successful but substantial losses of activity occurred. Therefore, the enzyme is apparently unstable at low pH. Adjusting buffer 2 to pH 7.5 (Table I) eliminated the loss of activity.

In the final step, in which the DEAE-Sephacel eluate was bound to and eluted from Cibacron Blue-agarose, a 26-fold purification was achieved. The simplicity of the purification procedure overall results from the high affinity of the protein for the blue-agarose dye matrix in this step. The high affinity permits the extreme conditions of high KCl concentration and high pH to be used to elute the majority of contaminating proteins. Adenosine, a competitive inhibitor of the *V. fischeri* CNP with a K_i of approximately 800 μM (data not shown), was used at a concentration of 1 mM in the wash buffer to elute CNP from the blue-agarose. SDS-PAGE analysis of the adenosine eluate revealed a single protein, even when the gel was heavily loaded (Fig. 1). The enzyme was estimated to account for approximately 0.025% of the total cellular protein and 0.07% of the periplasmic protein. Previously, the enzyme purified in this manner was subjected to microsequence analysis of the first 20 residues at the amino-terminal end, and the sequence for the mature protein matched that deduced from the cloned gene (7).

Molecular Mass of the Native CNP—The purified protein exhibited a monomeric molecular mass of approximately 34,000 Da on SDS-PAGE (Fig. 1), which is consistent with the mass of the protein calculated from the deduced amino acid sequence of the cloned *cpdP* gene (33,636 for the protein without its leader sequence; 7). To determine if the enzyme was active as a monomer, we used a calibrated Sephacryl gel filtration column to conduct size exclusion chromatography of the purified protein under non-denaturing conditions. The CNP activity in each of three trials eluted as a single molecular species, with an estimated molecular mass of 32,000 ± 800 Da (mean ± S.D.). A representative experiment is shown in Fig. 2. The similarity of the estimated masses under denaturing and
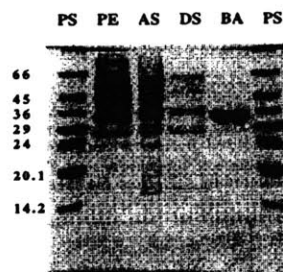
FIG. 1. **SDS-PAGE analysis during purification of the *V. fischeri* periplasmic CNP.** *PE*, periplasmic extract; *AS*, 40–70% ammonium sulfate fraction; *DS*, DEAE-Sephacel eluent; *BA*, Cibacron Blue-agarose eluent; and, *PS*, protein standards (66 kDa, bovine serum albumin; 45 kDa, ovalbumin; 36 kDa, glyceraldehyde-3-phosphate dehydrogenase; 29 kDa, carbonic anhydrase; 24 kDa, trypsinogen; 20.1 kDa, trypsin inhibitor; 14.2 kDa, α-lactalbumin).
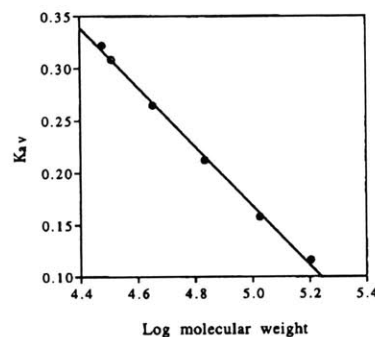
FIG. 2. **Native molecular weight determination of *V. fischeri* periplasmic CNP.** One of three experiments is shown in which proteins were separated on Sephacryl HR200. CNP (32.4 kDa, Log molecular weight = 4.51) purified as described in the text ($\bigcirc$) and protein size standards ($\bullet$) (160 kDa, aldolase; 106 kDa, 6-phosphogluconate dehydrogenase; 66 kDa, bovine serum albumin; 45 kDa, ovalbumin; 29 kDa, carbonic anydrase; 21 kDa, adenylate kinase).

non-denaturing conditions indicates that the native enzyme is active as a monomer and may be present in the periplasm as such.

cAMP Hydrolysis: Kinetics, pH, and Temperature Effects— Activity of the purified periplasmic protein was maximal at pH 8.25, with half-maximal activities at pH values of approximately 7.0 and 9.0 (Fig. 3). The enzyme obeys typical Michaelis-Menten kinetics and has a K_m for cAMP of 73 μM at pH 8.25 and 25 °C (Fig. 4A), as derived from a Hofstee plot (16). V_{max} at 25 °C, calculated from the Hofstee plot (Fig. 4A), was 3700 units/mg protein, and the measured rate at 2 mM cAMP was approximately 3400 units/mg. The corresponding turnover number using the calculated molecular mass of 33,636 is 1.24 × 10^5/min.
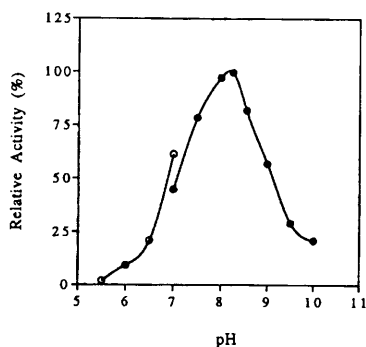
FIG. 3. **Effect of pH on hydrolysis of cAMP by V. fischeri periplasmic CNP.** Assay 2 was used, and the buffers were 50 mM glycine with 50 mM glycyl-glycine (●) and 100 mM MES (O). Data presented are the averages of duplicate assays. For each data point, the measured values differed by <5% from the average.
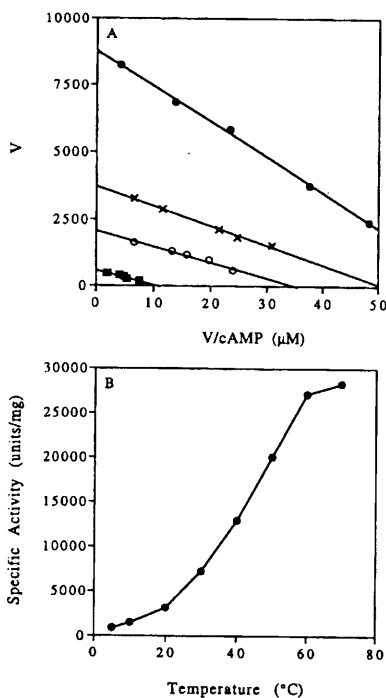
FIG. 4. **Effect of temperature on hydrolysis of cAMP by V. fischeri periplasmic CNP.** The assay mixture was adjusted to pH 8.25 at each temperature. Data presented are the averages of duplicate assays. A, Hofstee (16) plots were used to calculate values of K_m and V_{max} for cAMP hydrolysis at various temperatures: 5 °C, K_m = 53 μM and V_{max} = 588 units/mg (■); 15 °C, K_m = 59 μM and V_{max} = 2077 units/mg (O); 25 °C, K_m = 73 μM and V_{max} = 3730 units/mg (×); 35 °C, K_m = 132 μM and V_{max} = 8764 units/mg (●). For each data point, the measured values differed by <3% from the average. B, plot of measured specific activities at different temperatures. For each data point, the measured values differed by <5% from the average.

It was of interest to determine if the CNP exhibited maximum activity at a temperature that the bacterium experiences in seawater. Enzymatic reactions in V. fischeri, as in many marine plants and poikilothermic animals, generally occur at

TABLE II
Hydrolysis of 3′:5′-cyclic nucleotides by CNP

Assay 3 was used at a substrate concentration of 2 mM. Activity is expressed as a percentage of activity with cAMP as substrate.

3′:5′-Cyclic nucleotide	CNP activity
	%
cAMP	100
2′-deoxy-cAMP	36
cCMP	111
cGMP	61
cIMP	110
cTMP	27
cUMP	150

temperatures of approximately 27 °C or lower. Remarkably, the rate of the CNP-catalyzed reaction increases with temperature up to 60 °C (Fig. 4B) at which temperature the measured specific activity was about 27,000 units/mg, indicating that the CNP is active at temperatures much higher than it is expected to experience in nature. K_m and V_{max} were determined at 10 °C intervals between 5 and 35 °C (Fig. 4A). The K_m for cAMP increased from 53 μM at 5 °C to 132 μM at 35 °C, and V_{max} increased by 15-fold over the same range, giving a coefficient of 2.47/10 °C rise in temperature.

Substrate Specificity—Previously, we demonstrated that the V. fischeri periplasmic CNP is specific for 3′:5′-cyclic nucleotides (6). Consistent with that study, cGMP was utilized at approximately half the rate of cAMP (Table II). The rates observed with cCMP, cIMP, and cUMP were similar to or somewhat higher than that with cAMP, whereas those with the 2′-deoxy-cyclic nucleotides, 2′-deoxy-cAMP and cTMP, were 3-fold lower.

Metal Content—Our earlier observation that EDTA inhibits activity (6) suggested that the CNP might be a metalloenzyme or might require a divalent metal ion cofactor for full activity. Furthermore, sequence similarity of the V. fischeri enzyme to a known zinc enzyme, the low affinity CNP (PDE1) of S. cerevisiae, including the conservation of 4 histidine residues in the two sequences (7), suggested that the V. fischeri enzyme contains zinc. Therefore, we tested three chelators of zinc for their effects on CNP activity: EDTA, dithiothreitol, and 1,10-orthophenanthroline. The chelators, present in the reaction mixture at 1 mM, inhibited CNP activity 23, 64, and 98%, respectively. With regard to inhibition by dithiothreitol, a strong chelator of zinc (17), it should be noted that the mature CNP protein has no cysteine residues (7); thus, the inhibition by dithiothreitol cannot be due to effects on sulfhydryl groups or disulfide bonds. Subsequent analysis by ICPES detected 2.2 mol zinc/mol CNP, while none of the other 30 elements tested was detected in significant quantities. We conclude that the native V. fischeri CNP is a zinc-containing enzyme, with two atoms of zinc/peptide.

Reactivation of zinc-free apoenzyme with various metal ions supported this conclusion. The apoenzyme was prepared by dialysis of the purified protein against EDTA, followed by size exclusion chromatography to separate EDTA-metal complexes from the apoenzyme. 140 h of dialysis was required to reduce CNP activity to 1% that of a control sample dialyzed against buffer alone, which retained 94% of its original activity (Fig. 5A). After removal of EDTA from the sample, nine divalent metal ions, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}, were added to the apoenzyme to test their ability to restore activity. Four metal ions reactivated the apoenzyme to varying degrees (Fig. 5B). Zinc was the most effective; 1 μM restored 94% of the activity of the control sample. Concentrations above 1 μM were less effective or inhibitory. Copper (1 μM), magnesium (10 mM), and calcium (50 μM) restored 33, 47,
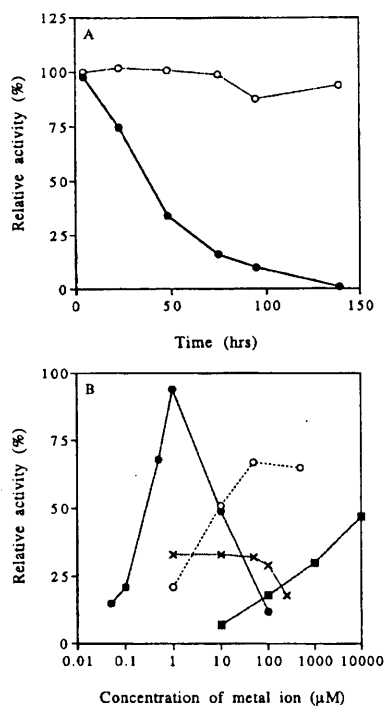
FIG. 5. Inactivation of the *V. fischeri* periplasmic CNP by EDTA and reactivation by metal ions. *A*, activity of CNP dialyzed against buffer containing 1 mM EDTA (●) and against buffer without EDTA (○). *B*, activity of EDTA-inactivated CNP upon addition of various metal ions (Zn^{2+} (●), Ca^{2+} (○), Cu^{2+} (×), Mg^{2+} (■)). Data presented are the average of duplicate assays. For each data point, the measured values differed by <8% from the average.

and 67% of the activity, respectively. Magnesium was still increasing enzyme activity at 10 mM, the highest concentration tested.

To test for the involvement of a loosely bound metal ion in CNP activity, metal ions were added to the reaction mixture with holoenzyme that had not been exposed to EDTA. Of the nine metal ions tested with the apoenzyme, none enhanced activity of the holoenzyme at a concentration of 100 μM, whereas Zn^{2+} and Cu^{2+} inhibited holoenzyme activity about 25% and Cd^{2+} inhibited 77%. No inhibition was observed with calcium or magnesium.

DISCUSSION

Purification of the the periplasmic CNP of *V. fischeri* to apparent homogeneity permitted the characterization of some of its properties. Several of these, including its narrow substrate specificity, stability, abundance in the cell, specific activity, and the ability of certain metal ions to reactivate apoenzyme are unusual and provide insight into the role of the enzyme in the biology of *V. fischeri* and the ecology of cyclic nucleotides in seawater.

The narrow substrate specificity of the *V. fischeri* CNP is atypical for a periplasmic nucleotidase. Other nucleotide degrading periplasmic phosphatases, such as alkaline phosphatase, acid phosphatases, 2':3'-cyclic phosphodiesterase:3'-nucleotidase, and UDP-glucose hydrolase:5'-nucleotidase, are each capable of hydrolyzing multiple types of phosphorylated compounds, but the CNP of *V. fischeri* is specific for 3':5'-cyclic

nucleotides; it shows no activity with 2':3'-cAMP, ADP, ATP, or non-nucleotide phosphate esters (6). Activity measurements with various 3':5'-cyclic nucleotides suggest that the 2'-oxygen of the ribose moiety is necessary for full activity of the enzyme, and that the enzyme does not discriminate between purines and pyrimidines (Table II).

Despite its narrow substrate specificity, the *V. fischeri* enzyme is similar to many periplasmic phosphatases in containing zinc. Alkaline phosphatase (18), 2':3'-cyclic phosphodiesterase:3'-nucleotidase (19), and UDP-glucose hydrolase:5'-nucleotidase (19) are zinc metalloenzymes. However, there appears to be no significant amino acid sequence similarity between the four proteins. Other than the low affinity CNP (PDE1) of *S. cerevisiae*, which also contains 2 atoms of zinc/ peptide (20), no eukaryotic CNPs have been shown to be metalloenzymes.

The mature CNP protein has no cysteine residues (7), and, therefore, no disulfide bonds to stabilize its tertiary structure. Nonetheless, several observations in this report indicate that it is a very stable protein. Full catalytic activity was retained after exposure of the enzyme to 60 °C (Fig. 4*B*) or to a pH of 10.3 (as used in the dye-affinity step of the purification procedure). In addition, the binding of one or both of the zinc ions is tight, since prolonged dialysis against EDTA was required to inactivate the CNP; after 24 h of dialysis, the activity was 75% of the starting value, and a period of 140 h was required to reduce the activity by 99% (Fig. 5*A*). A prolonged dialysis against EDTA is also required to inactivate the zinc-containing CNP of *S. cerevisiae* (20), and it is possible that the zinc ions in both the yeast CNP and that from *V. fischeri* are firmly bound to the same ligands (see below).

Copper, magnesium, and, calcium, are all unusual substitutes for zinc, but in metal substitution experiments with the *V. fischeri* enzyme, activity was partially restored to the apoenzyme by each of these metal ions. Copper has been used as a spectroscopic probe in metal substitution experiments, but with the notable exception of superoxide dismutase, copper-substituted zinc enzymes usually are inactive (21). Similarly, glyoxylase is one of a few zinc enzymes that is active when substituted with magnesium (22). Yeast enolase has been shown by x-ray diffraction to bind zinc or calcium at the same site as the native magnesium ion, but the calcium-substituted enzyme is inactive due to an alteration in the conformation of bound substrate (23). The difficulty in removing zinc from the *V. fischeri* CNP and the reactivation of the apoenzyme by calcium, which is surprising because calcium has a valence electronic configuration distinct from that of zinc, may indicate that zinc serves as a structural, rather than a catalytic, component in CNP.

However, contrary to the proposal that the zinc of CNP is structural, a recent analysis of zinc enzymes with known structures (24) suggests that the zinc might be catalytic. In that study, it was pointed out that non-catalytic zinc has 2 or more cysteine residues as ligands, of which the mature *V. fischeri* CNP has none. In addition, catalytic zinc often has 1 or more histidine residues as ligands, of which the CNP has 4 that, by sequence comparison, are evolutionarily conserved (7). Determination of the role of zinc in the CNP of *V. fischeri* will require further examination.

The rapid kinetics of the *V. fischeri* enzyme may make it an interesting enzyme for studies of catalytic mechanism. Using the turnover number and K_m determined at 25 °C (Fig. 4*A*), k_{cat}/K_m can be calculated to be 2.8×10^7 s^{-1} M^{-1}, which indicates that the rate of formation of the enzyme-substrate complex is nearly as fast as allowed by diffusion controlled encounters between CNP and cAMP (25). At 30 °C, the assay

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temperature used in studies of other CNPs, the *V. fischeri* enzyme has a specific activity of 7100 units (Fig. 4B), a value more than 20 times greater than any previously reported for a CNP that is specific for 3':5'-cyclic nucleotides, including that of the extracellular CNP of *D. discoideum* (26) and the low affinity CNP of *S. cerevisiae* (20) to which it has substantial amino acid sequence identity (7). The highest previously reported specific activity for a CNP is that from bovine brain and heart, 300 units/mg (27). The highest specific activity for catalysis of cAMP hydrolysis by a CNP from a bacterium other than *V. fischeri* is that from *Serratia marcescens*, 295 units/mg (28).

The pH/activity profile (Fig. 3) supports the involvement of 1 or more histidine residues in CNP activity. Although side chain ionizable groups on amino acid residues in proteins often have pK_a values that are different from those seen with free amino acids, and the ionizable groups involved in enzyme catalysis cannot be definitively identified from a pH/activity profile, such profiles can be suggestive, especially if additional information about the enzyme's structure and function is available. The *V. fischeri* CNP exhibits half-maximal activity at pH values of 7 and 9. Imidazole side chains of histidine residues have pK_a values in the range of 5–8 (29), and 4 histidines, which occur infrequently in bacterial proteins, are conserved in the CNPs of *V. fischeri*, *S. cerevisiae*, and *D. discoideum* (7). Such highly conserved residues often have essential roles in enzyme structure and/or catalysis. Thus, we assume that further study may show that 1 or more of the histidines in *V. fischeri* CNP must be in the unprotonated state for most efficient catalysis. Likewise with regard to the half-maximal activity at pH 7, zinc-bound water is a participant in many hydrolytic reactions, and it ionizes with a pK_a of about 7. Our data are also consistent with a role for zinc-bound water in the activity of CNP. The implications of the half-maximal activity at pH 9 are less clear. Protein α-amino groups, lysine ε-amino groups, and tyrosine phenolic hydroxyl groups generally ionize with pK_a values of about 9, and our results (Fig. 3) might indicate involvement of one or more of these groups in the CNP-catalyzed hydrolysis of cAMP.

The biochemical characteristics of the *V. fischeri* CNP are consistent with hypotheses for the biological role of the enzyme in the ecology and symbiosis of *V. fischeri*. The enzyme has been proposed to degrade cAMP free in the environment (6). Many organisms release cAMP (30, 31), but the concentration in the environment is low (31, 32). The CNP of *V. fischeri*, because of its periplasmic location and high specific activity, could function in a manner analogous to phosphate scavenging periplasmic enzymes in bacteria. Many bacteria have periplasmic 5'-nucleotidase activity, which degrades AMP to inorganic phosphate and adenosine prior to their transport into the cytoplasm and subsequent metabolism. The *V. fischeri* CNP, therefore, represents a novel class of enzyme that can catalyze the first step in the recovery of cAMP from the environment.

In the luminescent mutualism of *V. fischeri* with sepiolid squid and monocentrid fish (33, 34), the enzyme has been hypothesized to permit *V. fischeri*, exclusively, to utilize host-provided cAMP as a source of nutrition. According to the hypothesis, *V. fischeri* cells in the animal light organ elicit the overproduction and release of host cAMP by secreting a toxin that, through an ADP-ribosylating activity, interferes with the regulation of host adenylate cyclase in a manner analogous to the secretion and activity of cholera enterotoxin by *V. cholerae* in the human intestine (6, 7, 35). Recent evidence indicating that *V. fischeri* contains *toxRS* genes (36) is consistent with this hypothesis. Nonetheless, a direct test of the symbiosis hypothesis will require examination of the ability of a *cpdP* null mutant of *V. fischeri* to colonize its animal host.

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Chapter 3. Acyl-Homoserine Lactone Controlled Genes Define a Quorum Sensing Regulon in *Vibrio fischeri*

ABSTRACT

In *Vibrio fischeri* the luminescence system (*luxR luxICDABEG*) is regulated by the transcriptional activator LuxR and two homoserine lactone autoinducers (*luxI*-dependent $3OC_6$-HSL and the *ainS*-dependent C_8-HSL) in a cell-density dependent manner by a process termed quorum sensing. We have identified five additonal proteins whose production is positively regulated by quorum sensing. 2-D PAGE protein profiles of MJ-215 ($\Delta luxI$ *ainS*⁻) and MJ-208 ($\Delta luxR$) grown with and without the autoinducers were contrasted with protein profiles of MJ-100 (parent strain) to identify eight proteins whose production is regulated by $3OC_6$-HSL and *luxR*. N-terminal sequencing identified three proteins to be LuxE, LuxA, and LuxB. The remaining five proteins were of sizes inconsistent with those of the other Lux proteins, and N-terminal sequence analysis demonstrated that four were novel (the fifth was N-terminally blocked). Cell fractionation indicated one protein to be periplasmic, one to be in the cytoplasmic membrane, one to be in the outer membrane and one to be soluble in the cytoplasm. The periplasmic protein, termed QsrP, is encoded monocistronically, with a probable LuxR/autoinducer binding site in the promoter region and a strong stem-and-loop terminator downstream of the coding region. Consistent with its periplasmic location, the mature protein lacks a 19 amino acid canonical leader peptide. The predicted 129 amino acid sequence has no significant similarities to sequences in the NCBI database. A *qsrP* insertional mutant displayed no obvious phenotype in culture and by itself colonized its symbiotic host *Euprymna scolopes* to an extent similar to that of the parent strain. However, in competition with the parent strain, the ability of the mutant to colonize and persist in the symbiosis was reduced. A

second protein of 217 amiino acids is a homolog of RibB, the enzyme that catalyzes the transformation of ribulose 5-phosphate to 3,4-dihydroxy-2-butanone 4-phosphate, a precursor for the xylene ring of riboflavin. The promoter region of this second gene also contained a putative LuxR binding site. We propose that, along with the *lux* genes, the genes for these novel proteins are components of a LuxR/autoinducer-dependent regulon in *V. fischeri*.

INTRODUCTION

Quorum sensing has become recognized recently as an important mechanism by which bacteria regulate gene expression in response to population density and host association [1]. Classically defined in Gram-negative bacteria, quorum sensing involves the LuxI family of synthases [2], which produce membrane permeant acyl-homoserine lactone (acyl-HSL) signals [3], and the LuxR family of transcriptional regulatory proteins, which mediate the response to those signals [1, 4]. Currently, over 25 species of bacteria, including several plant and animal pathogens, are known to use quorum sensing to regulate activities as diverse as luminescence, conjugative plasmid transfer, and the production of antibiotics and extracellular enzymes [1, 4]. In many of these species, quorum sensing regulates transcription from several unlinked genetic loci to control multiple functions [4]. *Pseudomonas aeruginosa*, for example, controls the production of elastase, chitinase, cyanide, and rhamnolipid biosurfactant via a complex quorum sensing regulatory network [5-7].

As one of the first and perhaps the most thoroughly studied of these systems, quorum sensing in *Vibrio fischeri* has become a model for quorum sensing in other bacteria [1]. In *V. fischeri* N-3-oxohexanoyl-L-HSL (3OC$_6$-HSL) serves as an intercellular signal to regulate transcription of the luminescence (*lux*) genes in a population density-dependent manner [8]. The *lux* operon consists of seven genes, *luxICDABEG* [9-11]. The first gene of the operon, *luxI*, encodes the synthase that catalyzes the production of 3OC6-HSL from S-adenosylmethionine and an acylated acyl carrier protein, presumably from the fatty acid biosynthesis pathway [2]. The genes *luxA* and *luxB* encode the two subunits of luciferase, which oxidize fatty aldehyde and FMNH$_2$ to generate light [12]; *luxC, luxD,* and *luxE* encode subunits of a fatty acid reductase complex that is necessary for

regeneration of fatty aldehyde substrate [9, 13]; and *luxG* apparently encodes a flavin reductase [14].

The promoter region of the *lux* operon reveals the multiple levels of control that are involved in the regulation of luminescence. Centered 44 nucleotides upstream of the *lux* operon transcriptional start site is a 20-bp region of DNA with dyad symmetry, the *lux* box, which is necessary for induction of luminescence [15]. In *V. fischeri* the location of the *lux* box relative to a putative -10 Pribnow box suggests that it occurs in place of a typical σ^{70} -35 polymerase recognition site. Upstream of the *lux* box is a CRP binding site which positively regulates transcription of the divergently transcribed gene *luxR* and simultaneously inhibits transcription from the *lux* operon[16-19]. Presumably, this inhibition prevents the runaway induction of luminescence that is inherent in a positive feedback loop in the absence of such inhibition. LuxR and RNA polymerase bind to the *lux* box and surrounding promoter DNA in a synergistic fashion [20]. Genetic dominance experiments in *Escherichia coli* containing wild-type and mutant alleles of *luxR* suggest that LuxR functions as a multimer [21]. The LuxR protein consists of two domains, an N-terminal DNA-binding domain and a C-terminal domain that binds $3OC_6$-HSL [22, 23]. In the absence of $3OC_6$-HSL, the N-terminal domain is thought to inhibit the activator function of the C-terminus. Binding of $3OC_6$-HSL has been proposed to relieve this inhibition and promote transcription.

A *luxI* mutant of *V. fischeri*, which is deficient in the production of $3OC_6$-HSL, still induces luminescence, but to a lesser extent than the parent strain. This finding led to the discovery of a second autoinducer, N-octanoyl-L-HSL (C_8-HSL) [24]. Production of C_8-HSL is directed by *ainS*, which specifies an acyl-HSL synthase distinct from LuxI [22, 25]. C_8-HSL can influence *lux* operon transcription by competitively inhibiting the interaction between $3OC_6$-HSL and LuxR; in vivo C_8-HSL apparently operates to limit

premature *lux* operon induction [26, 27]. The construction of a strain of *V. fischeri* defective in both *luxI* and *ainS* eliminated the ability of cells to produce both the LuxI-dependent and the AinS-dependent acyl-HSLs [27], thereby setting the stage for the identification of quorum-regulated genes distinct from *lux* in this species. In this study, we have identified five proteins that are distinct from Lux whose production, like that of the Lux proteins, is dependent on $3OC_6$-HSL and LuxR. The genes that encode two of these proteins, *qsrP* and *ribB*, have been characterized.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* Novablue and SM10-λpir were grown on LB medium [28] at 37°C with antibiotics as appropriate (ampicillin, 100 µg/ml; chloramphenicol, 30 µg/ml). *Vibrio fischeri* MJR1 is a derivative of MJ-1 [29] that contains a spontaneous mutation that confers resistance to rifampicin. Strains of *V. fischreri* were maintained on LBS agar [30] with the appropriate antibiotics (chloramphenicol, 3 µg/ml; naladixic acid, 20 µg/ml; neomycin, 200 µg/ml; and rifampicin, 100 µg/ml). VFM is a minimal salts media that has been previously described [31]. For protein isolation, cells were grown with aeration in 3 ml cultures of liquid LBS without antibiotics at 27°C to an A_{660} of 0.4 (mid-exponential phase), 0.8 (late exponential phase), or 1.2 (early stationary phase). Cells from 1 ml volumes were pelleted at 4°C, washed twice with an equal volume of ice-cold Artificial Sea Water [32], and stored frozen as a pellet at -70°C until use.

Culture density was determined by measuring the absorbance at 660 nm (A_{660}). Under the conditions used, an A_{660} unit corresponded to approximately 10^9 cells/ml. Synthetic autoinducers were added to culture tubes as solutions in chloroform to yield a final concentration of 100 nM. The chloroform was removed by evaporation with a stream of sterile air prior to addition of the medium and bacteria.

Cell fractionation. Cells were separated into outer membrane, inner membrane, and cytoplasmic/periplasmic (i. e., soluble) fractions as described for *Vibrio cholerae* [33]. Cells were treated with chloroform to isolate periplasmic proteins [34].

Protein isolation for 2-D PAGE. All procedures were conducted at 4°C or on ice. Frozen cell pellets were resuspended in 80 µl sample buffer 1 (SB1; 40 mM Tris-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characterisitics	Source or reference
E. coli		
Novablue	*endA hsdR17*(r_{K12}⁻m_{K12}⁺) *supE thi recA gyrA relA lac*[F' *proA⁺B⁺ lacI𝑞ZΔM15*::Tn*10*(Tc^r)]	Novagen
SM10-λ*pir*	*thi thr leu supE tonA lacY recA*::RP4-2-Tc::Mu Km^r λ::*pir*	[33]
V. fischeri		
ES114	Wild type	[35]
ESR1	ES114, Rf^r	[36]
MJ-1	Wild type	[29]
MJR1	MJ-1, Rf^r	This study
MJ-100	MJ-1, Nx^r	[37]
MJ-208	MJ-100, Δ*luxR* (450-bp nonpolar deletion)	[24]
MJ-211	MJ-100, Δ*luxI* (~250-bp nonpolar deletion)	[24]
MJ-215	MJ-211, *ainS* (2-bp insertion)	[27]
MJ-216	MJ-100, *ainS*::*neo* (1.8-kb Nm^r cassette)	[25]
MJ-10X	MJR1, *qsrP*::p300-10XM	This study
ES-10X	ESR1, *qsrP*::p300-10XE	This study
MJ-ribBX	MJR1, *ribB*::p300-12XM	
Plasmids		
pT7Blue	cloning vector; Ap^r	Novagen
pBluescript SK+	Cloning vector, Ap^r	Stratagene
pGFPuv	Source of GFP reporter protein	Clontech
pBsGFP	pBluescript containing a *Hind*III/*Eco*RI GFP insert	This study
pSupGFP	pSup102 containing *Hind*III/*Bam*HI GFP insert	This study
pQsrPGM	MJR1 QsrP translational fusion in pSupGFP	This study
pQsrPGE	ESR1 QsrP translational fusion in pSupGFP	This study
pGP704	*oriR6K mobRP4* Ap^r	[33]
pSMC300	pGP704; Δ*bla*, cm^r	This study
p300-10XE	pSMC300; *qsrP* 119-269 from ESR1	This study
p300-10XM	pSMC300; *qsrP* 119-269 from MJ-100	This study
p300-12XM	pSMC300; *ribB* 194-450 from MJ-100	This study

HCl [pH 8.0], 200 mM dithiothreitol, and 0.3% SDS) and boiled for 2 min. After cooling, 8 μl of sample buffer 2 (0.5 M Tris-HCl [pH 8.0], 50 mM MgCl$_2$, 1 mg ml^{-1} DNAse I, and 0.25 mg ml^{-1} RNAse A) was added to the mixture, which was incubated for 30 min. Proteins were precipitated by the addition of acetone to 80%, collected by centrifugation, and resuspended in 80 μl SB1. After a second acetone precipitation, the pellet was air-dried for 30 min, resuspended in 60 μl SB1 and 240 μl sample buffer 3 (9.9 M urea, 100 mM dithiothrietol, 4% triton X-100, and 2.2% polyampholytes [40% w/v, pH 3-10; ESA, Inc.]) and stored at -70°C until use.

2-D PAGE. Proteins were separated essentially as described by O'Farrell [38] using the 2-D Investigator system from Millipore and reagents from Oxford Glycosystems. First dimension gels contained 4.1% acrylamide, 9.5 M urea, 5 mM CHAPS, 2% triton X-100 and 5.8% polyampholytes. 10 μl of sample was loaded to the basic end of the gel for analytical gels or 50 μl for preparative gels and electrophoresed for 18,000 volt-hours. After completion of the run, gels were incubated in equilibration buffer (0.375 M Tris-HCl [pH 9.2], 50 mM dithiothreitol, and 0.01% bromophenol blue) for 2 min or stored frozen in 10% glycerol at -70°C.

Second dimension separation was performed with continuous gels of 12.5% acrylamide (Duracryl) at 4°C. Analytical gels were stained with silver and stored in 10% glycerol. Preparative gels were electroblotted to polyvinylidiene difluoride (PVDF) membrane (Millipore) and stained with Coomassie Brilliant Blue. Individual protein spots were excised for microsequence analysis.

Peptide sequencing. For amino-terminal sequencing, excised proteins were subjected to automated Edman degradation on an Applied Biosystems Model 477A (Foster City, CA) protein sequencer with an on-line Model 120 phenylthiohydantion amino acid analyzer at the Massachusetts Institute of Technology Biopolymers Laboratory. For internal sequencing, protein bound to PVDF membrane was digested in situ with

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endoproteinase LysC (Wako) [39], and the resulting peptide mixture was separated by microbore high performance liquid chromatography using a Zorbax C18 1.0 mm by 150 mm reverse-phase column on a Hewlett-Packard1090 HPLC/1040 diode array detector at the Harvard Microchemistry Facility. Optimum fractions from the chromatagram were chosen based on differential UV absorption at 205 nm, 277 nm and 292 nm, peak symmetry and resolution. Peaks were further screened for length and homogeneity by matrix-assisted laser desorption time-of-flight mass spectrometry on a Finnigan Lasermat 2000 (Hemel, England); and selected fractions were submitted to automated Edman degradation. Details of strategies for the selection of peptide fractions and their microsequencing have been previously described [40].

PCR amplification, cloning, and sequencing of DNA. For QsrP and RibB, two peptide sequences each were used to design degenerate PCR primers for isolation of DNA in the region of the genome encoding the proteins. Because the relative positions of the sequenced peptides in the protein were unknown, both forward and reverse primers were designed from each peptide sequence. For QsrP, primers corresponding to regions of peptides 10-PK12 and 10-PK39 (10-12F, 10-12R, 10-39F, and 10-39R; Table 2) were combined as appropriate in two separate PCR reactions using MJ-100 chromosomal DNA as the template. From the reaction using 10-12F and 10-39R as primers, a single product of 209 bp was obtained. For RibB, primers corresponding to regions of peptides 12-PK32 and 12-PK78 (12-32F, 12-32R, 12-78F and 12-78R; Tables 2 and 3) were used to amplify a portion of the genome that codes for the protein. From the reaction using 12-32F and 12-78R as primers, a single product of 215 nucleotides was obtained. Both PCR products were cloned into pT7-Blue and sequenced.

PCR amplifications were performed in an Idaho Technology's Rapidcycler (Idaho Falls, ID) using 50 μl glass capillary tubes. The oligonucleotides used as primers are

TABLE 2. N-terminal peptide sequences

Peptide or oligonucleotide	Sequence	Approx. MW (kD)
LuxA	MKFGN	40
LuxB	- KFGL	37
LuxE	- - V - TEY	44
6	[A]PRVGL	22
7	NH2-terminus blocked	15
8	- [D]NAPVKGGFT	14
10 (QsrP)	KNTYS	12
12	- - KLNQ	24

Brackets denote low confidence amino acid designations.

TABLE 3. Internal peptide sequences and oligonucleotides

Peptide: internal fragment	
10-PK12	NVPQIGDTYK
10-PK39	DRVSIPPIYVELSDNRFSGLHVSGMK
10-PK51	YGNLFFELIK
12-PK32	KGVTTGVSATDR
12-PK78	LAELTPAGVLCEVTN
Oligonucleotide	
10-12F*	AAYGTNCCNCARATHGGNGAYACRTA
10-12R*	TAYGTRTCNCCDATYTGNGGNACRTT
10-39F*	ATHTAYGTNGARCTNAGYGAYAAYCG
10-39R*	CGRTTRTCRCTNAGYTCNACRTADAT
12-32F*	ACIACIGGDGTWAGYGCWACIGAYAG
12-32R*	CTRTCIGTWGCTCTWACHCCIGTIGT
12-78F*	GCWGGDGTWTTDTGYGARGTWACIAA
12-78R*	TTIGTWACYTCRCAHAAWACHCCWGC
10-210F	CTGTAGAGGCTTGCTCTATTGGATCT
10-210R	TCAGAAGAAGCTCTCTACGACCCTTG
10-826F	ATCAAGAAACCTCGACGAGATAAACG
10-826R	GTCCTAAAGAGGAAATGCTAAGTGGT
QsrP-pH3	CCCAAGCTTGAAACCTCGACGAGATAAAC
QsrP-pK	AAAGGTACCGGGTCTAACTTTATAAGTATC
R6KR	GGCTTTTAAAGCTTTTAAGGTTTAACGG
sp12-101R	CGACAGTCCCTTCTGTATGTCCTC

* For degenerate primers: Y=T, C; R=A, G; H=A, T, C; D=A, T, G; N=A, T, C, G

described in Table 3. Standard conditions were used except for the initial reactions with degenerate primers. In this case, conditions included 4 mM $MgCl_2$ in the buffer and 30 cycles were performed with denaturation at 94°C for 2 s, annealing at 40°C for 10 s, and elongation at 50°C for 10 s. PCR products were purified from agarose gels with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into pT7-Blue using the Perfectly Blunt cloning kit (Novagen, Madison, WI). DNA sequences were determined by the dideoxy-nucleotide chain termination method of Sanger et al. [41] using dye terminator labelling at the CRC DNA Sequencing Facility, University of Chicago.

Construction of a QsrP::GFP translational fusion. To examine regulation of QsrP production, a translational fusion was made between the N-terminus of QsrP and the Green Fluorescent protein of *Aequorea victoria*. The source of the gene for GFP was pGFPuv (Clontech, Palo Alto, CA) which encodes a LacZ::GFP fusion protein. The coding region for GFP was cloned as a *Hind*III/*Eco*RI fragment into pBluescript to yield pBsGFP. Cloning into pBluescript provided a downstream *Bam*HI restriction site which was used to clone the gene for GFP into pSup102 [42] as a *Hind*III/*Bam*HI fragment to yield pSupGFP. The resulting vector can be mobilized into *V. fischeri* by conjugation, and it contains unique *Hind*III and *Kpn*I restriction sites that can be used to insert the promoter and 5'-coding region of a gene in frame with the GFP initiation codon, which is located 11 bp downstream of the *Kpn*I site. For the fusion with QsrP, primers QsrP-pH3 and QsrP-pK (Table 3) were used to PCR amplify the promoter regions and the first 45 amino acids coded by *qsrP* in MJ-100 and ESR1. These fragments were ligated into pSupGFP to yield pQsrPGM and pQsrPGE, respectively. Each vector contains the first 45 amino acids of QsrP fused in frame with the GFP initiation codon. The vector pQsrPGM was transferred to MJ-100 and MJ-215, and pQsrPGE was transferred to ESR1 by conjugation from *E. coli* strain SM10-λ*pir*.

Construction of *qsrP* and *RibB* mutants. Because *V. fischeri* is resistant to ampicillin, the suicide plasmid pSMC300 was constructed by replacing the ampicillin resistance on pGP704 [33] with resistence to chloramphenicol. pGP704, which contains the R6K origin of replication and the *mob* region of pRP4, was digested with *Pst*1, and the 1.1 kb Cat gene supplied as a control insert in the pCR-Script cloning kit (Stratagene, La Jolla, CA) was blunt-end cloned in place of the 5'-end of the gene for ß-lactamase. The resulting vector can be conjugated from *E. coli* SM10-λ*pir* to *V. fischeri*, but it cannot replicate independently; it confers resistance to chloromphenicol only if it has recombined into the chromosome. To promote recombination between the vector and *qsrP*, a 150 bp PCR fragment corresponding to nucleotides 119-269 of the coding regions from MJ-100 and ESR1 were cloned into the *Xba*I and *Sac*I sites of pSMC300 to yield p300-10XM and p300-10XE respectively. For *ribB*, a 257 bp PCR fragment that corresponds to nucleotides 194-450 of the coding region was cloned into the *Xba*I and *Sph*I sites of pSMC-300 to yield p300-12XM. Conjugation of the vectors from SM10-λ*pir* to *V. fischeri* strains MJR1 and ESR1were performed as previously described [32]. Transconjugants were selected for their ability to grow on LBS supplemented with rifampicin and chloramphenicol. Disruption of the *qsrP* and *ribB* genes was confirmed by PCR analysis. For the *qsrP* mutants, MJ10X and ES10X, primer R6KR, which spans the *Hind*III site of the R6K origin and reads towards the multiple cloning site of pSMC300, and 10-826R were used to PCR MJ10X and ESR10X chromosomal DNA. Both reactions yielded the approximately 700 bp product that is anticipated if the plasmid has integrated into the coding region of *qsrP* (data not shown). To confirm the integrity of the *ribB* mutant, MJ-ribBX, primers R6KR and SP12-101R, the latter of which corresponds to DNA sequence 26 nucleotides downstream of the sequence cloned into the suicide plasmid, was used to PCR MJ-ribBX DNA. The reaction yielded a 420 bp fragment that is anticipated if the vector had inserted into the chromosome as intended.

In vivo assay for colonization of *E. scolopes* by strains of *V. fischeri*. Colonization of the juvenile squid light organs was performed essentially as described by Ruby [43]. Juveniles were maintained individually in 10 ml of filter sterilized Instant Ocean water (Aquarium Systems) in glass scintillation vials. Within 24 hours of hatching, bacterial cells grown to mid-exponential phase in VFM media with 20 mM ribose as the sole carbon source were added to a final concentration of 10^4 cells/ml per strain. Luminescence was used as an indicator of development of the symbiosis. 48 hours postinoculation the individual colonized squid were rinsed in sterile Instant Ocean water and homogenized to release the bacterial symbionts. Dilutions of the homogenate were spread on LBS agar containing rifampicin. Colonies were transferred to LBS agar containing rifampicin and chloramphenicol to distinguish between strains ESR1 (chloramphenicol sensitive) and ES-10X (chloramphenicol resistant). For the in vitro growth competition, the ratio of the mutant to the parent strain was determined from cultures grown in triplicate to an absorbance of $A_{660} = 0.6$.

RESULTS

Production of non-Lux, quorum sensing-regulated proteins in

V. fischeri. *V. fischeri* induces the production of proteins encoded by the *lux* operon

(*luxICDABEG*) in late exponential to early stationary phase of growth via transcriptional

control by LuxR and $3OC_6$-HSL. We used 2-D PAGE to compare the cellular protein

patterns of strains MJ-100 and MJ-215 to determine whether *V. fischeri* produces other

proteins, distinct from those encoded by the *lux* operon, whose production is also regulated

by quorum sensing. MJ-100 is wild-type for autoinducer synthesis, whereas MJ-215 is

defective in the two *V. fischeri* autoinducer synthase genes, *luxI* and *ainS*, and therefore

makes neither $3OC_6$-HSL nor C_8-HSL [24, 25].

Protein patterns of cells grown to late exponential (A_{660} = 0.8) and early stationary

(A_{660} = 1.2) phases revealed seven proteins produced by MJ-100 that were not produced

by MJ-215 and one protein that was produced to a lesser extent by MJ-215 (Fig. 1A, B).

In MJ-100, all eight proteins were more abundant in cells grown to early stationary phase

compared to late exponential phase (Appendix A, Fig. A1B). At mid-exponential phase

(A_{660} = 0.4), however, these proteins were not detected on 2-D PAGE from MJ-100 cells,

and at that population density the protein patterns of MJ-100 and MJ-215 did not exhibit

obvious differences (Appendix A, Fig. A1C and data not shown). These results

demonstrate the efficacy of 2-D PAGE for revealing population density-dependent

production of proteins in *V. fischeri*, and they suggest that production of the eight

differentially produced proteins requires autoinducer.

Amino terminal sequence analysis and size estimates ([10], Table 2) indicated that

five of these eight proteins were not Lux proteins. To distinguish between Lux and non-

Lux proteins, we extracted the eight proteins from gels, had their amino termini sequenced,

and compared those sequences with the amino termini sequences of *V. fischeri* Lux

A | Acidic   Basic

— 66

LuxE

LuxB   LuxA

— 45

12

— 31

6

— 21.5

7

8

— 14.5

10

— 6.5

B
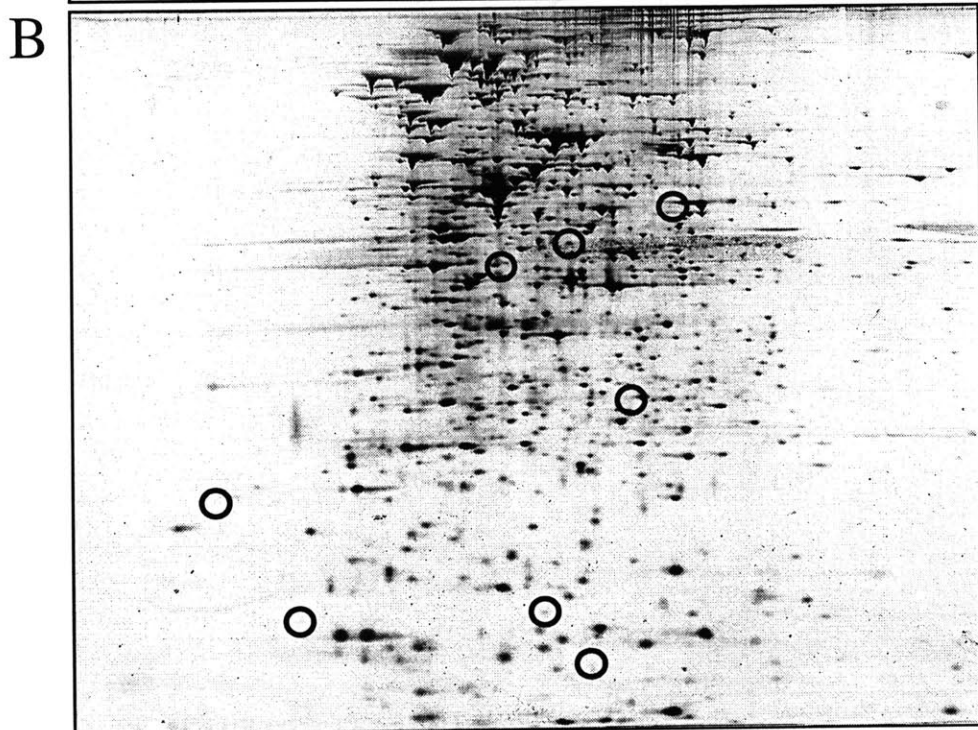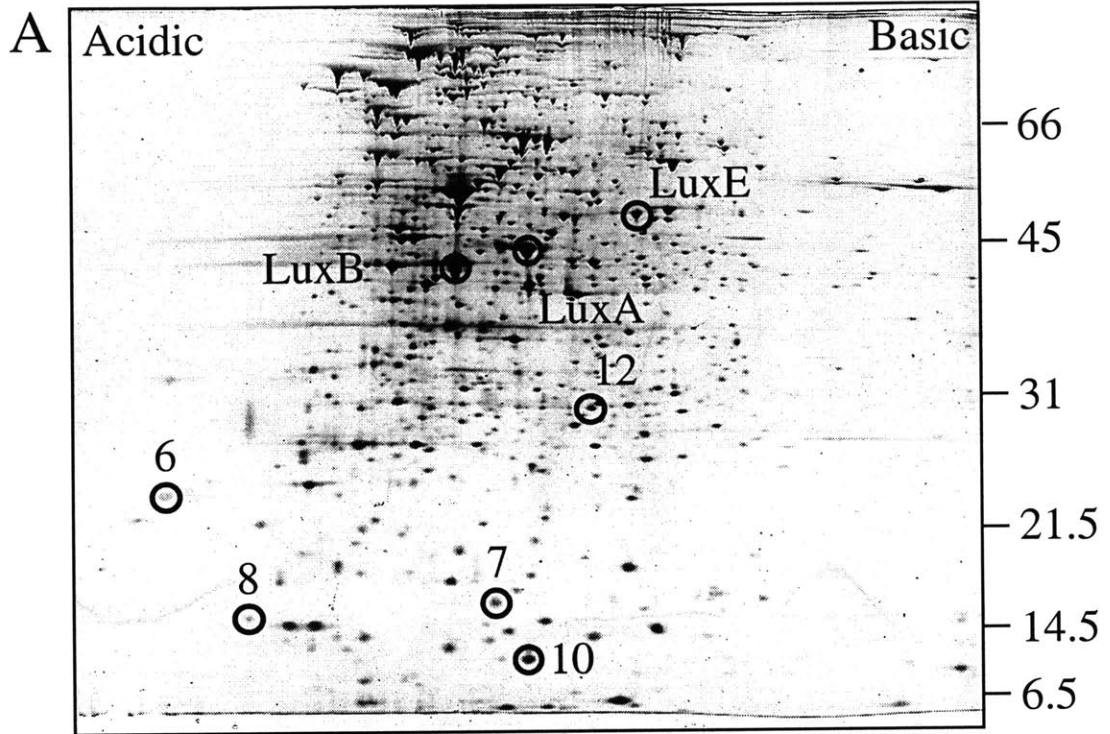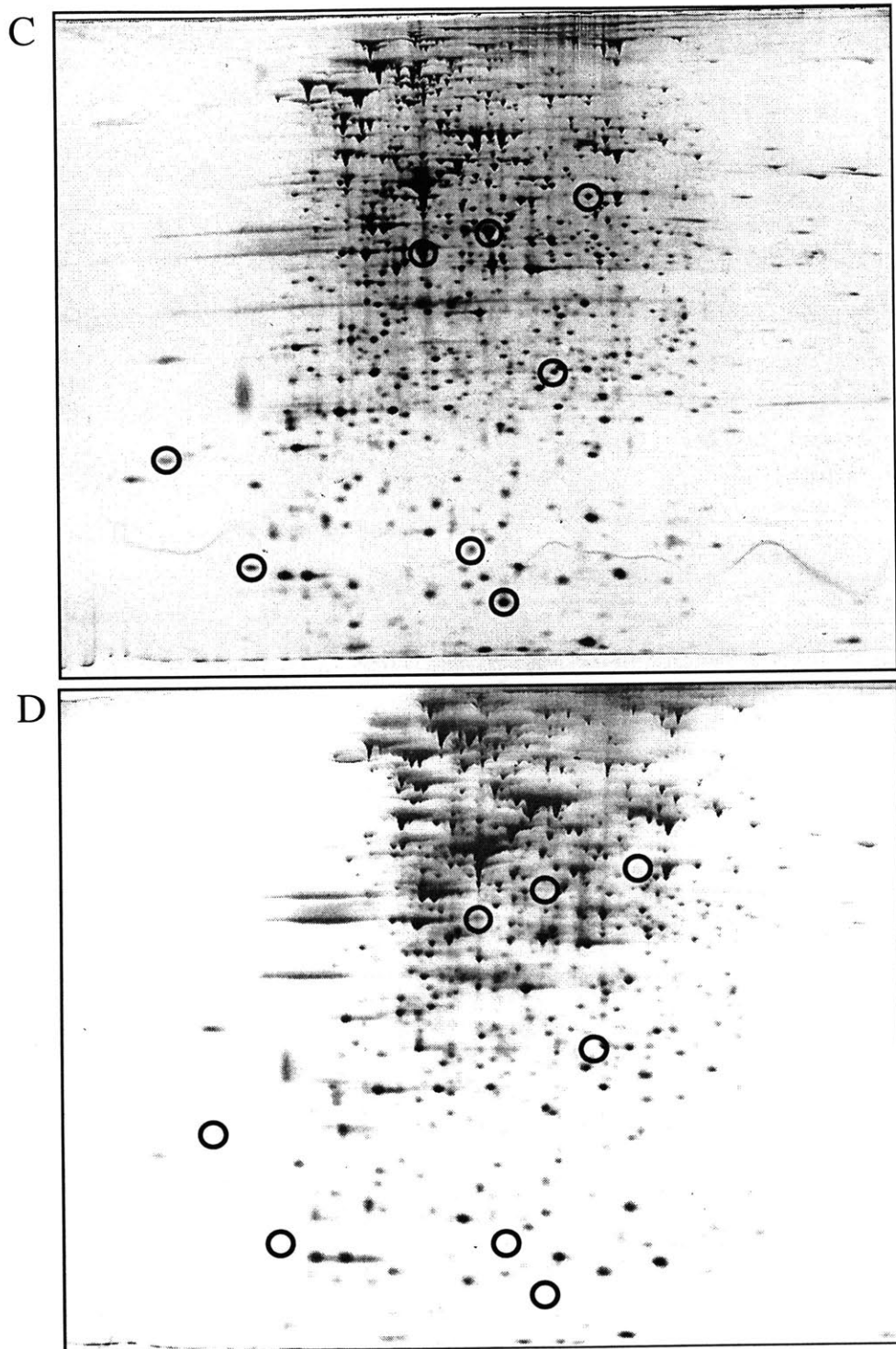
Figure 1. Two dimensional PAGE of whole-cell protein preparations from different strains of *V. fischeri*. (A) MJ-100, wild-type for both quorum sensing systems. (B) MJ-215, Δ*luxI, ainS⁻*. (C) MJ-215 grown with 100 nM 3OC$_6$-HSL added exogenously to the media. (D) MJ-208, Δ*luxR*.

proteins [10]. Three of the eight proteins were found to be LuxA, LuxB, and LuxE, whereas four of the other proteins were distinct from Lux proteins. The amino terminus of the fifth protein (Fig. 1A, #7) apparently was blocked; however, its estimated size, about 15 kD, is smaller than that of any of the Lux proteins, the smallest of which, LuxI, is approximately 25 kD [9]. These results demonstrate the presence in *V. fischeri* of five proteins (#6, #7, #8, #10, and #12) distinct from those encoded by the *lux* operon and whose production requires autoinducer (quorum sensing regulated proteins; QSR proteins). Cell fractionation experiments (Materials and Methods) localized these proteins to the cytoplasmic membrane (QSR 6) the outer membrane (QSR 7), the periplasm (QSR 10), and the cytoplasm (QSR 12) (Appendix A, Figs. A5-A8). QSR 8, though readily observed in whole cell extracts on 2-D PAGE (Fig 1.) was not detected in any of the cell fractions.

Requirement for $3OC_6$-HSL and LuxR. We next carried out experiments which demonstrated that production of the five QSR proteins was dependent on $3OC_6$-HSL and LuxR. Because MJ-215 is a double autoinducer synthase mutant, defective in both *luxI* and *ainS*, the lack of production of the five QSR proteins in MJ-215 could be due to the absence of either $3OC_6$-HSL or C_8-HSL, or possibly to the absence of both signal molecules. To distinguish between the effects of the two signals, we compared the proteins of MJ-211 (Δ*luxI*), which fails to produce $3OC_6$-HSL, with those of MJ-216 (*ainS⁻*), which fails to produce C_8-HSL. The three Lux proteins and the five QSR proteins were not produced by MJ-211, whereas all eight proteins were produced by MJ-216 (Appendix A, Figs. A3A and B). These results indicate a requirement for $3OC_6$-HSL for production of the proteins.

To confirm the apparent requirement for $3OC_6$-HSL, we assessed the ability of synthetic, pure autoinducers to restore production of the eight proteins in MJ-215. Cells of MJ-215 were grown in the presence of saturating levels (100 nM) of $3OC_6$-HSL or C_8-

HSL to early stationary phase, and the proteins were extracted and examined by 2-D PAGE. The presence of $3OC_6$-HSL restored production of the three Lux and the five QSR proteins, whereas the presence of C_8-HSL did not restore the production of these proteins (Fig. 1 C, and AppendixA, Fig. A2C). Therefore, the induction of the eight proteins is dependent on $3OC_6$-HSL, and the results demonstrate that the presence of a high level of C_8-HSL cannot substitute for $3OC_6$-HSL. Furthermore, we did not detect any protein whose expression is regulated by C_8-HSL. Specifically, the protein patterns on gels from MJ-100 were indistinguishable from those of MJ-216, and the protein patterns of MJ-215 grown in the presence of C_8-HSL were identical to those of MJ-215 grown in the absence of C_8-HSL to early stationary phase.

$3OC_6$-HSL operates in *V. fischeri* through LuxR, the receptor/transcriptional activator of the *lux* operon. To determine whether LuxR, along with $3OC_6$-HSL, is required for production of the five QSR proteins, we compared the protein pattern of MJ-208, a *luxR* deletion mutant, grown to stationary phase, with that of MJ-100. MJ-208, which does not induce *lux* operon transcription [24], failed to produce the three Lux proteins and also failed to produce the five QSR proteins (Fig. 1D). Thus, LuxR, along with O-C_6-HSL, is required for production of these proteins.

QSR proteins and Lux proteins are produced at lower population density in strain MJ-216. During exponential phase growth, C_8-HSL negatively modulates luminescence in *V. fischeri* [27]. MJ-216 emits approximately 100-fold the amount of light made by the parent strain MJ-100 during mid-exponential phase growth $(A_{660}=0.4)$. To determine if C_8-HSL also modulates production of the five QSR proteins, we compared 2-D PAGE gels of strains MJ-100 and MJ-216 collected at mid-exponential phase. Each of the QSR proteins was detected from MJ-216 and absent from MJ-100 (Appendix A, Figs. A1C and D). Their abundance in MJ-216 was similar to that in MJ-100 grown to late exponential phase. In addition, the three Lux proteins were detected in

gels from MJ-216. Therefore, we conclude that C_8-HSL inhibits production of the QSR

proteins during mid-exponential phase growth.

Isolation of *qsrP*, the gene encoding QSR 10. To gain insight into the

identity and functions of the QSR proteins, we isolated the genes for two of them, QSR 10

and QSR 12. QSR 10 was chosen because of our interest in acyl-HSL-regulated proteins

in the symbiosis of *V. fischeri* with the sepiolid squid *Euprymna scolopes*. A protein of

similar size with a more basic isoelectric point is one of the more abundant proteins we

have observed on 2-D PAGE from *V. fischeri* cells taken from the light organ of *E.*

scolopes (Appendix A, Fig. A9). Its absence in the same strain grown in laboratory culture

suggests that it is produced preferentially during the symbioisis. (S. M. Callahan and P. V.

Dunlap, unpublished data).

A PCR approach was used to isolate the gene for QSR 10 and flanking DNA.

QSR 10 was extracted from gels and subjected to internal proteolytic digestion. Three of

the resulting peptides were separated by reversed-phase HPLC, and sequenced (Table 2;

10-PK12, 10-PK39, and 10-PK51). From two of the three peptide sequences (Materials

and Methods), forward and reverse PCR primers were designed and used to amplify from

MJ-100 chromosomal DNA a 209-bp portion corresponding to 69 amino acid residues

within QSR 10. The amino-terminus of peptide 10-PK39, which was not encoded by the

primer, was contained in the 69 amino acid translation, demonstrating that the PCR product

represented a portion of the protein's coding region.

Attempts to use the 209-bp portion as a probe to isolate the complete gene for QSR

10 from a plasmid-borne genomic library of MJ-100 DNA [31] were unsuccessful,

possibly because of underrepresentation of this DNA region in the library. As an

alternative, we used PCR to amplify the DNA flanking the 209 bp region and subsequently

PCR-amplified the intact gene using primers designed from the flanking DNA sequence.

To facilitate PCR amplification of the DNA flanking the 209 bp fragment, MJ-100

chromosomal DNA was digested to completion with *Cfo*I, the four bp recognition sequence of which is not present in the fragment. The chromosomal fragments were then circularized by ligation, and primers 10-210R and 10-210F (Table 1 and Fig. 2) were used to amplify the regions flanking the 209 bp fragment, which were joined at the *Cfo*I sites, making them contiguous on the circular molecule. The resulting 760 bp fragment was cloned and sequenced. From the sequences adjacent to the *Cfo*I site, primers 10-826F and 10-826R were designed and used to PCR amplify the intact gene, designated here as *qsrP* (quorum sensing regulon, periplasm), and the flanking DNA.

Characteristics of the *V. fischeri qsrP* gene. The nucleotide sequence of *qsrP* and its deduced translation are shown in Figure 2. Neither the nucleotide sequence nor the deduced amino acid sequence exhibited significant similarity with genes or gene products in the NCBI databases: apparently, *qsrP* is a novel gene. The 129 amino acid QsrP precursor protein has a calculated molecular weight of 14,746 Da, which includes a 19 amino acid sequence typical of prokaryotic leader peptides [44]. Consistent with the presence of the leader peptide and with localization of the protein to the periplasm, the amino terminus of the mature protein (Table 2) begins at K20 of the deduced translation product. The mature protein is composed of 110 amino acids and has a calculated molecular weight of 12,660 and a calculated isoelectric point of 4.9. These attributes are consistent with the mobility of the mature protein on 2-D PAGE (Fig. 1A).

Transcription of the *qsrP* gene is monocistronic and appears to be regulated directly by $3OC_6$-HSL and LuxR. Centered 73 nucleotides upstream of the *qsrP* coding region is a *lux* box (Fig. 2), a 20-bp region with dyad symmetry identified in the promoter of the *lux* operon as the binding site for the $O-C_6$-HSL/LuxR transcritional activator complex [10, 20, 45]. The *qsrP lux* box shares 14 of its 20 nucleotides with the *lux* operon *lux* box (Fig. 3). In the *lux* operon promoter, the *lux* box replaces the -35 region [16]. With that positioning

```
                    primer 10-826F
   1 AAGAAACCTC GACGAGATAA ACGAGCATTC ACCATATTGC TAAAATCAGA TTCTTTCTCT ACAAATTTAT


  71 TCATCATACT TCCCTTTTGA AACTGTTATT TTTATAAGTG TATTTTTACA ATCAGAAGTA GACTAAAGGT


 141 GCTTTGTGAC AGAAATATTG AAAGGAGATG ACGATTTTAT TGCGTATTTT TATTATCACT CTATATAAAA

                                   Lux Box                               -10
 211 AATAATATTA ATAATTAAAT ATAAACCTGT AATAAGTTAC AGGAACATAA TTACAACCGT TAATATAAAA

                      R.B.
 281 TTCTCTGGAA ACATATAGAA ATATAAATGG GGATTTTATG AAA AAA ATA TCA GCG TTA TTA CTT
                                                   M   K   K   I   S   A   L   L   L    9

                                            ↓
 345 TTC ACA CTT ATA TCA CCA CCA ATT TTT GCC AAA AAC ACA TAC TCA ATC AAG TCA TTA
      F   T   L   I   S   P   P   I   F   A   K   N   T   Y   S   I   K   S   L   28

                            primers 10-12F(>) and R(<)          <primer 10-210F
 402 AGC AAC CTA AAA AAT GTT CCC CAA ATT GGT GAT ACC TAT AAG TTA GAT CCA ATA GAG>
      S   N   L   K   N   V   P   Q   I   G   D   T   Y   K   L   D   P   I   E   47
                           peptide 10-PK12


 459 CAA GCC TCT ACA GAA CTT TTT TGT TGG GAG AAA GGA GAG AAT GAT GAT TTT TCT GCA
      Q   A   S   T   E   L   F   C   W   E   K   G   E   N   D   D   F   S   A   66

                                      primer 10-210R>
 516 TGT CAA ATT GTA ATT AAC TAT TCA GAA GAA GCT CTC TAT GAC CCT TGT TTA CAG GTA
      C   Q   I   V   I   N   Y   S   E   E   A   L   Y   D   P   C   L   Q   V   85

                                    primers 10-39F(>) and R(<)
 573 AAA GAT AGA GTT TCC ATT CCT CCT ATT TAT GTC GAA TTA AGT GAT AAT AGA TTT TCT
      K   D   R   V   S   I   P   P   I   Y   V   E   L   S   D   N   R   F   S>  104
                                    peptide 10-PK39


 630 GGG TTG CAT GTC TCT GGA ATG AAA ATT AAA TTT AAA GAA ATA AAG TAT GGA AAC TTA
      G   L   H   V   S   G   M   K   I   K   G   K   E   I   K   Y   G   N   L>  123


 687 TTT TTT GAA CTG ATC AAG TAA CTTTGGATAT TATATCTTAA TAAAAAAGCC TCTATTGCAG>
      F   F   E   L   I   K   *                          stem and loop          126
            peptide 10-PK56


 748 AGGCTTTTTA GCTTTTATTT AATCTTAACA ACCGCTGGTG GTTGCCAAGT ACCACTTAGC ATTTCCTCTT>
                                                            <primer 10-826R

 818 TAGGAC 823
```

Figure 2. Nucleotide sequence and deduced amino acid translation product of the *qsrP* gene. Putative *lux* box, -10 region of the putative σ^{70} promoter, ribosome binding site, and stem and loop terminator sequences are indicated. Also indicated are peptides determined by microsequencing and primers used to isolate the intact gene.

```
luxI MJ-1    1  A C C T G T A G G A T C G T A C A G G T 20
luxI 7744    1  A C C T G T A G G A T C G T A C A G G T 20
luxI H905    1  G G C T G T A G G A T A G T A C A G G T 20
luxI ES114   1  A G C T G T A G G A T G G T A C A G G T 20
luxI EM30    1  G C C T G T A G G A T C G T A C A G G T 20
qsrP MJ-1    1  A C C T G T A A T A A G T T A C A G G A 20
qsrP ES114   1  A C C T G T A A T A A A C G A C A G G A 20
ribB MJ-1    1  A C C T G C A A T A A T T T A C A G T A 20
                A C C T G T A G G A T   G T A C A G G T
```

Fig. 3. Comparison of the *lux* box sequences in the promoter regions of *V. fischeri* quorum-regulated genes. The *lux* boxes preceding *qsrP* and *ribB* are compared to the *lux* boxes preceding *luxI* from different strains of *V. fischeri* [15, 16, 46]. The last sequence represents a consensus sequence. The shaded and boxed nucleotides indicate regions of >50% identity.

as a guide, a reasonable -10 Pribnow box can be discerned in the putative *qsrP* promoter region, 27 nucleotides downstream from the center of the *lux* box. A putative Shine-Dalgarno ribosomal binding site was found 5 bases upstream of the ATG translation initiation codon. Downstream of the coding region, 23 bases after the translational stop codon, a probable rho-independent transcriptional terminator was identified. The stem and loop structure consists of a perfect 11 base stem and a 5 base loop followed by a poly-T region. Thus, the *qsrP* coding region was bounded by a promoter with good similarity to that of the *lux* operon and a strong putative transcriptional terminator.

Comparison of the *qsrP* genes from MJ-1 and ES114. To assess the degree of similarity between *qsrP* genes from two ecologically distant *V. fischeri* strains, we isolated the *qsrP* gene from *V. fischeri* strain ESR1, a derivative of ES114. In contrast to MJ-1 and its derviatives, ESR1 is fully competent to colonize the squid *E. scolopes* [47]. Furthermore, MJ-1 is brightly luminescent in laboratory culture, whereas ES114 and its derivatives are not visibly luminescent under the same conditions [35]. The *qsrP* gene was isolated from ESR1 by PCR-amplification of the relevant chromosomal DNA using

oligonucleotides 10-826F and 10-826R. Sequence analysis of the cloned PCR product revealed that the DNA flanking the QsrP coding region was essentially identical in MJ-100 and ESR1, whereas the QsrP coding region differed substantially. Of 234 nucleotides preceding the *lux* box, 233 were identical in the two strains. Furthermore, 17 of the 20 nucleotides identifed as a *lux* box-like region were the same in the two strains. For the QsrP coding region, however, only 84% of the nucleotide sequence and 78% of the deduced amino acids were identical. Notable differences in the coding region included the addition of amino acid residues R and S between D63 and F64 of the MJ-100 sequence (data not shown). Furthermore, the mature QsrP protein from ESR1 had a calculated molecular weight of 12,908, and a calculated isoelectric point of 7.0. The higher pI of the protein from ESR1 is consistent with its position on 2-D gels of proteins from bacteria isolated from the squid light organ, as mentioned previously.

QsrP translational fusions with GFP. To examine the production of QsrP during growth of ESR1 in the light organ of *E. scolopes*, we created a vector encoded translational fusion between QsrP and the Green Fluorescent Protein (GFP) of the jellyfish *Aequorea victoria* (Materials and Methods). Plasmids pQsrPGM and pQsrPGE contain the promoter regions and the coding regions for the first 43 amino acids of *qsrP* from MJ-100 and ESR1, respectively, joined in frame with the initiation codon for GFP. To test if the fusion construct could be used as a reporter of QsrP production in cells grown in laboratory culture, pQsrPGM was mobilized into MJ-100 and MJ-215 by conjugation. Transconjugants of MJ-100 grown on solid LBS were green when examined with ultra violet light at a wavelength of 365 nm (Data not shown). Transconjugants of MJ-215, on the other hand, were similar to the same strain without the fusion construct and to MJ-100 carrying pSupGFP, which lacks the *qsrP* region. The difference in GFP production between the strains demonstrated the efficacy of this approach for monitoring the production of QsrP.

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ESR1 containing pQsrPGE grown on solid LBS shows now signs of GFP when examined with ultra violet light. We are currently examining ESR1 cells carrying the reporter construct from the light organ of *E. scolopes* to monitor production of QsrP during the symbiosis.

Construction and characterization of *qsrP* mutants of *V. fischeri*.
Mutants defective in *qsrP* were constructed in both MJR1 and ESR1 by a plasmid integration procedure (Materials and Methods) and analyzed under a variety of growth conditions in laboratory culture to assess their phenotype. Regardless of the growth condition (e.g., minimal versus rich medium, various carbon sources), or the attribute tested (e.g., growth rate, light production, regulation of light production, gelatinase production), both mutant strains, MJ10X and ES10X, grew and behaved similarly to their respective parental strains. The absence of an obvious phenotype in culture for the *qsrP* mutants and the lack of a match in the database for QsrP makes ascribing a function for QsrP problematic at this time.

To determine if QsrP is involved in the symbiotic relationship between *V. fischeri* and its invertebrate host *E. scolopes*, ES-10X was used both alone and in competition with the parent strain in squid colonization assays (Materials and Methods). By itself, the *qsrP* mutant colonized the squid light organ to levels equivalent to those of the parent strain, ESR1. However, when both strains were used to coinoculate juveniles at a 1:1 ratio in an in vivo competition assay, the mutant represented a small fraction of the cells recovered from the light organ 48 h postinoculation (Table 4). The colonization ratio of ES-10X to ESR1 was 0.01. A similar competition in laboratory media between the two strains yielded an in vitro ratio of 1.17, which suggests that the competitive advantage of ESR1 over the *qsrP* mutant is specific to conditions in the host light organ. Although the *qsrP* locus is not

Table 4. In vitro and in vivo competition assays between ESR1 and ES-10X

In vitro, replicate	Ratio ES-10X:ESR1
1	27:23 = 1.17
2	27:23 = 1.17
3	27:23 = 1.17
	Average in vitro ratio = 1.17

In vivo, squid	Ratio ES-10X:ESR1
1	0:192 = 0
2	2:190 = 0.01
3	2:190 = 0.01
4	10:182 = 0.05
5	0:192 = 0
	Average in vivo ratio = 0.01

necessary for the symbiosis, *qsrP* is necessary for efficient colonization of the *E. scolopes* light organ.

Isolation of *ribB*, the gene encoding QSR 12. The second gene examined was that encoding QSR 12. To isolate the relevant chromosomal DNA, QSR 12 was digested and internal fragments were sequenced. As with QsrP, degenerate primers based on two peptide sequences were used to PCR amplify a portion of the coding region from the chromosome of MJ-100 (Materials and Methods and Table 2). Sequencing of the resultant 215 nucleotide fragment confirmed that it was part of the coding region for QSR 12. The intact gene was then isolated from a plasmid-borne library of MJ-1 chromosomal DNA in *E. coli* [31] using PCR analysis as a screen. Plasmids from 2000 colonies

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representing the library were initially pooled in groups of 50, and DNA from these 40

groups served as the template for individual PCR reactions using primers 12-32F and 12-

78R. One of the reactions generated a 215 bp fragment. Each plasmid from the pool of 50

for that reaction was then screened individually to identify a single positive clone, which

contained approximately 7.5 kb of chromosomal insert DNA. The sequence of the region

containing the QSR 12 open reading frame was obtained using a primer walk strategy (Fig.

4).

Analysis of the deduced translation sequence revealed a high degree of similarity to

the sequences of LuxH from the marine luminous bacterium *Vibrio harveyi* [48], and RibB

from *E. coli* [49] and *Haemophilus influenzae* [50]. In *E. coli*, RibB has been shown to be

3,4-dihydroxy-2-butanone 4-phosphate synthase, which catalyzes an initial step of

riboflavin synthesis [49]. In *V. harveyi*, *luxH* is located downstream of *luxG* as the last

gene of the *lux* operon [48], whereas in *V. fischeri* the *lux* operon ends after *luxG* with a

strong bi-directional rho-independent terminator [11]. In accord with the enzymatic

characterization of the protein from *E. coli* and because the *V. fischeri* gene is not part of

the *lux* operon, we designate the *V. fischeri* protein RibB. The deduced *V. fischeri* RibB

sequence is 65% identical to LuxH of *V. harveyi* and 56% and 55% identical to RibB of

Haemophilus influenzae and *E. coli*, respectively (Fig. 5).

The *ribB* gene from *V. fischeri* encodes a 217 amino acid protein of 23,616 Da that

has an estimated pI of 5.26. Amino-terminal sequencing indicates that the protein begins at

the Met residue indicated in Figure 4 and not at a possible alternate Met residue five codons

upstream (Table 2). Like *qsrP*, transcription of *ribB* from *V. fischeri* appears to be

regulated directly by LuxR/3OC$_6$-HSL in a monocistronic fashion; a *lux* box palindromic

element is centered 133 nucleotides upstream of the coding region and a putative stem and

loop rho-independent terminator begins 128 nucleotides after the coding region (Fig. 4).

The *lux* box shares 12 of its nucleotides with the same region from the *lux* operon and 18

```
  1  TCATTAAACT ATTTAACATC AATTACCTTT CAAAAGTGGC CTAACGCCTG CATAACACGT TTGCTACCAT
 71  GCAGATTAAG CTCAAATTTA CCACTTAATA CGGTAAACTC AAAGTAAACC TAGAATGCCG AATGTAGCAA
                                                                      lux box
141  ATCGTGTTGA TGCATTTGTT ATACCTTTTA TATAATTGGC TAAAAGCCTA ACCTGCAATA ATTTACAGTA
                 -35                                -10
211  ATAACATAAC TAAAATTGTC ATAAACTCAT TATCAATCAT TAAATATGAA TAGATAAATG AAATATCTAA
                                                   R.B.
281  TTTCTTAATT TGTCATTAAA TTTTTAATTA ACCATTATAT GGAGTTTGTGGTA ATG AAG TTA AAT
                                                               M   K   L   N     4

346  CAA GAT TCA CTA ACT AGT AAA TTT GGA TCT CCT ATA GAG CGT GTC GAA CAA GCA ATA
     Q   D   S   L   T   S   K   F   G   S   P   I   E   R   V   E   Q   A   I   23

403  AAA TCC GTT CAA CAA GGT AAT GGT GTT TTA TTA CTT GAT GAT GAG TCC CGT GAA AAT
     K   S   V   Q   Q   G   N   G   V   L   L   L   D   D   E   S   R   E   N   42

460  GAG GGA GAC CTT ATA TAT TCA GTA GAT CAC CTA ACT TCA GAA CAA ATG GCT TTA ATG
     E   G   D   L   I   Y   S   V   D   H   L   T   S   E   Q   M   A   L   M   61

517  ATC CGT TCT TGC AGC GGT ATT GTA TGT TTA TGT CTT ACT GAG GAT AAA GCT GAT TAC
     I   R   S   C   S   G   I   V   C   L   C   L   T   E   D   K   A   D   Y   80

574  CTT GAA CTT TCA CCA ATG GTT GAA CAT AAT GAA AGT GTA AAT CAA ACA GCC TTT ACA
     L   E   L   S   P   M   V   E   H   N   E   S   V   N   Q   T   A   F   T   99

631  ATT TCA ATA GAG GCC AAA AAA GGA GTT ACT ACA GGT GTA TCA GCC ACA GAT AGA GTA
     I   S   I   E   A   K   K   G   V   T   T   G   V   S   A   T   D   R   V   118

688  ACA ACG ATT AAA ACG GCT TGT AAA AAT GGC GCC CTT CCA CAT GAA CTT GCA AAA CCT
     T   T   I   K   T   A   C   K   N   G   A   L   P   H   E   L   A   K   P   137

745  GGA CAT GTT TTC CCT TTA AGG GCA AAA GAT GGC GGT GTT TTA ACT AGA CGA GGA CAT
     G   H   V   F   P   L   R   A   K   D   G   G   V   L   T   R   R   G   H   156

802  ACA GAA GGG ACT GTC GAC TTA ATG CGA CTA GCA GAG TTA ACT CCT GCT GGC GTT TTA
     T   E   G   T   V   D   L   M   R   L   A   E   L   T   P   A   G   V   L   175

859  TGC GAA GTA ACC AAC TCA GAT GGT TCA ATG GCT AAA ACC CCT GAA ATA ATT ATT TTT
     C   E   V   T   N   S   D   G   S   M   A   K   T   P   E   I   I   I   F   194

916  GCT GAA CAA CAT CAT TTA CCA GTC TTG ACT GTT AAT GAT ATT GCT TTA TAT CGA AAG
     A   E   Q   H   H   L   P   V   L   T   V   N   D   I   A   L   Y   R   K   213

973  GAA AAG AGC AAC TAAACTTAAT CTAGGGCATC ACTTCAAGAT CGCCTTAGGT ATAACGCCTG
     E   K   S   N   *                                                         217

                          stem and loop terminator
1035 CATAACACGT TTGCTACCAT GTAGATTAAG CTCAAATTTA CCACTTAATA CAGTAAACTC AAAGAAACCT

1105 AGAATGCCAA ATGTAGCAAA TCGTGTTGAT GCATTTGTTA TGCGACCAAT TAATACTAGA TTAATTTTAC
```

Figure 4. Nucleotide sequence and deduced amino acid translation product of the *ribB* gene. Putative *lux* box, -35 and -10 regions of the σ^{70} promoter, and ribosomal binding site are indicated. Stop codon (*) and possible stem and loop terminator are also indicated.

```
V. f. RibB    1   M K L N Q D S L T S K F G S P I E R V E Q A I K S V Q Q G N   30
V. h. LuxH    1     M S S T S L L D E F G T P V Q R V E R A I E A L K N G L     28
E. c. RibB    1     M N Q T L L S S F G - T P F E R V E N A L A A L R E G R     27
H. i. RibB    1     M N Q S I L S P F G N T A E E R V L N A I N A F K N G T     28


V. f. RibB    31  G V L L L D D E S R E N E G D L I Y S V D H L T S E Q M A L   60
V. h. LuxH    29  G V L L M D D E D R E N E G D L I F S A Q H L T E A Q M A L   58
E. c. RibB    28  G V M V L D D E D R E N E G D M I F P A E T M T V E Q M A L   57
H. i. RibB    29  G V L V L D D E D R E N E G D L I F P A E T I T P E Q M A K   58


V. f. RibB    61  M I R S C S G I V C L C L T E D K A D Y L E L S P M V E H N   90
V. h. LuxH    59  M I R E G S G I V C L C L T E E R A N W L D L P P M V K D N   88
E. c. RibB    58  T I R H G S G I V C L C I T E D R R K Q L D L P M M V E N N   87
H. i. RibB    59  L I R Y G S G I V C L C I T D E R C Q Q L D L P P M V E H N   88


V. f. RibB    91  E S V N Q T A F T I S I E A K K G V T T G V S A T D R V T T   120
V. h. LuxH    89  C S K N Q T A F T V S I E A K E G V T T G V S A K D R V T T   118
E. c. RibB    88  T S A Y G T G F T V T I E A A E G V T T G V S A A D R I T T   117
H. i. RibB    89  N S V N K T A F T V T I E A A K G V S T G V S A A D R V T T   118


V. f. RibB    121 I K T A C K N G A L P H E L A K P G H V F P L R A K D G G V   150
V. h. LuxH    119 V K T A T Y F D A Q P E D L A R P G H V F P L V A K T N G V   148
E. c. RibB    118 V R A A I A D G A K P S D L N R P G H V F P L R A Q A G G V   147
H. i. RibB    119 I Q T A I A D N A V L T D L H R P G H V F P L R A A N G G V   148


V. f. RibB    151 L T R R G H T E G T V D L M R L A E L T P A G V L C E V T N   180
V. h. LuxH    149 L A R R G H T E G T I D L M Y L A N L V P S G I L C E L T N   178
E. c. RibB    148 L T R G G H T E A T I D L M T L A G F K P A G V L C E L T N   177
H. i. RibB    149 L T R R G H T E A S V D L A R L A G F K E A G V I C E I T N   178


V. f. RibB    181 S D G S M A K T P E I I I F A E Q H H L P V L T V N D I A L   210
V. h. LuxH    179 P D G T M A K L P E T I E F A R R H G M P V L T I E D I V D   208
E. c. RibB    178 D D G T M A R A P E C I E F A N K H N M A L V T I E D L V A   207
H. i. RibB    179 D D G T M A R A P E I V E F A K K F G Y S V L T I E D L V E   208


V. f. RibB    211 Y R K - - - - - - - E K S N                 217
V. h. LuxH    209 Y R T G I D L R N E Y K S G L V R E V S W S   230
E. c. RibB    208 Y R Q A H E R K A S                         217
H. i. RibB    209 Y R L A H N I                               215
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Figure 5. Alignment of RibB form*V. fischeri* with similar proteins. *V. h.*, *Vibrio harveyi* LuxH [48]; *E. c.*, *Escherichia coli* RibB [49]; *H. i.*, *Haemophilus influenzae* RibB [50]. Conserved residues are boxed. Light shading indicates a region of sequence similarity, and areas of sequence identity are indicated by heavy shading.

of 20 nucleotides from that preceeding *qsrP* (Fig 3). In contrast to the promoter regions for the *lux* operon and *qsrP*, the *lux* box from *ribB* does not replace or overlap the -35 polymerase binding site, but instead is in addition to a putative -35 region. 28 nucleotides downstream of the *lux* box is a reasonable -35 region (TTGTCA) followed by a -10 region (AAATAT) 26 nucleotides further downstream. Consistent with initiation of translation at the Met residue indicated, a likely Shine-Dalgarno ribosomal binding site is positioned 8 bases upstream of the proposed ATG translation initiation codon. No Shine-Dalgarno sequence is present upstream of the alternate translation initiation codon. Thus, like *qsrP*, the *ribB* coding region is bounded by a promoter region that contains a putative binding site for LuxR and a probable trascriptional terminator.

Attempts to isolate portions of the *ribB* gene from *V. fischeri* ESR1 were unsuccessful. The primers used to screen the library for the gene from MJR1 (Materials and Methods; Table 2) and several combinations of primers used in sequencing of the gene all gave negative results when used with ESR1 chromosomal DNA as the template. In contrast, all primer combinations tested were positive with MJR1 DNA (data not shown).

Construction and characterization of a *V. fischeri ribB* mutant. The involvement of the *E. coli* RibB protein in riboflavin synthesis and the utilization of reduced flavin mononucleotide as a substrate in the luminescence reaction suggested that RibB might play a role in light production in *V. fischeri*. To gain insight into this possibility, we constructed a mutant of MJR1 defective in *ribB* by a plasmid integration procedure (Materials and Methods). However, the *ribB* mutant grew normally, produced a high level of light, and induced luminescence in a fashion similar to that of the wild-type. Therefore, RibB apparently is not required for normal light production in *V. fischeri*.

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DISCUSSION

Prior to this report, only the luminescence system (*luxR luxICDABEG*) was known to be regulated by quorum sensing in *V. fischeri*. Here we have demonstrated that *V. fischeri* produces several proteins under quorum sensing control that are distinct from those encoded by the *lux* operon. 2-D PAGE patterns of total cellular proteins from mutants defective in the acyl-HSL synthases, LuxI and AinS, and in the acyl-HSL receptor protein, LuxR, were compared to the proteins made by the parent strain to reveal five additional proteins under quorum regulation. These proteins, like the Lux proteins, were produced at high population density and required both $3OC_6$-HSL and LuxR for their production. The genes, *qsrP* and *ribB*, for two of the QSR proteins were isolated and characterized. The identification of these proteins and genes initiates the description of a $3OC_6$-HSL/LuxR-dependent quorum sensing regulon in *V. fischeri*.

Substantial precedent exists in other bacterial species for multiple functions controlled by quorum sensing. For example, *Pseudomonas aeruginosa*, an opportunistic pathogen of humans, produces several extracellular virulence factors, including proteases, elastases, and exotoxin under the control of a complex hierarchical quorum sensing network (e.g., [51]). Similarly, in the plant pathogen, *Erwinia carotovora*, the antibiotic carbapenem and several extracellular enzymes involved in virulence are subject to quorum sensing control (e.g., [52]). Furthermore, the marine luminous bacterium, *V. harveyi*, regulates both light production and synthesis of poly-β-hydroxybutyrate by quorum sensing [53]. In *V. fischeri*, the quorum sensing regulon is likely to be composed of additional components beyond those identified in this study. Under our conditions of cell growth and 2-D PAGE, we detected only three of the seven proteins encoded by the *lux* operon. Presumably, the other four are produced but were not detected. Our inability to

detect four proteins known to be regulated by quorum sensing suggests that additional proteins whose production is regulated by quorum sensing in *V. fischeri* await discovery.

C_8-HSL has been shown to delay the onset of luminescence, most likely by competitively inhibiting the effect of $3OC_6$-HSL [27]. We report here that C_8-HSL also delays the production of the five newly discovered QSR proteins until late exponential and early stationary phase. Inhibitory processes contribute to quorum regulation in other species of bacteria as well. In *V. harveyi* LuxO acts as a repressor of transcription [54]. In the model for induction of luminescence in *V. harveyi,* two distinct acyl-HSL signals act via a phosphorelay network to inactivate the repressor function of LuxO and potentiate the *lux* operon for transcriptional activation [55]. *Agrobacterium tumefaciens* also employs an inhibitor of quorum regulated genes to insure their transcription only at high population density [56]. Inhibition of transcription at low population density appears to be an emergent theme in quorum regulation.

Because C_8-HSL potentially represents a second acyl-homoserine signalling system with distinct regulatory targets, we had expected to see a difference between the proteins made by MJ-215 grown with and without the exogenous addition of C_8-HSL. Surprisingly, we did not see any affect of C_8-HSL on protein expression with 2-D PAGE analysis. The C-terminal half of AinS, the synthase responsible for production of C_8-HSL, is similar to a putative protein in *V. harveyi,* which is necessary for production of one of the two acyl-HSL signals in that organsim [25]. Downstream of *ainS* is another gene, *ainR,* which shows similarity to *luxN,* a gene similarly located in *V. harveyi.* LuxN is believed to possess both the sensor kinase and response regulator functions for the response to acyl-HSL signal [57]. By analogy, the possible interaction between C_8-HSL and AinR is intriguing [25]. C_8-HSL-regulated proteins may exist in *V. fischeri*, but the conditions necessary for their detection may differ from the conditions used in this study.

The presence of a *lux* box in the promoter regions of *qsrP* and *ribB* is consistent with the requirement for $3OC_6$-HSL and LuxR for production of QsrP and RibB, and it suggests direct transcriptional activation of these genes by $3OC_6$-HSL/LuxR. The *V. fischeri* MJ-1 *lux* operon *lux* box, to which the *qsrP* and *ribB lux* boxes are very similar (Fig. 3), is essential for the $3OC_6$-HSL/LuxR-dependent activation of *lux* operon transcription [15], and the synergistic binding of the C-terminal domain of LuxR and RNA polymerase to the *lux* box in the *lux* operon promoter region has been demonstrated in vitro [20]. Identical or similar sequences have been identified in the *lux* operon promoters of various *V. fischeri* strains, in the *ainSR* promoter region in *V. fischeri* MJ-1, as well as in the promoters controlling expression of *lasB* and *rhlI* in *P. aeruginosa*, *traA* and *traI* of the octopine-type Ti plasmid, and *traI* of the nopaline-type plasmid, respectively, of *Agrobacterium tumefaciens*, *solI* of *Ralstonia solanacearum*, and *cepI* of *Burkholderia cepacia* [25, 46, 58-63]. Thus, both within *V. fischeri* (Fig. 3) and among various Gram-negative bacteria, the *lux* box represents a conserved regulatory sequence. Its presence upstream of a bacterial gene is interpreted as consistent with autoinducer-mediated control of that gene [46]. With respect to $3OC_6$-HSL/LuxR-dependent expression of *qsrP* and *ribB*, the *qsrP* promoter region resembles that of the *lux* operon, with the *lux* box replacing the -35 element of a σ^{70}-type promoter. That arrangement, by analogy with the *lux* operon [16] suggests regulation of *qsrP* solely by quorum sensing. In contrast, the *ribB* promoter region contains a likely -35 region adjacent to the *lux* box, suggesting both quorum and "housekeeping" regulation of *ribB*.

A *qsrP* mutant exhibits a reduced competence phenotype when placed in competition with the parent strain in host colonization assays. The deduced QsrP sequence does not match sequences in the databases, and *qsrP* mutants of *V. fischeri* do not exhibit an obvious phenotype in laboratory culture, so the molecular basis for reduced colonization

by the *qsrP* mutant is unknown at this time. The periplasmic location of QsrP suggests that QsrP may represent a novel nutrient-scavenging pathway or facilitate communication between the bacterium and *E. scolopes* . Consistent with this notion, many of the quorum-regulated genes identified in other species of bacteria mediate interactions between the bacterium and higher organisms [4]. The high level of sequence similarity between RibB from *V. fischeri* and proteins of *V. harveyi, E. coli* and *H. influenzae*, besides permitting a designation for the *V. fischeri* protein, allows a possible function for it to be ascribed. The *E. coli* protein, RibB, plays a key role in riboflavin synthesis, catalyzing the conversion ribulose-5-phosphate to 3,4-dihydroxy-2-butanone 4-phosphate [49]. Because riboflavin is a precursor of flavin mononucleotide (FMN), which in reduced form ($FMNH_2$) is a substrate for the luminescence reaction [64], the *V. fischeri* RibB protein could play a role in the production of light in this species. The *V. fischeri luxG* gene product, based on sequence similarities, is thought to encode a flavin reductase [14]. The coordinate regulation of *ribB* and genes of the *lux* operon by quorum sensing seems logical because fully induced levels of luminescence would presumably require more $FMNH_2$ than necessary for normal cellular processes. Perhaps the dual needs for riboflavin, in luminescence and in other cellular processes, accounts for the structure of the *ribB* promoter, which contains both quorum-response and "housekeeping" elements. The absence of an obvious phenotype for the *ribB* mutant of MJ-1, a brightly luminous strain, and the apparent lack of *ribB* in ES114, however, would appear to argue against this scenario, unless another gene can complement the luminescence related function of *ribB* in its absence. Because growth of the RibB mutant on rich medium that is not supplemented with riboflavin is similar to that of the wild-type, a second RibB-like protein must be present in MJ-1. It should be noted that in two other bioluminescent bacteria, genes for riboflavin synthesis are part of, or are closely linked to, the *lux* operon. In *V. harveyi*, *luxH*, the gene encoding the RibB homolog, is the last gene of the *lux* operon

(*luxCDABEGH*), and in *Photobacterium leiognathi,* the *ribEBHA* genes occur immediately after *luxG* [65]. It seems likely that other genes involved in riboflavin synthesis may be members of the quorum sensing regulon in *V. fischeri,* whether or not they are closely linked to the *lux* operon.

Quorum regulation of luminescence in *V. fischeri* serves as a model, aiding the identification and characterization of quorum sensing systems in other Gram-negative bacteria. From the information presented in this study, it is now apparent that luminescence is just one of several activities in *V. fischeri* subject to quorum regulation by acyl-HSLs and LuxR. On-going studies to characterize the genes encoding QSR 6, 7, and 8, and to identify other genes regulated by $3OC_6$-HSL and LuxR, will further define the quorum sensing regulon of this bacterium.

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Chapter 4. Quorum regulation controls *acfA* and *qsrV*, which comprise a two gene operon that may encode a pilus protein.

ABSTRACT

In the previous chapter the production of five proteins was shown to be regulated by LuxR and $3OC_6$-HSL in a cell-density dependent manner. The genes for two of these QSR proteins were isolated and characterized at the sequence level. In this section the genes for two additional proteins, QSR 6 and QSR 8, are examined. QSR 6 is a homolog of AcfA (accessory colonization factor A) and OmpW (outer membrane protein W) from *Vibrio cholerae*. Based on recent evidence that OmpW is a pilus subunit, we suspect that QSR 6 is part of a pilus made by *Vibrio fischeri*. A leader peptide and a sequence motif at the C-terminus indicative of outer membrane proteins is consistent with it being a pilin. The mature 193 amino acid polypeptide has a calculated molecular weight of 21,548 Da and a predicted isoelectric point (pI) of 4.0, which are in agreement with its position on 2-D PAGE gels from Chapter 3. Based on sequence similarity, we have designated QSR 6 to be AcfA from *V. fischeri*. The gene that encodes QSR 8 follows *acfA* and appears to be part of the same operon. Like AcfA, the predicted QSR 8 polypeptide has a leader peptide at the N-terminus that is absent from the mature protein. The mature 101 amino acid protein has a calculated molecular weight of 10,826 and a pI of 4.1. The predicted pI agrees with its position on 2-D PAGE gels, but the molecular weight is less than expected. QSR 8 has been redesignated QsrV, pending a determination of its function in the cell. Preceding *acfA*, a characteristic *lux* box replaces a -35 σ^{70} polymerase recognition site, suggesting that the operon is regulated at the transcription level by LuxR. An insertion into the *acfA* gene designed to disrupt production of the corresponding protein conferred an

altered colonization phenotype to the mutant in a competition assay with the parent strain.

We are currently examining these mutants for altered pilus production.

INTRODUCTION

Many species of pathogenic bacteria produce adhesins that allow them to bind to specific receptors that are present on host cells. Recognition of host structures is highly tuned and is believed to contribute to the specificity of pathogen-host interactions [1]. Adhesins in bacteria often take the form of pili, hair-like structures that protrude from the outer membrane much like flagella but lacking the rotational machinery. Several classes of pili have been established based on phenotypic traits and, more recently, on sequence similarities between the major and minor protein subunits that comprise the various pili [2]. In some cases the adhesin properties of the pilus are attributed to the major subunit, which serves as the building block for the structure, and in others a minor pilin subunit is involved. In *Escherichia coli* the elaboration of pili is often regulated as part of a global network that responds to environmental factors. For instance, the production of pili has been shown to be influenced by carbon source via CRP, aliphatic amino acids via Lrp, iron concentration via Fut, and respiratory status via Fnr [2].

In the previous chapter we showed that, in addition to the *lux* operon, LuxR and $30C_6$-HSL regulate the production of five additional proteins. The genes for two of these proteins, *qsrP* and *ribB*, were characterized and shown to have sequences in their promoter region that were similar to the consensus *lux* box sequence. Apparently both are regulated directly by the quorum sensing system. QSR protein 7 is approximately 15 kD, has a relatively neutral isoelectric point, and its N-terminus was found to be blocked during a sequencing attempt. The remaining two proteins, QSR 6 and 8, are approximately 22 and 14 kD proteins, respectively, and appear to have acidic isoelectric points. Here we describe the isolation and characterization of the genes for QSR proteins 6 and 8. QSR protein 6 is similar in sequence to a pilin subunit from *Vibrio cholerae* and may be the major subunit of

a pilus elaborated by *V. fischeri* in response to high population density.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are described in Table 1. *E. coli* Novablue and SM10-λ*pir* were grown on LB medium [3] at 37°C with antibiotics as appropriate (ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml). *Vibrio fischeri* MJR1 is a derivative of MJ-1 [4] that contains a spontaneous mutation that confers resistance to rifampicin. Strains of *V. fischeri* were maintained on LBS agar [5] with the appropriate antibiotics (chloramphenicol, 3 μg/ml for resistance conferred by a chromosomal insertion and 15 μg/ml for plasmid encoded resistance; naladixic acid, 20 μg/ml; neomycin, 200 μg/ml; and rifampicin, 100 μg/ml).

Isolation of the DNA encoding *acfA* and *qsrV*. Samples of QSR protein 6 were isolated by excision from 2-D PAGE gels of MJ-100 grown to stationary phase and sequenced internally as described for QsrP and RibB in the Materials and Methods section of Chapter 3. The two peptide sequences, 6-PK80 and 6-PK122 (Table 2), were used to design degenerate PCR primers for isolation of DNA in the region of the genome encoding the protein. Because the relative positions of the sequenced peptides in the protein were unknown, both forward and reverse primers were designed from each peptide sequence. For QSR protein 6, degenerate primers corresponding to regions of peptides 6-PK80 and 6-PK122 (6-80FI, 6-80RI, 6-122FI, and 6-122RI; Table 2) were combined as appropriate in two separate PCR reactions using MJ-100 chromosomal DNA as the template. From the reaction using 6-122FI and 6-80RI as primers, a single product of 152 bp was obtained.

Attempts to use the 152 bp portion as a probe to isolate the complete gene for protein #10 from a plasmid-borne genomic library of MJ-100 DNA [6] were unsuccessful, possibly because of under representation of this DNA region in the library. As an

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
Novablue	*endA hsdR17*($r_{K12}^- m_{K12}^+$) *supE thi recA gyrA relA* *lac*[F' *proA*$^+$*B*$^+$ *lacI*^{q}*ZΔM15*::Tn*10*(Tc^r)]	Novagen
SM10-λ*pir*	*thi thr leu supE tonA lacY* *recA*::RP4-2-Tc::Mu Km^r λ::*pir*	[7]
V. fischeri		
ES114	Wild type	[8]
ESR1	ES114, Rf^r	[9]
MJR1	MJ-1, Rf^r	Chapter 3
MJ-100	MJ-1, Nx^r	[10]
MJ-215	MJ-211, *ainS* (2-bp insertion)	[11]
MJacfX	MJR1 *acfA*::pSMC300	This study
ESacfX	ESR1 *acfA*::pSMC300	This study
Plasmids		
pT7Blue	Cloning vector, Ap^r	Novagen
pSMC300	Suicide vector, Cm^r	Chapter 3
p300-525	pSMC300 with a 525 bp fragment of *acfA*	This study
p5'acfA	pSMC300 with 15 kb of DNA upstream of *acfA*	This study
p3'acfA	pSMC300 with 2 kb of DNA downstream of *acfA*	This study
pAcfGM	pSUP102 *acfA*::GFP (*acfA* from MJR1)	This study
pAcfGE	pSUP102 *acfA*::GFP (*acfA* from ESR1)	This study
pAcfXMJ	pSMC300 with 258 internal *acfA* fragment of MJR1	This study
pAcfXES	pSMC300 with 258 internal *acfA* fragment of ESR1	This study

TABLE 2. Internal peptide sequences and oligonucleotides

Internal peptide fragments	
6-PK80	FDSSANTEYEN-L
6-PK122	ERE[C]DADLDATQW[T][F]K
Oligonucleotides	
6-80FI*	GAYWSIWSIGCIAAYACIGARTAYGA
6-80RI*	TCTTAYTCIGTRTTIGCISWISWRTC
6-122FI*	GARMGIGARTGYGAYGCIGAYYTIGA
6-122RI*	TCIARRTCIGCRTCRCAYTCICKYTC
6-outF	CAGGTTTAAAGGTAAATTGAGTTGC
6-outR	CAATTAAAAACAGGCTTAGGTTTGAC
6-525Fsph	CCCGCATGCGGTTTAAAGGTAAATTGAGTTG
6-525Rsac	CCCGAGCTCTAAAAACAGGCTTAGGTTTGAC
Acf-pH3	TTTAAGCTTGGCTAATTGCTGGTTGAGAG
Acf-pK	AAAGGTACCGTTAGGCCATATTCTAAACC
acfXFxba	CCCGCTAGACTCCTGATGATTCTAGTTCTGC
acfXRsph2	CCCGCATGCTAGTATAAGTCAAACCTAAGC
R6Kr	GGCTTTTAAAGCTTTTAAGGTTTAACGG
MD29S-3	TTCTTGTGATGCATTTAATACTGACG

* For degenerate primers: I=inosine; K=T, G; M=A, C; R=A, G; S=C, G; W=A, T; Y=T, C. Brackets around an amino acid denote a low confidence designation.

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alternative, we used PCR to isolate a 525 bp fragment that included DNA flanking the original 122 bp fragment. To facilitate PCR amplification of the DNA flanking the 152 bp fragment, MJ-100 chromosomal DNA was digested to completion with *Sau*3A, the four bp recognition sequence of which is not present in the fragment. The chromosomal fragments were then circularized by ligation, and primers 6-outF and 6-outR (Table 2) were used to amplify the regions flanking the 152 bp fragment, which were joined at the *Sau*3A sites, making them contiguous on the circular molecule. The resulting 492 bp fragment was cloned and sequenced. From the sequences adjacent to the *Sau*3A site, primers 6-525Fsph and 6-525Rsac were designed and used to PCR amplify the predicted 525 bp fragment. Cloning of the fragment into the *Sph*I and *Sac*I sites of plasmid pT7Blue (Table 1) and subsequent sequencing verified it's authenticity.

The DNA encoding QSR protein 6 and flanking DNA was isolated by inserting the suicide plasmid pSMC300 (Chapter 3, Materials and Methods) into the chromosome of MJ-100 and reisolating the vector and flanking DNA in *E. coli*. Homologous recombination between the vector and QSR 6 DNA was driven by the 525 bp PCR product described above, which was cloned into pSMC300 to yield p300-525. After mobilization of the vector into *V. fischeri* strain MJR1 by conjugation from *E. coli* strain SM10-λ*pir*, transconjugants were selected for resistance to rifampicin and chloramphenicol. Chromosomal DNA from one transconjugant was digested with *Sph*I and *Sac*I in separate reactions, recircularized by ligation, and used to transform *E. coli* strain DH5α- λ*pir*. p5'acfA, which resulted from the *Sph*I digest/ligation, consisted of pSMC300 and an approximately 15 kb insert that represents the original 525 bp insert plus upstream DNA with respect to the QSR 6 coding region. p3'acfA, which resulted from the *Sac*I digest/ligation, contained approximately 2 kb of DNA downstream of the 525 bp insert in addition to the vector and PCR product. Primers 6-outF and 6-outR were used to begin

sequencing of insert DNA from p3'acfA and p5'acfA, respectively, and a primer walk strategy was used thereafter.

Construction of translational fusions between AcfA and GFP. For the fusion of AcfA with GFP, primers AcfA-pH3 and AcfA-pK were used to PCR amplify the promoter regions and the first 28 amino acids coded by *acfA* in MJ-100 and ESR1. These fragments were ligated into pSupGFP (Chapter 3, Materials and Methods) to yield pAcfGM and pAcfGE, respectively. Each vector contains the first 28 amino acids of AcfA fused in frame with the GFP initiation codon. The vector pAcfGM was transferred to MJ-100 and MJ-215, and pAcfGE was transferred to ESR1 by conjugation from *E. coli* strain SM10-λ*pir*.

Construction of *acfA/qsrV* mutants. A polar mutation was created in the *acfA* gene using the same plasmid integration procedure described for creation of the *qsrP* mutation in Chapter 3. Using MJR1 and ESR1 chromosomal DNA as template in separate reactions, primers acfXFxba and acfxRsph2 were used to PCR amplify the region corresponding to nucleotides 381-638 in Figure 1. The resulting 258 bp fragments were cloned into the *Xba*I and *Sph*I sites of pSMC300 to yield plasmids pAcfXMJ and pAcfXES. The two plasmids were mobilized into *V. fischeri* strains MJR1 and ESR1 by conjugation from *E. coli* strain SM10λ-*pir*, and transconjugants were selected for growth on rifampicin and chloramphenicol. The resulting strains were designated MJacfX and ESacfX. Correct insertion of the vector into *acfA* was verified by PCR using primer MD29s-3, which corresponds to a region 558 bp upstream of the *acfA* coding region (See Chapter 5, *qsrR*), and primer R6Kr, which corresponds to sequence in the origin of pSMC300 (See Chapter 3, Materials and Methods).

PCR amplification and sequencing of DNA. PCR amplifications were performed in an Idaho Technology's Rapidcycler (Idaho Falls, ID) using 50 µl glass

capillary tubes. The oligonucleotides used as primers are described in Table 2. Standard conditions were used except for the initial reactions with degenerate primers. In this case, conditions included 4 mM $MgCl_2$ in the buffer and 30 cycles were performed with denaturation at 94°C for 2 s, annealing at 40°C for 10 s, and elongation at 50°C for 10 s. PCR products were purified from agarose gels with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into pT7-Blue using the Perfectly Blunt cloning kit (Novagen, Madison, WI). DNA sequences were determined by the dideoxy-nucleotide chain termination method of Sanger et al. [12] using dye terminator labeling at the CRC DNA Sequencing Facility, University of Chicago.

RESULTS

QSR protein 6 is a homolog of AcfA and OmpW from *Vibrio*

cholerae. After having isolated and characterized the genes that coded for the quorum-regulated proteins QsrP and RibB in Chapter 3, we decided to focus our attention on QSR protein 6 and isolate the corresponding gene. To isolate the gene, we first used PCR to obtain a portion of the coding region, and then recovered DNA flanking a suicide plasmid that had been introduced into the chromosome by homologous recombination (Materials and Methods). Each of the reisolated suicide plasmids contained two-thirds of the gene for Qsr 6 and corresponding upstream or downstream flanking DNA. The nucleotide sequence and deduced amino acid sequence for QSR 6 are shown in Figure 1. The 211 amino acid precursor protein has a molecular mass of 23,549 Da and a predicted pI of 4.2. The N-terminus of the mature protein was shown in Chapter 3 to start with the sequence APYVGL indicating that the first 18 amino acids constitute a leader peptide that is absent from the mature protein. These first 18 amino acids have a sequence typical of a prokaryotic leader peptide [13]. The mature 193 amino acid protein has a predicted molecular mass of 21,548 Da and a predicted pI of 4.0. The size of the translated amino acid sequence is in good agreement with the molecular mass estimate of 22 kDa made in Chapter 3.

The amino acid sequence of QSR 6 was used to look for similar sequences in the NCBI database. Substantial sequence identity (32% and 19%, respectively) and similarity (53% and 31%, respectively) was observed between QSR 6 and two proteins from *V. cholerae*, AcfA and OmpW (Figure 2). Based on similar mutant virulence phenotypes, AcfA, AcfB, AcfC, and AcfD have been proposed to work together to produce an accessory colonization factor in *V. cholerae* [14]. It was also speculated that the *acf* locus encodes a new pilus or a nutrient-scavenging pathway in *V. cholerae*. Along those lines, it was recently reported that the N-terminal 20 amino acids of a 20 kDa pilus protein

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  1 TATCACGTAAGACCAAGATTAAATGTATTACTATCGTATTGAATTAGTAAAGAAAATAACCTTTATCCCA
          lux box                                     -10
 71 TTATGCCCTGTAAGTTATTACAGCTGTAAAAGTATTCGGAATGAATGTAATTATGAGCTGTAAATTTATA

141 TGAAAGTGCTCTTCACACTTTCTTCACGATCTCTTCTTTATAGTAGCGACATTAACGAATTAACACCAAG

                   R.B.              M  K  K  Y  L  F  L  L  M  A  A  T  S    13
211 CTCAACATCATAACTATAGGATTGAATTACAATGAAAAAGTATTTATTTTTGCTAATGGCTGCCACTTCA
                       ↓
     T  A  T  I  A  A  P  Y  V  G  L  E  Y  G  L  T  N  M  S  H  D  Y  S    36
281 ACAGCAACAATTGCTGCCCCATATGTAGGTTTAGAATATGGCCTAACAAACATGAGTCACGATTACTCAA

     T  T  F  S  N  D  N  V  S  L  T  P  D  D  S  S  S  A  F  G  G  F  V  G  60
351 CGACTTTTTCAAATGACAATGTGTCATTAACTCCTGATGATTCTAGTTCTGCTTTTGGTGGTTTTGTTGG

     Y  R  F  N  E  F  G  L  E  F  G  Y  K  K  F  E  S  D  D  S  R  S  Q    83
421 CTATCGTTTTAATGAATTTGGCCTTGAGTTCGGCTACAAAAAATTTGAGAGTGATGATTCTCGTAGTCAA

     M  L  V  S  D  K  P  G  Y  T  K  E  R  E  W  D  A  D  L  D  A  T  Q   106
491 ATGCTAGTGTCTGATAAACCTGGTTATACTAAAGAGCGTGAGTGGGATGCTGATTTAGATGCAACTCAAT

     F  T  F  K  P  V  Y  F  Y  N  I  N  E  K  V  Q  L  K  T  G  L  G  L  T 130
561 TTACCTTTAAACCTGTGTATTTTTATAACATCAATGAAAAAGTACAATTAAAAACAGGCTTAGGTTTGAC

     Y  T  Q  Y  K  F  D  S  S  A  N  T  E  Y  E  N  D  L  T  D  H  E  F   153
631 TTATACTCAATATAAATTTGATTCATCTGCGAATACTGAATATGAAAATGACCTAACAGATCATGAATTC

     T  Q  D  Y  V  A  G  D  S  K  S  E  S  V  F  G  G  I  A  S  V  G  I   176
701 ACACAAGATTATGTTGCTGGTGATTCAAAATCTGAAAGCGTATTCGGCGGTATTGCAAGCGTAGGTATTG

     E  Y  M  V  I  P  N  L  A  L  G  A  S  A  S  Y  Q  V  D  S  I  A  N  S 200
771 AATACATGGTAATTCCTAACCTTGCTTTAGGTGCATCAGCAAGCTACCAAGTAGATAGTATTGCTAATTC

     T  S  F  M  L  S  S  A  Y  Y  F  *                                     211
841 TACTTCGTTCATGTTGAGTTCAGCTTACTACTTTTAATATAGCTCCATCGAATAGTTAAAGATTACAAAT

                   R.B.              M  K  K  I  V  L  V  S  A  L  V  F  A  S  S  S  16
911 CTAAGTTAATTGGACATAATAAAATGAAAAAAATCGTATTAGTTAGTGCATTAGTTTTCGCTTCTTCAAG
                       ↓
     V  F  A  A  Q  N  A  P  V  K  G  G  F  T  G  P  S  S  V  S  V  S  T    39
981 TGTTTTTGCTGCTCAGAACGCACCAGTTAAAGGTGGTTTTACTGGCCCTAGTTCAGTTTCTGTTAGTACG

     V  R  A  A  L  D  S  D  D  D  A  A  V  V  L  T  G  Y  I  T  S  S  L    62
1051 GTTCGAGCTGCACTTGATTCTGATGATGATGCAGCGGTTGTATTAACTGGCTACATTACGTCTTCTTTAG

     G  N  E  E  Y  Q  F  K  D  A  T  G  E  V  R  I  E  I  D  N  K  D  W  N  86
1121 GTAATGAAGAATACCAATTTAAAGATGCGACGGGTGAAGTGAGAATCGAAATTGATAATAAAGATTGGAA

     G  I  E  A  T  P  E  T  K  L  V  I  H  G  E  V  D  K  E  W  T  E  T   109
1191 CGGTATTGAAGCTACACCAGAAACGAAATTAGTTATTCATGGTGAAGTAGATAAAGAGTGGACTGAGACA

     T  I  D  V  N  T  V  Q  L  A  K  *                    Stem and        120
1261 ACAATTGACGTAAATACAGTTCAATTAGCTAAATAATTACTCGTTATTTTACTTATAAAAGCCAGAGTTT>

     loop terminator
1331 TTCTACTCTGGCTTTTTTGTTTTTATTTTATGCGCTGCTTAACGGCATCAATTTTTTTAGAGTCCGTCAC
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Figure 1. Nucleotide sequence and deduced translation products of the *acfA* (upper) and *qsrV* (lower) genes. Putative *lux* box, -10 Pribnow box, stem and loop transcriptional terminator, and ribosomal binding sites are indicated. Arrows indicate the end of the signal sequence and the beginning of the mature protein for each peptide

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V. f. AcfA     1  M K K Y L F L L M A A T S T A T I A A P Y V G L E Y G L T N   30
V. c. AcfA     1  M Q K T L S A I F L F T T L S A N A A P Y I G L E L G I G T   30
V. c. OmpW     1  M K Q T I C L A V L A A L L A A P V F A H Q E G D F I V R A   30

V. f. AcfA    31  M S H D Y S T T F S N D N V T L T P - - - - D D S S A F G   56
V. c. AcfA    31  A N H S F E T N Y Q S D A V S L N P - - - - N M E D M F L G   56
V. c. OmpW    31  G I A S V V P N D S S D K V L N T Q S E L A V N S N T H L G   60

V. f. AcfA    57  G F V G Y R F N - E F G L E F G Y K K F E S D D S R S Q M L   85
V. c. AcfA    57  G L I G Y K F N D N F S F E I N Y S S Y K L E D Q Y S K F I   86
V. c. OmpW    61  L T L G Y M F T D N I S F E V L A R T P F S H K I S T S G G   90

V. f. AcfA    86  V S D K P G - - - Y T K E R E W D A D L D A T Q F T F K P V   112
V. c. AcfA    87  G I E S L N N Q Q Y R K E H E W S S E I E A K Q L A L I S V   116
V. c. OmpW    91  E L G S L G - - - - - - - - - D I G E T K H L P P T F M V Q Y   112

V. f. AcfA   113  Y F Y N I N E K V Q L K T G L G L T Y T Q Y K F D S A N T   142
V. c. AcfA   117  Y S Y P I H Q Q L K T N I K V G V T H T Q Y E A Y S G K Y E   146
V. c. OmpW   113  Y F G E A N S T N R P Y V G A G L N Y T T F F D E S F N S T   142

V. f. AcfA   143  E Y E N D L T D H E F T Q D Y V A G D S K - - - - - - S E S   166
V. c. AcfA   147  E L E L L L N D D I E I R K A L L E T G L - - - - - K K N   170
V. c. OmpW   143  G T N N A L S D L K L D D S W G L A A N V G F D Y M L N D S   172

V. f. AcfA   167  V F G G I A S V G I E Y M V I P N L A L G A S A S Y Q V D S   196
V. c. AcfA   171  R F G A L F S L G L D Y N L I P E L S V G T E L R Y Q F D K   200
V. c. OmpW   173  W F L N A Y V W Y A N I E T T A T Y K A G A D A K - S T D V   201

V. f. AcfA   197  I A N S T S F M L S S A Y Y F   211
V. c. AcfA   201  Y N D V L S I S L N S K Y Y F   215
V. c. OmpW   202  E I N P W V F I I A G G Y K F   216
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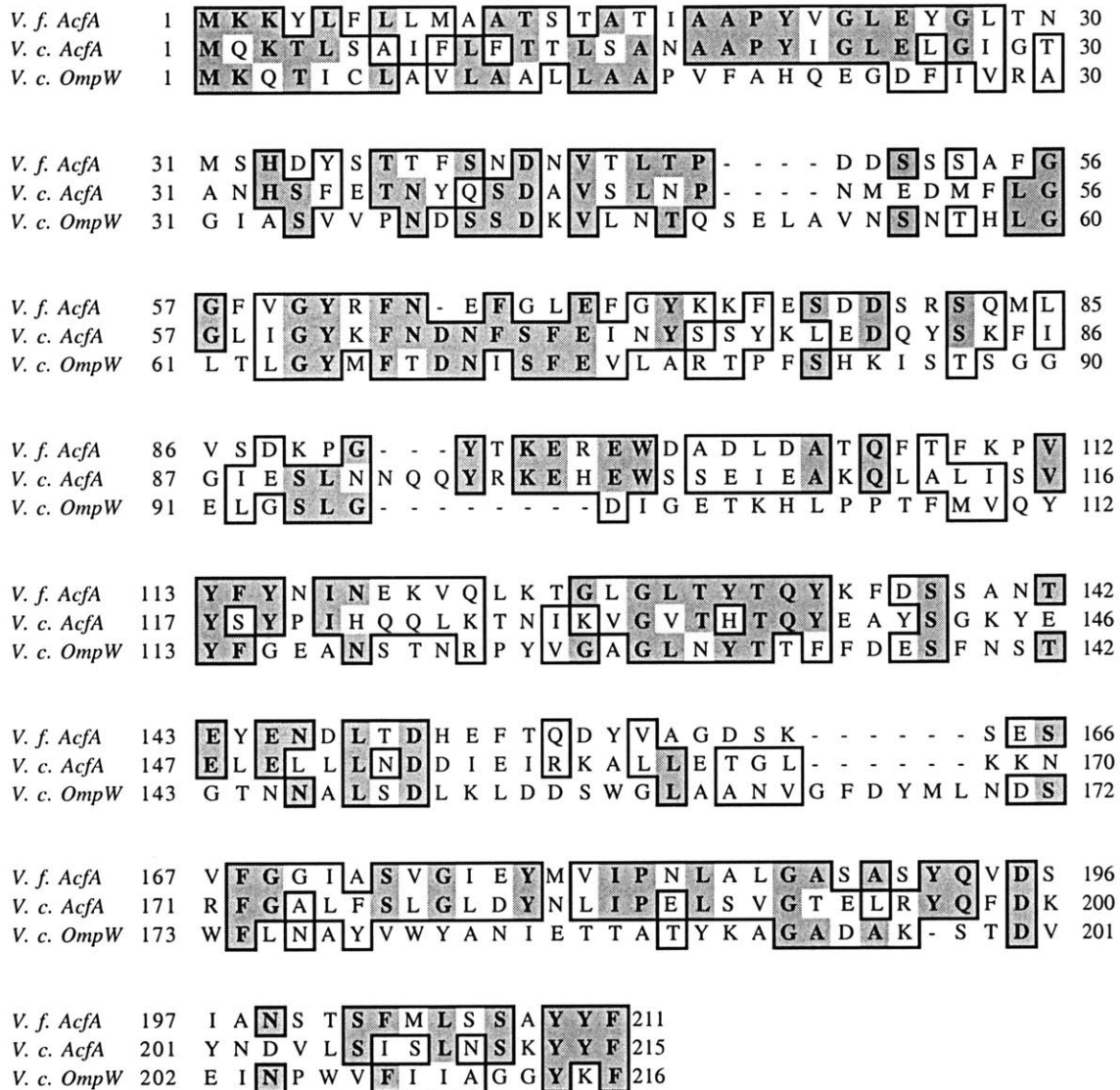
Figure 2. Sequence alignment of AcfA from *V. fischeri* with AcfA [15] and OmpW [16] from *V. cholerae*. Identities are boxed and shaded dark, and similarities are boxed and shaded light.

from *V. cholerae* matched those of OmpW [17]. All three similar sequences possess a YXF motif at the C-terminus, which is indicative of outer membrane proteins [15, 18]. Based on amino acid sequence similarity, we have designated QSR protein 6 to be AcfA from *V. fischeri*.

The genes for AcfA and QSR 8 appear to be part of a two-gene operon. 57 nucleotides downstream from the translation stop codon of AcfA is another open reading frame that codes for a 120-amino acid protein with a calculated molecular mass of 12,805 Da and a predicted pI of 4.3. Amino acids 20-30 of this second translated sequence are AQNAPVKGGFT which closely resembles the N-terminus of QSR protein 8 which was determined in Chapter 3. From N-terminal sequencing, the first amino acid for QSR 8 could not be determined, and a low confidence designation of D instead of Q was made for the second amino acid. We assume that the difference in sequences is due to an error in the microsequence results and that this second open reading frame, designated *qsrV*, encodes the protein referred to as QSR 8 on 2-D PAGE gels. The mature 101-amino acid QsrV protein has a molecular mass of 10,826 Da and an estimated pI of 4.1. This calculated molecular mass differs substantially from the estimate of 14 kD based on mobility on 2-D PAGE in Chapter 3. QsrV is similar in sequence to two hypothetical proteins from *E. coli* and *Haemophilus influenzae* (Figure 3). Both of these hypothetical proteins were discovered in the course of genome sequencing projects. The function of neither protein has been described, so we are unable to determine by inference the function of QsrV.

Characteristics of the *acfA/qsrV* operon. *acfA* and *qsrV* appear to be cotranscribed as part of a two-gene operon that is regulated directly by LuxR and $3OC_6$-HSL. Centered 156 nucleotides upstream of the *acfA* coding region is a putative binding site for LuxR that shares 13 nucleotides with the 19 bp consensus *lux* box described in Chapter 3 (Figure 1). In the *lux* operon promoter, the *lux* box replaces the -35 region.
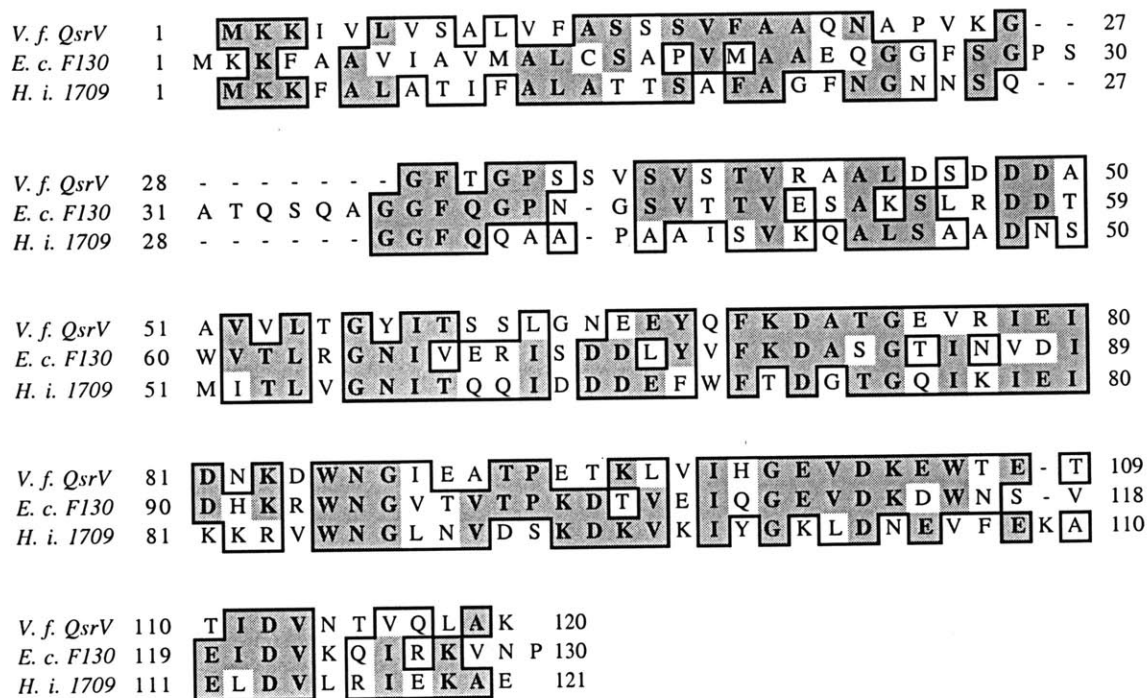
Figure 3. Sequence alignment of QsrV from *V. fischeri* with F130 from *E. coli* [19] and HI1709 from *H. influenzae* [20]. Identities are boxed and shaded dark, and similarities are boxed and shaded light.

With that positioning as a guide, a reasonable -10 Pribnow box can be discerned 31

nucleotides downstream from the center of the *acfA lux* box. Putative Shine-Dalgarno

ribosomal binding sites are indicated in Figure 1 for *acfA* and *qsrV*. Beginning 21

nucleotides downstream of the qsrV translation termination codon there is a putative stem

and loop transcriptional terminator. It consists of a perfect 12 bp stem and a 6 bp loop

followed by a poly-T region. Thus, the two-gene operon is bound on one side by a

promoter that is similar to that of the *lux* operon and *qsrP*, and on the other side by a strong

putative terminator of transcription.

AcfA translational fusions with GFP. To examine the production of AcfA

during growth of ESR1 in the light organ of *E. scolopes*, we created a plasmid-borne

translational fusion between AcfA and the Green Fluorescent Protein of the jellyfish

Aequorea victoria (Materials and Methods). Plasmids pAcfGM and pAcfGE contain the

promoter regions and the coding regions for the first 28 amino acids of *acfA* from MJ-100

and ESR1, respectively, joined in frame with the initiation codon for GFP. To test if the

fusion construct could be used as a reporter of AcfA production in cells grown in

laboratory culture, pAcfGM was mobilized into MJ-100 and MJ-215 by conjugation.

Transconjugants of MJ-100 grown on solid LBS were green when examined with ultra

violet light at a wavelength of 365 nm (Data not shown). Transconjugants of MJ-215, on

the other hand, were similar to the same strain without the fusion construct. The difference

in GFP production between the two strains demonstrated the efficacy of this approach for

monitoring production of AcfA in *V. fischeri*.

As expected, ESR1 containing pAcfGE grown on solid LBS shows no signs of

GFP when examined with ultra violet light. The apparent absence of AcfA production by

ESR1 is consistent with the greatly reduced production of $3OC_6$-HSL by this strain in

Table 3. In vitro and in vivo competition assays between ESR1 and ESacfX

In vitro, replicate	Ratio ESR1:mutant
1	26:24 = 1.08
2	28:22 = 1.27
3	24:26 = 0.92
average in vitro ratio = 1.09	

In vivo, squid	Ratio ESR1:mutant
1	0:192 = 0
2	0:192 = 0
3	53:139 = 0.38
4	46:146 = 0.32
5	0:192 = 0
average in vivo ratio = 0.14	

laboratory culture. We are currently examining ESR1 cells carrying the reporter construct in the light organ of *E. scolopes* to monitor production of AcfA during the symbiosis.

Mutational analysis of the *acfA/qsrV* operon. Mutants defective in *acfA* were constructed in both MJR1 and ESR1 by a plasmid integration procedure to yield MJacfX and ESacfX, respectively, which appeared to be unaffected by the mutation. Because of the similarity in sequence between *acfA* and *ompW*, which is believed to encode a pilus subunit, we are in the process of examining MJacfX grown in laboratory culture for altered pilus formation. To determine if QsrP is involved in the symbiotic relationship between *V. fischeri* and its invertebrate host *E. scolopes*, ESacfX was used both alone and in competition with the parent strain in squid colonization assays as

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described for *qsrP* in Chapter 3. By itself, the ESacfX colonized the squid light organ to levels equivalent to those of the parent strain, ESR1. However, when both strains were used to coinoculate juveniles at a 1:1 ratio in an in vivo competition assay, ESR1 represented a small fraction of the cells recovered from the light organ 48 h postinoculation (Table 3). The colonization ratio of ESR1 to ESacfX was 0.14. A similar competition in laboratory media between the two strains yielded an in vitro ratio of 1.09, which suggests that the competitive advantage of the mutant over the parent strain is specific to conditions in the host light organ. The *acfA/qsrV* locus is not necessary for the symbiosis, and the mutant strain appears to be enhanced in its ability to colonize *E. scolopes*.

DISCUSSION

In this report we further define the quorum-sensing regulon of *V. fischeri*. The genes for two of the proteins identified by 2-D PAGE, *acfA* and *qsrV* (coding for proteins previously denoted as QSR 6 and QSR 8), were isolated and shown to be arranged in a manner that suggests they are cotranscribed as a two gene operon. A *lux* box sequence in the promoter region of the operon is consistent with the requirement for $30C_6$-HSL and LuxR for production of QSR 6 and QSR 8 and suggests that regulation of transcription is mediated directly by the quorum-sensing transcriptional activator complex. Like the promoter regions of *qsrP* and the *lux* operon, the *lux* box replaces a typical -35 σ^{70} polymerase recognition site. A probable rho-independent stem and loop terminator of transcription closely followed the stop codon of *qsrV*.

DNA sequence similarity between OmpW and AcfA from *V. fischeri* and *V. cholerae* suggests that the AcfA proteins from both species are pilins. Ghose and colleagues have shown that the N-terminal 20 amino acids of a 20-kD pilus protein isolated from a non-01 clinical isolate of *V. cholerae* are identical to the N-terminus of OmpW [17], an immunogenic outer membrane protein from the same species [16]. Antiserum raised against the 20-kD protein recognized the hair-like pilus structures, and inhibition by the antisera suggested that the 20-kD protein has haemagglutination and intestinal adherence properties [21]. Colonization efficiency in a mouse intestinal colonization system was also inhibited by pretreatment of the bacteria with the antisera prior to challenge. This 20 kDa pilin is antigenically distinct from the well-characterized toxin-coregulated pilus that is composed of a major protein subunit, TcpA, of approximately the same size.

In Chapter 3 AcfA was localized to the cytoplasmic membrane fraction of the cell. The designation was based on solubility in *N*-lauroylsarcosine, which solubilizes the inner,

but not the outer membranes of bacterial cells. This method is applicable for differentiating integral membrane proteins, but may not apply for proteins that are attached peripherally to the membrane, such as pilus subunits. Fractionation with the detergent-soluble inner membrane may be an artifact of the solubilization of pilus subunits by N-lauroylsarcosine.

In *Vibrio cholerae* the ACF cluster (*acfA-D*) is encoded on a chromosomal pathogenicity island (PAI) that is present in epidemic and pandemic strains, but absent from nonpathogenic strains [22]. The 39.5-kb PAI is believed to be of bacteriophage origin, and as such has a low %G+C (35%) compared to that of the entire chromosome of *V. cholerae* (47-49%). The finding that *V. fischeri* has a homolog of the *acfA* gene led us to speculate that *acfA* from *V. fischeri* might also be encoded on a region of the chromosome analogous to the *V. cholerae* pathogenicity island. The %G+C of the *acfA/qsrV* operon is similar to the PAI of *V. cholerae*, but because the %G+C content of *V. fischeri* (approx. 39% [23]) is similar to that of the *V. choerae* PAI, %G+C could not be used as an indicator of a PAI in this case.

Cotranscription of *acfA* and *qsrV* suggests that QsrV might also be involved in pilus biogenesis. For QsrV there is an obvious discrepancy between the molecular weight estimation made in Chapter 3 by mobility on SDS-PAGE gels and that of the calculated translation product. QsrV appears to be larger than QsrP on the 2-D PAGE gels; the estimated molecular mass of QsrV was 14 kD compared to 12 kD for QsrP. Yet, the calculated molecular mass of the mature protein for QsrP is 12,660 Da, and that of QsrV is 10,826 Da, nearly 2 kD less than QsrP. Estimates of size by SDS-PAGE are only approximations and properties other than size are sure to influence migration through the gel matrix, but the discrepancy in this case is almost 40% of the size of the protein. The leader sequence of QsrV suggests that it is an exported protein, but unfortunately, cell fractionation experiments in Chapter 3 failed to localize the protein. Post-translational modifications of pilus proteins have been shown to decrease their mobility in SDS-PAGE.

Loss of the digalactosyl portion of a trisaccharide pilin modification in *Neisseria meningitidus* resulted in a 1.5-2.0 kD change in the molecular mass estimation by SDS-PAGE [24]. For this reason, we suspect that QsrV may contain a covalently-linked substituent not predicted by the gene sequence.

Demonstration of quorum-sensing regulation of a putative pilus subunit protein adds population density to the many environmental factors that are already known to influence pilus production via global regulatory networks in other bacteria. In *E. coli*, pilus production is influenced by carbon source via CRP, aliphatic amino acids via Lrp, iron concentration via Fut, and respiratory status via Fnr [2]. Elaboration of the toxin-coregulated pilus by *V. cholerae* is regulated directly by ToxT, which in turn is regulated by ToxR in response to pH and oxygen tension [14, 25]. In *Pseudomonas aeruginosa*, it was recently demonstrated that two autoinducer signals play a role in maintaining type 4 pilus function, and that one of the two signals is necessary for assembly of pilin into functional pili, but not for the synthesis of pilin subunits [26]. The target genes of the quorum-sensing system that are necessary for pilus formation in *P. aeruginosa* are unknown at this time. Pilus function appears to be another of the many virulence determinants that are regulated by acyl-HSL signals in Gram-negative bacteria.

Some of the activities that have been shown to be mediated by pili lead us to speculate about the possible roles of AcfA and pili in the symbiosis between *V. fischeri* and its eukaryotic host, *E. scolopes*. For a long time, pili have been known to have adhesive properties that allow bacteria to bind to other bacteria and to eukaryotic cells. Recent studies have investigated the possibility of pili-mediated signal transduction to both the bacterium and to the eukaryotic cell. In uropathogenic *E. coli*, P-pili binding to host cells is followed by transcriptional activation of a sensor-regulator protein that is essential for virulence gene activation in the bacterium [27]. The timing of the two events suggests that pilus mediated attachment triggers virulence gene expression. Host cell response to

pilus attachment has also been demonstrated. Type 4 pili of the bacterial pathogen *Neisseria gonorrhoeae* have been shown to initiate a signal that is transduced into human epithelial cells and triggers an increase in cytosolic free calcium [28]. Presumably, signaling between *V. fischeri* and its squid host is involved in generating the distinct morphologies of the host light organ and bacterium during symbiotic growth.

The finding that strain ESacfX has an enhanced ability to colonizee the squid light organ was unexpected given the number of pathogens that have been shown to use pili in the course of a pathogenic interaction. The adhesive properties of pili and their involvement in pathogenesis have been well documented [1]. In the *Vibrio-Euprymna* symbiosis a diurnal pattern of bacterial expulsion and regrowth of the remaining population has been reported [29]. Adhesion of *V. fischeri* to the epithelial cells lining the light organ crypts or to other bacterial cells may determine the partitioning of the expelled and retained portions of the population. Diurnal expulsion of *V. fischeri* cells from the light organ combined with the possible differences in adhesive properties between the mutant and the parent strain may account for the super-coloniztion phenotype observed in the mutant. It is also possible that pili do not play a role in the symbiosis, but are instead involved in another ecological niche that also involves high popultion density.

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Chapter 5. Bi-directional *lux* boxes: open reading frames oriented divergently from *qsrP* and *acfA* code for hypothetical proteins and putative transcriptional activators.

ABSTRACT

In this section, genes that are transcribed divergently from *acfA* and *qsrP* are described. The dyadic structure of *lux* boxes suggests that they have the potential to function bi-directionally. DNA upstream of *acfA*, *qsrP*, and *ribB* was sequenced to identify open reading frames that could potentially be regulated by quorum sensing. Upstream of *acfA* and *qsrP*, but not *ribB*, promoter elements consistent with divergent transcription were found, and in each case, open reading frames were oriented opposite the quorum regulated genes. A single open reading frame that codes for a polypeptide with sequence similarity to a hypothetical protein from *Vibrio cholerae* was found opposite *qsrP*. Opposite *acfA*, a potential operon with three open reading frames was identified. The deduced amino acid sequences from two of these open reading frames are similar to members of the LysR family of transcriptional regulators. To investigate quorum regulation of both divergent sets of open reading frames, translational GFP fusions were made with each and examined in *V. fischeri* strains positive and negative for quorum regulation.

INTRODUCTION

In the previous two chapters we identified and characterized four genes from *V. fischeri* that appear to be regulated directly by the transcriptional activator LuxR and the coinducer $3OC_6$-HSL. In the promoter regions of *qsrP, ribB,* and the operon containing *acfA* and *qsrV*, putative *lux* box sequences have been identified. In Chapter three, sequences from several different *lux* boxes were used to derive a consensus sequence for *lux* boxes in *V. fischeri*. The double-stranded consensus sequence is almost a perfect dyad; in other words, the sequence and its reverse complement are almost identical. The dyadic nature of the *lux* box sequence suggests that genes transcribed divergently from those already identified may also be targets of LuxR and $3OC_6$-HSL regulation.

Like LuxR, the LysR family of transcriptional regulators (LTTRs) affect transcription in several diverse species of bacteria by binding to dyadic sequences in the promoter regions of target genes [1]. In this case, the target gene is often transcribed divergently from the gene encoding the LTTR. Transcription of the target gene is usually activated, and that of the LTTR gene is repressed. The dyadic LTTR binding sites differ from the *lux* box in two important ways. First, the LTTR sites are an interrupted dyad - much like the sequence of a stem and loop terminator sequence - that contains a six or seven nucleotide dyad separated by three to nine nucleotides. The *lux* box, on the other hand, is a continuous 20 bp dyad. Second, the LTTR binding sites are present as a pair of dissimilar dyads in the promoter regions of target genes. DNase I footprint analysis and gel-shift DNA binding assays with LTTRs from several species of bacteria have shown that one site, usually located at about -65 with respect to the start of transcription, is a recognition site and is bound by the LTTR in the absence of coinducer. The second site, the activation site, often overlaps a -35 region and requires the presence of the coinducer for binding. Although the individual binding sites are dyadic, the presence of two

dissimilar binding sites in the promoter regions of LTTRs suggests directional control of transcription. This inherent directionality has not been demonstrated in promoters regulated by LuxR and its homologs.

In this section we describe genes that are transcribed divergently from two of the three newly-identified *lux* boxes. Divergent open reading frames were detected opposite *acfA* and *qsrP*. Two of these open reading frames encode members of the LysR family of transcriptional regulators. To investigate quorum regulation of both divergent regions, translational GFP fusions were made with each and examined in *V. fischeri* strains positive and negative for quorum regulation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and growth conditions used have been described in Chapter 3 of this work.

Isolation of DNA upstream of *qsrP*. DNA upstream of *qsrP* was isolated from strain MJ-10X, which has the vector pSMC300 inserted in a known orientation into the chromosome of MJR1 in order to interrupt the *qsrP* gene (Chapter 3, Materials and Methods). Chromosomal DNA from MJ-10X was cut with the restriction enzyme *Sac*I, ligated, and used to transform *E. coli* strain DH5α-λ*pir*. Cells that had received pSMC300 and flanking DNA were selected for growth on chloramphenicol. The four clones examined carried approximately 15 kb of *V. fischeri* DNA in the *Sac*I site of pSMC300, which was designated p5'qsrP.

GFP fusion construction. Translational GFP fusions were constructed by PCR amplifying MJR1 chromosomal DNA with the appropriate primers and cloning the product into the *Kpn*I and *Hind*III sites of pSupGFP (Chapter 3, Materials and Methods). Primers qsrQ-pKpn (5'-AAAGGTACCAAGAAACCTCGACGAGATAA) and qsrQ-pH3 (5'-GGGAAGCTTGGTGATATAAGTGTGAAAAG) were used to construct pQsrQ-GFP, and primers qsrR-pKpn (5'-TTTGGTACCGCTTGGCTAATTGCTGGTTG) and qsrR-pH3 (5'-GATAAGCTTGTTAGGCCATATTCTAAACC) were used to construct pQsrR-GFP.

DNA sequencing. A primer walk strategy was used in each case for sequencing of DNA. Forward and reverse strands of DNA were sequenced in all cases except for qsrQ, for which only the forward strand was determined. DNA sequences were determined by the dideoxy-nucleotide chain termination method using dye terminator labeling at the CRC DNA Sequencing Facility, University of Chicago.

RESULTS

A hypothetical protein, QsrQ, is encoded divergently from QsrP. In
Chapter 3 the gene encoding QsrP was isolated by PCR from the chromosome of *V.
fischeri* strain MJ-100. The 826-bp PCR product included very little flanking DNA. To
isolate DNA upstream of *qsrP*, we isolated an approximately 19-kb fragment that conferred
resistance to chloramphenicol from the chromosome of strain MJ-10X (Materials and
Methods), which had been constructed by inserting pSMC300 into *qsrP*. The resulting
plasmid, p5'qsrP, consisted of pSMC300 and approximately 15 kb of DNA upstream of
qsrP. Sequencing from the promoter of *qsrP* upstream revealed an open reading frame
that begins 168 bp from the center of the *lux* box preceding *qsrP* (Figure 1). The spacing
between the *lux* box and the coding region is consistent with the 156 and 133 nucleotides
that separate the *lux* box from the *acfA* and *ribB* coding regions, respectively. The open
reading frame codes for a 423-amino acid protein with a molecular mass of 46,677 Da that
we have designated QsrQ until a function can be ascribed to the putative protein. As with
the promoter regions of *qsrP, acfA,* and *ribB*, the *lux* box appears to replace a -35 site in
the promoter of *qsrQ*; 28 nucleotides downstream from the center of the *lux* box is a
reasonable -10 Pribnow box (TATTAT). Using the canonical sequences for a -35 σ^{70}
polymerase recognition sequence (TTGACA) and a -10 Pribnow box (TATAAT) , we were
unable to identify any alternate promoter elements. A reasonable ribosomal binding site
(AAGG) located 5 nucleotides from the translational start site is indicated in Figure 1.

We did not detect any proteins in the NCBI protein database with a sequence similar
to QsrQ. On the other hand, a blastn search of the *V. cholerae* genome in the TIGR
Microbial Genome database identified a region of DNA with substantial identity to *qsrQ*
(data not shown). When this region of the *V. cholerae* genome was analyzed,

```
  1 CTGATATTTTTTTCATAAAATCCCCATTTATATTTCTATATGTTTCCAGAGAATTTTATATTAACGGTTG
    S  I  K  K  M   <<qsrP
               lux box                                    -10
 71 TAATTATGTTCCTGTAACTTATTACAGGTTTATATTTAATTATTAATATTATTTTTTATATAGAGTGATA

141 ATAAAAATACGCAATAAAATCGTCATCTCCTTTCAATATTTCTGTCACAAAGCACCTTTAGTCTACTTCT

                                      qsrQ>>  R.B.       M  M  N  K  F  V  E  K    8
211 GATTGTAAAAATACACTTATAAAAATAACAGTTTCAAAAGGGAAGTATGATGAATAAATTTGTAGAGAAA

      E  S  D  F  S  N  M  V  N  A  R  L  S  R  R  G  F  L  M  G  T  A  A   31
281 GAATCTGATTTTAGCAATATGGTGAATGCTCGTTTATCTCGTCGAGGTTTCTTGATGGGAACTGCGGCGG

      A  G  A  G  A  F  L  A  L  N  P  V  A  K  A  I  A  G  N  M  N  N  A  L   55
351 CAGGTGCTGGCGCTTTTTTGGCGTTAAACCCAGTAGCAAAAGCCATCGCTGGTAATATGAATAATGCGTT

      L  N  F  E  A  I  P  T  S  T  S  D  S  I  V  L  P  K  G  Y  S  W  N   78
421 ATTAAACTTTGAAGCCATTCCCACCTCTACAAGTGACTCTATTGTGCTTCCCAAAGGGTACAGTTGGAAT

      T  L  M  S  W  G  D  P  I  F  A  N  A  P  K  F  N  P  N  G  K  Q  D   101
491 ACATTAATGTCTTGGGGAGACCCTATCTTTGCTAACGCTCCGAAATTTAATCCAAATGGAAAGCAAGATT

      S  K  A  Q  A  L  Q  F  G  D  N  T  D  G  M  S  A  F  P  L  S  N  D  R 125
561 CAAAAGCGCAAGCATTGCAATTTGGTGATAACACAGATGGTATGAGTGCATTCCCATTATCAAACGATCG

      A  I  L  A  V  N  N  E  Y  T  N  Y  E  F  L  F  A  H  Q  G  K  E  M   148
631 AGCGATTCTTGCAGTTAATAATGAATATACTAATTATGAATTTCTGTTTGCTCACCAAGGCAAAGAAATG

      T  A  D  D  V  A  K  A  Q  A  A  V  G  I  T  I  V  E  I  V  K  K  N   171
701 ACTGCGGATGATGTAGCTAAAGCACAGGCGGCCGTGGGAATTACCATTGTTGAAATTGTAAAAAAGAACG

      G  Q  W  T  M  D  S  A  G  K  A  N  R  R  I  T  A  N  T  E  M  E  V  T 195
771 GTCAATGGACGATGGATTCAGCAGGCAAGGCAAATCGCCGTATTACAGCAAATACTGAAATGGAAGTGAC

      G  P  A  A  G  H  D  L  L  K  T  K  A  D  P  T  G  K  K  V  L  G  T   218
841 TGGTCCTGCTGCGGGTCATGATTTACTAAAAACCAAAGCCGATCCTACGGGTAAAAAAGTGTTAGGTACC

      F  N  N  C  A  N  G  E  T  P  W  G  T  Y  L  T  C  E  E  N  F  N  D   241
911 TTTAATAATTGTGCAAATGGTGAAACGCCTTGGGGTACTTATTTAACGTGTGAAGAAAATTTTAATGATT

      F  F  G  S  Q  Q  N  A  E  L  T  P  D  D  K  R  Y  G  F  K  A  E  Q  S 265
981 TTTTTGGTTCACAACAAAATGCAGAATTAACACCTGATGATAAGCGTTATGGTTTTAAAGCAGAGCAAAG

       D  Y  Q  W  H  N  F  D  D  R  F  D  V  A  K  N  P  N  E  P  N  R  F  288
1051 TGATTATCAATGGCATAATTTTGATGATCGTTTTGATGTAGCGAAAAATCCAAATGAACCGAATCGTTTT

       G  W  V  V  E  I  D  P  N  D  P  T  S  T  P  L  K  R  T  A  L  G  R   311
1121 GGGTGGGTGGTTGAAATTGATCCAAATGATCCAACATCAACGCCATTAAAACGAACGGCACTAGGTCGAT

       F  K  H  E  N  A  A  L  V  V  N  D  D  G  H  V  V  V  Y  L  G  D  D  E 335
1191 TTAAACATGAAAATGCTGCACTGGTTGTNAATGATGATGGCCATGTTGTGGTGTATCTTGGTGATGACGA

       R  G  E  H  L  Y  K  F  V  S  K  D  K  Y  Q  E  G  N  N  R  A  N  R  358
1261 ACGTGGTGAACACCTTTATAAGTTCGTATCAAAAGATAAATATCAAGAAGGGAATAACAGAGCAAATCGT

       N  L  L  E  E  G  T  L  Y  V  A  K  F  A  M  K  D  D  K  L  E  G  D   381
1331 AATCTCCTTGAAGAGGGCACCTTGTACGTTGCTAAATTTGCAATGAAAGACGATAAGTTAGAAGGTGATG

       G  Q  W  L  E  L  T  Y  G  K  N  G  L  T  R  E  N  G  F  N  S  Q  A  E 405
1401 GCCAGTGGCTAGAGTTGACTTATGGTAAAAATGGGTTAACCAGAGAAAATGGGTTCAATAGCCAAGCTGA

       V  M  I  F  A  R  R  A  A  T  Q  S  G  G  N  H  N  G  *         423
1471 AGTGATGATTTTTGCTCGCCGTGCGGCAACCCAAAGTGGGGGCAACCACAATGGATAGACCTGAATGGG  1539
```

Figure 1. Translated sequence of *qsrQ*. Ribosomal binding site, -10 Pribnow box, and
lux box are indicated.

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an open reading frame of 624 amino acids was revealed. The sequence of QsrQ is 73% identical and 79% similar to the N-terminus of this hypothetical protein from *V. cholerae* (Figure 2). To our knowledge, a function has not been ascribed to the hypothetical protein from *V. cholerae*, so we are unable to propose a function for QsrQ by sequence analogy.

Two proteins with similarity to members of the LysR family of transcriptional activators are encoded divergently from *acfA*. In Chapter 4, *V. fischeri* chromosomal DNA upstream of *acfA* was isolated on plasmid p5'acfA. Sequence upstream of the promoter of *acfA* revealed three open reading frames that appear to be transcribed as an operon oriented divergently from *acfA* (Figure 3). For the purpose of discussion, the putative product of the first open reading frame was designated QsrR, and the ensuing ORFs were designated QsrS and QsrT to reflect their order in the proposed operon. The promoter region of the operon resembles those of *acfA* and *qsrQ*; the *lux* box is located 152 nucleotides upstream of the coding region of *qsrR*, and a sequence (TATTTT) resembling the canonical -10 Pribnow box begins 28 nucleotides from the center of the *lux* box. Putative ribosome-binding sites were identified for *qsrR* (TAAGGA), *qsrS* (AGG), and *qsrT* (GGA). Although a stem and loop structure exists between *qsrS* and *qsrT* (7 bp stem and 10 bp loop), the lack of a recognizable promoter region and the sequence similarity between *qsrR* and *qsrT* (see below) suggests that the three genes may be transcribed as a single operon. A probable stem and loop terminator that begins 42 nucleotides after the translation termination codon of *qsrT* consists of an imperfect (1 mismatch) 12 bp stem and a 10 bp loop that is followed by a poly-T region.

Sequence similarity between the 309 and 307 deduced amino acid translation products of *qsrR* and *qsrT* and several transcriptional regulators suggests that QsrR and

V. c. QsrQ 1   M W K R D E Q E S Q F S E M I E A Q L S R R H F L A G S A A V S A G A F L A L   39
V. f. QsrQ 1   M M N K F V E K E S D F S N M V N A R L S R R G F L M G T A A A G A G A F L A L   40

V. c. QsrQ 40   N P V A S A V A A P A T S N L L N F S A I P V S T E D K V I V P K G Y K A T P L   79
V. f. QsrQ 41   N P V A K A I A G N M N N A L L N F E A I P T S T S D S I V L P K G Y S W N T L   80

V. c. QsrQ 80   M S W G D P I F A N A P E F D Q S G K Q D S K A Q E K Q F G D N T D G M S F F P   119
V. f. QsrQ 81   M S W G D P I F A N A P K F N P N G K Q D S K A Q A L Q F G D N T D G M S A F P   120

V. c. QsrQ 120 I S E D R G V L A I N N E Y T N Y E Y L F D H Q G K A M T A D D V R K A Q A A V   159
V. f. QsrQ 121 L S N D R A I L A V N N E Y T N Y E F L F A H Q G K E M T A D D V A K A Q A A V   160

V. c. QsrQ 160 G V T I V E V V R K N G Q W M V D R Q G E R N R R I T A Y T P M M M T G P A A G   199
V. f. QsrQ 161 G I T I V E I V K K N G Q W T M D S A G K A N R R I T A N T E M E V T G P A A G   200

V. c. QsrQ 200 H D L L K T A E D P S G L K V L G T F N N C A N G E T P W G T Y L T C E E N F D   239
V. f. QsrQ 201 H D L L K T K A D P T G K K V L G T F N N C A N G E T P W G T Y L T C E E N F N   240

V. c. QsrQ 240 D F F G A D Q E G S V D A D Q K R Y G I A A E P S D Y Q W H K H D A R F D I T K   279
V. f. QsrQ 241 D F F G S Q Q N A E L T P D D K R Y G F K A E Q S D Y Q W H N F D D R F D V A K   280

V. c. QsrQ 280 N P K E P N R F G W V V E I D P H N P N S T P L K R T A L G R F K H E N A A L V   319
V. f. QsrQ 281 N P N E P N R F G W V V E I D P N D P T S T P L K R T A L G R F K H E N A A L V   320

V. c. QsrQ 320 I N N D G H V V V Y L G D D E R G E H L Y K F V S K H R Y Q A G N D Q Q N R N L   359
V. f. QsrQ 321 V N D D G H V V V Y L G D D E R G E H L Y K F V S K D K Y Q E G N R A N R N L   360

V. c. QsrQ 360 L E E G T L Y V A K F D I N E N E L K G S G R W M E L S F G K N G L T P E N G F   399
V. f. QsrQ 361 L E E G T L Y V A K F A M K D D K L E G D G Q W L E L T Y G K N G L T R E N G F   400

V. c. QsrQ 400 K D Q A E V L I F A R R A A T Q V G A T T M D R P E W V A V H P D K K H V F C T   439
V. f. QsrQ 401 N S Q A E V M I F A R R A A T Q S -   417

V. c. QsrQ 440 L T N N K R G K E G Q P V G G P N P R E K N N Y G Q I V R W M P A Q G D H T S   479
V. f. QsrQ 418 - - - - - - - - - - - - - G G - N -   420

V. c. QsrQ 480 D V F A W D L Y L I A G N P T V H K G T L Y A G S E N I S A D N M F N S P D G I   519
V. f. QsrQ 421 - - - - - - - - - - - - - - - H N G   423

V. c. QsrQ 520 G F D T A G R L W I Q T D G N Y S N Q G D F A G Q G N N Q M L C G D P I T G E V   559
V. f. QsrQ 424   423

V. c. QsrQ 560 K R F L T G P I A C E I T G L T F S P D H K T M F V G V Q H P G E E A A P S H F   599
V. f. QsrQ 424   423

V. c. QsrQ 600 P Y G G T S K P R S T I M M I T R E D G G V I G A 624
V. f. QsrQ 424   423

Figure 2. Comparison of QsrQ from *V. fischeri* and a hypothetical protein derived from the minus strand of nucleotides 4727-6598 of contig asm 821 in the TIGR Microbial Genome *V. cholerae* database. Identical amino acids are boxed and shaded dark, and similar amio acids are boxed and shaded light.

```
  1 TAAATACTTTTTCATTGTAATTCAATCCTATAGTTATGATGTTGAGCTTGGTGTTAATTCGTTAATGTCG
    L   Y   K   M  <<acfA
                                 interrupted dyad
 71 CTACTATAAAGAAGAGATCGTGAAGAAAGTGTGAAGAGCACTTTCATATAAATTTACAGCTCATAATTAC
    < -10                 ------>             <------
                                   lux box                        -10
141 ATTCATTCCGAATACTTTTACAGCTGTAATAACTTACAGGGCATAATGGGATAAAGGTTATTTTCTTTAC

211 TAATTCAATACGATAGTAATACATTTAATCTTGGTCTTACGTGATAATATAAGTACTATAGAAAGAGCCC

                       qsrR>> R.B.            M   A   K   D   L   F   A   T   L      9
281 TAAAGGAAACACACCAAATTATTTATAAGGAAATAAGTAAATATGGCAAAAGATCTATTCGCAACATTGG
                       ----->     <-----
        D   L   N   L   L   R   T   F   L   I   L   S   Q   E   L   N   M   R   K   A   S   E   R   L   33
351 ATTTAAATTTGCTGAGGACATTTTTAATTCTTTCCCAAGAATTAAATATGCGAAAAGCCTCTGAACGCTT

        F   V   S   Q   P   A   I   S   Q   A   L   Q   K   L   R   N   H   F   N   D   E   L   F   56
421 ATTTGTCTCTCAACCAGCAATTAGCCAAGCATTGCAAAAATTACGAAACCACTTCAATGATGAACTGTTT

        I   K   V   R   H   G   L   K   P   T   P   F   A   E   E   L   A   E   N   I   Q   P   Y   79
491 ATTAAAGTTCGCCATGGCCTTAAACCAACCCCCTTTGCCGAAGAATTAGCGGAAAATATCCAACCTTATT

        L   D   G   L   S   S   V   L   N   A   S   Q   E   F   Q   P   K   N   L   N   K   T   L   K   103
561 TAGATGGTCTATCGTCAGTATTAAATGCATCACAAGAATTCCAACCAAAAAATTTAAATAAGACGTTGAA

        I   A   L   A   P   Q   V   L   T   C   L   S   G   A   L   F   H   E   I   R   E   K   A   126
631 GATTGCTTTAGCACCTCAAGTATTAACCTGTTTGTCTGGTGCTTTATTTCATGAAATTAGAGAAAAAGCA

        P   N   V   D   L   Q   L   V   N   W   S   E   S   T   F   D   D   L   S   K   G   K   L   149
701 CCCAATGTTGATTTACAACTGGTTAATTGGTCAGAATCTACCTTTGATGATCTTAGCAAGGGTAAATTAG

        D   I   A   I   S   Y   E   Y   D   K   F   P   K   E   L   L   S   K   S   L   I   T   I   T   173
771 ATATCGCAATCAGTTACGAATATGACAAATTTCCTAAAGAATTACTTTCAAAAAGTCTAATAACAATAAC

        G   C   V   I   V   R   K   D   H   P   I   E   K   M   I   A   T   P   Y   D   F   E   G   196
841 TGGATGCGTCATTGTTAGAAAAGATCACCCAATAGAAAAAATGATTGCAACACCATATGACTTTGAAGGA

        Y   E   L   A   S   L   I   I   P   G   W   N   D   N   K   S   L   A   S   E   L   L   T   219
911 TATGAACTGGCTTCTTTAATTATTCCTGGTTGGAACGATAACAAAAGTTTAGCAAGTGAGCTTCTAACCA

        K   M   N   I   E   H   K   I   G   F   R   S   E   L   P   M   A   L   V   D   V   V   Q   H   243
981 AGATGAATATTGAACATAAGATAGGGTTTCGTTCAGAGCTACCAATGGCGCTAGTTGATGTTGTTCAGCA

        T   D   M   Y   F   P   S   T   S   L   F   P   I   H   Q   Y   S   G   L   R   R   I   D   266
1051 TACAGACATGTATTTCCCTTCCACCTCCTTATTTCCAATTCATCAATACTCAGGTTTAAGACGAATCGAT

        L   S   L   D   D   V   P   L   N   Y   P   I   Y   S   Y   F   H   Q   R   H   K   K   N   289
1121 TTATCTCTTGATGATGTGCCATTAAATTACCCAATCTATAGTTATTTCCACCAAAGACATAAAAAAAATG

        A   K   H   A   W   V   N   Q   L   I   T   N   L   L   S   E   A   N   I   K   *   309
1191 CACTGCACGCTTGGGTTAATCAGCTTATTACGAATTTATTATCAGAAGCAAATATCAAATAAGTATGCCT

                                                          qsrS>> R.B.
1261 TATGAGCTACATAATGAGAGATGATTGGTTCACTATCATAAGTATTTATATTATCTAAACCAGTCAACAG>

        M   I   F   K   R   I   T   T   V   T   A   I   F   T   L   S   R   S   D   E   V   F   R   23
1331 GAATGATATTCAAGCGTATTACTACTGTTACTGCGATATTTACTTTATCAAGGAGTGATGAAGTTTTTAG

        I   G   F   I   A   T   L   L   S   V   F   D   I   Y   R   *         stem and   39
1401 AATAGGCTTTATTGCAACTCTACTTTCCGTCTTTGACATTTACCGTTAGGTAACTTCGCCTCTGTTCTCT
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Figure 3. Nucleotide sequences and deduced translation products of QsrR, QsrS, and QsrT. Converging arrows underscore interrupted dyads.

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1471 CATC<u>CAGAGGC</u>TTTTTTTTCTTTACCCTTATCAATTTCTTTGCTCCAATAAAGAAAACGTCAAC<u>GGA</u>TTGA

    M A K D L F S N L D L N L L R T F I I L H Q   22
1541 TATTATGGCTAAAGATTTATTCTCTAATCTAGATTTAAACTTATTACGGACATTCATTATCCTTCATCAA

    E R N M R K A S E R L F V S Q P A I S K A L Q   45
1611 GAAAGAAATATGCGTAAAGCATCTGAACGCTTATTTGTTTCCCAACCAGCAATCAGTAAAGCCTTACAAC

    R L R D H F S D E L F V K T H H G L R A T E Y A   69
1681 GCTTACGTGACCATTTCAGTGATGAATTATTTGTAAAAACCCATCATGGACTACGAGCTACAGAGTACGC

    N Q L A E S I A P I L D E L S S T L N E S N E   92
1751 CAATCAGCTTGCTGAAAGTATTGCGCCAATCCTTGATGAATTATCCTCCACATTAAATGAAAGTAATGAG

    F D P K K L D G V I K L A L S P F I L S A I A   115
1821 TTTGATCCTAAAAAAACTTGATGGCGTCATTAAACTTGCTTTATCCCCTTTTATTTTAAGTGCTATTGCAA

    N K L F Q A I R T E A P N V Q V Q L L N W S K S 139
1891 ATAAGCTATTTCAAGCAATCAGAACTGAAGCTCCAAATGTTCAAGTACAATTATTGAATTGGTCTAAATC

    T M T D I I N N E V Q L G L N Y E I S H A P K 162
1961 AACCATGACTGACATTATTAATAATGAGGTACAACTCGGTCTTAATTATGAAATCAGCCACGCACCAAAA

    E L I Q K P I T Q D R F T G Y V R K D H P Y K 185
2031 GAACTCATTCAAAAACCGATAACTCAAGACCGTTTTACTGGGTATGTACGTAAAGATCACCCTTATAAGG

    G T E I E V K D G V N F E L A T V I A V D W N S 209
2101 GGACTGAAATAGAAGTAAAAGATGGCGTTAATTTTGAATTAGCTACCGTAATTGCTGTTGATTGGAATTC

    H Q S L A E K I L K I R G L E A N I G F R S E 232
2171 TCATCAATCTTTAGCTGAAAAAATATTAAAGATCAGAGGATTAGAAGCTAATATTGGCTTTAGGTCAGAG

    L P S A V I D V I H N S D M M F P A S K F L E 255
2241 CTTCCTTCGGCGGTTATTGATGTCATTCATAACTCAGACATGATGTTTCCAGCATCTAAATTTTTAGAAA

    I D N N H Q L R S L K I L F D N Q D I N P K V C 279
2311 TAGATAATAACCATCAGTTACGAAGCCTTAAAATACTCTTTGATAATCAAGATATTAACCCAAAGGTTTG

    A Y Y H H K N R N N P T M L W L K N I L N S L 302
2381 TGCTTATTACCACCATAAAAATCGCAATAATCCAACCATGTTGTGGTTAAAAAATATTCTTAATTCATTA

    L I D N P *   stem 307
2451 CTCATTGATAACCCATAGTTATTACGGCTATTCTTCTCTCTCATTAATAACTCAACGTT<u>AAAGCCTCTAT></u>

and loop terminator
2521 <u>T</u>CTTTTAAGGG<u>ATTAGAGGCTTT</u>TTTGTACACTTCATATACTTCTCTCATCGGCTAGGAACGCCATCAAC

2591 AAGCACTG 2598

Continuation of Figure 3.

Figure 4. Comparison of QsrR and QsrT amino acid sequences from *V. fiascheri* with that of NahR from *Pseudomonas putida* [2]. Identical amino acids are boxed and shaded dark, and similar amino acids are boxed and shaded lightly.

```
V. f. QsrR    1   MAKDLFATLDLNLLRTFLILS QELNMRKAS        30
V. f. QsrT    1   MAKDLFSNLDLNLLRTFIILH QERNMRKAS        30
Ps. p. NahR   1     MELRDLDLNLLVVFNQLL VDRRVSITA         27

V. f. QsrR   31   ERLFVSQPAISQALQKLRNHFNDELFIKVR         60
V. f. QsrT   31   ERLFVSQPAISKALQRLRDHFSDELFVKTH         60
Ps. p. NahR  28   ENLGLTQPAVSNALKRLRTSLQDPLFVRTH         57

V. f. QsrR   61   HGLKPTPFAEELAENIQPYLDGLSSVLNAS         90
V. f. QsrT   61   HGLRATEYANQLAESIAPILDELSSTLNES         90
Ps. p. NahR  58   QGMEPTPYAAHLAEPVTSAMHALRNALQHH         87

V. f. QsrR   91   QEFQPKNLNKTLKIALAPQVLTCLSGALFH        120
V. f. QsrT   91   NEFDPKKLDGVIKLALSPFILSAIANKLFQ        120
Ps. p. NahR  88   ESFDPLTSERTFTLAMTDIGEIYFMPRLMD        117

V. f. QsrR  121   EIREKAPNVDLQLVN-WSESTFDDLSKGKL        149
V. f. QsrT  121   AIRTEAPNVQVQLLN-WSKSTMTDIINNEV        149
Ps. p. NahR 118   VLAHQAPNCVISTVRDSSMSLMQALQNGTV        147

V. f. QsrR  150   DIAISYEYDKFPKELLSKSLITITGCVIVR        179
V. f. QsrT  150   QLGLNYEISHAPKELIQKPITQDRFTGYVR        179
Ps. p. NahR 148   DLAVG-LLPNLQTGFFQRRLLQNHYVCLCR        176

V. f. QsrR  180   KDHPIEKMIATPYDFEGYELASLIIPGWND        209
V. f. QsrT  180   KDHPYKGTEIEVKDGVNFELATVIAVDWNS        209
Ps. p. NahR 177   KDHPVTREPLTLERFCSYGHVRVIAAGTG-        205

V. f. QsrR  210   NKSLASELLTKMNIEHKIGFRSELPMALVD        239
V. f. QsrT  210   HQSLAEKILKIRGLEANIGFRSELPSAVID        239
Ps. p. NahR 206   -HGEVDTYMTRVGIRRDIRLEVPHFAAVGH        234

V. f. QsrR  240   VVQHTDMYFPSTSLFPIHQYSGLRRIDLSL        269
V. f. QsrT  240   VIHNSDMMFPASKFLEIDNNHQLRSLKILF        269
Ps. p. NahR 235   ILQRTDLLATVPIRLADCCVEPFGLSALPH        264

V. f. QsrR  270   DDVPLNYPIYSYFHQRHKKNALHAWVNQLI        299
V. f. QsrT  270   DNQDINPKVCAYYHHKNRNNPTMLWLKNIL        299
Ps. p. NahR 265   PVVLPEIAINMFWHAKYHKDLANIWLRQLM        294

V. f. QsrR  300   TNLLSEANIK   309
V. f. QsrT  300   NSLLIDNP     307
Ps. p. NahR 295   FDLFTD       300
```

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QsrT are members of the LysR family of transcriptional regulators (LTTRs). In Figure 4 it is shown that both QsrR and QsrT are approximately 24% identical and 42% similar to the sequence of NahR from *Pseudomonas putida*, a LysR-type transcriptional activator of genes that encode the enzymes for metabolism of napthalene and salicylate as carbon and energy sources [3]. Sequence conservation is greatest for all three proteins in the first 66 amino acids (using the NahR residue numbering), which is believed to encode a helix-turn-helix DNA binding domain that is highly conserved in all LTTRs [1]. After this N-terminal region, sequence similarity with NahR decreases dramatically, but the similarity between QsrR and QsrT remains high. The C-termini of LTTRs are generally poorly conserved and are believed to mediate binding of the various coinducers of transcription [1]. The sequences of QsrR and QsrT are 46% identical and 66% similar overall.

QsrS is a putative 39-amino acid protein encoded between QsrR and QsrT. The sequence of the protein had no significant similarity to sequences in the NCBI or TIGR *V. cholerae* databases.

GFP fusions to QsrQ and QsrR indicate regulation by quorum sensing. The promoter regions of *qsrQ* and *qsrR* suggest that they may be regulated by LuxR and $3OC_6$-HSL. To investigate this possibility, and as a prelude to investigating production of the putative proteins in the squid symbiosis, we constructed translational fusions between the translational start codon of GFP and the first 26 and 42 amino acids of QsrQ and QsrR, respectively (Materials and Methods). The promoter regions of *qsrQ* and *qsrR* were intact on the plasmid-borne constructs pQsrQ-GFP and pQsrR-GFP. Each plasmid was introduced separately into *V. fischeri* strains MJ-100 and MJ-215 (Δ*luxI*, *ainS*). Quorum-regulated GFP expression was assessed by visually comparing colonies of each plasmid-carrying strain grown on LBS agar illuminated by ultra-violet light at a

wavelength of 365 nm. Strain MJ-100 with pQsrQ-GFP was green when examined with ultra-violet light, whereas MJ-215 carrying the same construct lacked green coloring (data not shown). On the other hand, MJ-100 with pQsrR-GFP appeared the same as MJ-215 carrying the same construct. We interpret these results to be an indication that production of QsrQ is under quorum regulation.

DISCUSSION

The dyadic structure of the *lux* box and its potential to function bi-directionally prompted us to examine DNA upstream of the promoter regions of genes that encode proteins known to be regulated by the LuxR/3OC$_6$-HSL quorum-sensing system. For two of the three *lux* boxes discovered previously in this study, divergent open reading frames were identified. A single open reading frame denoted *qsrQ* was identified opposite the *qsrP* promoter region. The proposed promoter region of *qsrQ* resembles that of the other quorum-regulated genes. The deduced 423-amino acid translation product was similar in sequence to the N-terminal two-thirds of a hypothetical protein from *V. cholerae*.

Opposite the promoter of *acfA*, three open reading frames that have been proposed to comprise an operon were identified. The first and third putative genes of the operon, *qsrR* and *qsrT*, encode prospective members of the LysR family of transcriptional activators (LTTRs). In between *qsrR* and *qsrT* is a short open reading frame, *qsrS*, which codes for a 39-amino acid peptide and is followed by a possible stem and loop structure. The location of this putative terminator of transcription before the start of *qsrT* suggests that anti-termination may be involved in the regulation of *qsrT*. Examination of the region upstream of *ribB* revealed an open reading frame that was oriented in the same direction as *ribB* and was followed by a stem and loop structure (data not shown). The deduced translation product of this open reading frame was not similar to sequences in the NCBI database, and it was not pursued further.

Qualitative analysis of GFP translational fusions to the putative products of *qsrR* and *qsrQ* indicated that QsrQ is probably regulated by quorum sensing. Because MJ-215 is deficient in the production of both 3OC$_6$-HSL and C$_8$-HSL, either signal molecule could be

Table 1. Comparison of quorum-regulated *lux* boxes and promoter regions.

Gene	*lux* box sequence	Position relative to: -10 Pribnow	Translation Start site
acfA	CCCTGTAAGT TATTACAGCT	-31	-156
luxI	ACCTGTAGGA TCGTACAGGT	-29	-62
qsrP	ACCTGTAATA AGTTACAGGA	-27	-73
qsrQ	TCCTGTAACT TATTACAGGT	-28	-168
qsrR	AGCTGTAATA ACTTACAGGG	-28	-152
ribB	ACCTGCAATA ATTTACAGTA	-51	-133

necessary for transcription of the gene. The translational fusion to QsrR, on the other hand, did not show evidence of quorum regulation. LuxR may have no effect on transcription of QsrR, it may activate transcription under conditons different from those on solid laboratory media, or our reporter system may not be sensitive enough to monitor changes in transcription of QsrR. Currently, we are introducing the fusion constructs into the squid symbiont *V. fischeri* strain, ESR1, to investigate regulation of *qsrR* and *qsrQ* during growth in the squid light organ.

The two additional quorum-regulated promoters described here bring the total number of *lux* box-containing promoter regions in *V. fischeri* to six. The position of the *lux* box relative to the translation start sites and proposed -10 Pribnow boxes shows some similarities between the different promoter regions (Table 1). Replacement of a -35 hexanucleotide appears to be a common theme with the *lux* boxes of *V. fischeri*. In five of the six promoters a reasonable -10 Pribnow box is located about 28 nucleotides

downstream of the center of the *lux* box. In the promoter of *ribB*, the presence of a -35 σ^{70} polymerase recognition site in addition to the *lux* box suggests that another level of regulation may exist (Chapter 3). For *luxI* and *qsrP*, the *lux* boxes are located at -62 and -73 relative to the start of translation. In the promoter regions of *acfA, qsrQ, qsrR,* and *ribB* the *lux* boxes are located further upstream at positions -156, -168, -152, and -133 respectively. The apparent grouping of the genes by position of the *lux* box may be an artifact of the small sample number, or it may reflect differences in regulation between the two. The extra spacing between the *lux* box and the start of translation may facilitate a second level of transcriptional regulation.

In many cases, the target of LTTRs are divergently transcribed genes [1], which in this case would be *acfA* and *qsrV*. Because the binding sites for LTTRs are usually interrupted dyad sequences, we searched the mutual promoter region of *acfA* and *qsrR* for such sequences. In Figure 3 two such regions are indicated that are perfect dyads (GAAAGTGtgaagagCACTTTC and TTATTTataaggAAATAA). The T-N_{11}-A motif exhibited in the binding site for many LTTRs [4] is present in one of these, located between *acfA* and the *lux* box. A potential -10 Pribnow box (TATCAT) also begins 28.5 bp from the center of this *acfA* proximal dyadic region. This spacing between the two regions is consistent with the observation that the activation binding sites of many LTTRs overlap the -35 sites in the promoter regions of target genes. By sequence analysis, it appears that *acfA* and *qsrV* may be regulated by QsrR and/or QsrT. In chapter 4 it was stated that the presence of the *lux* box in the promoter region of *acfA* was a likely indicator of direct transcriptional regulation by LuxR, but it may be QsrR and QsrT that is primarily regulated by LuxR, and *acfA* is regulated indirectly by quorum sensing through QsrR and/or QsrT.

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Chapter 6. Use of transposon mutagenesis to identify quorum-regulated genes.

ABSTRACT

Expression of the luminescence operon (*luxICDABEG*) in *Vibrio fischeri* is controlled in a population density-responsive manner through the activities of LuxR protein and two different acyl-homoserine lactone (acyl-HSL) signal molecules, the *luxI*-dependent *N*-3-oxohexanoyl-L-HSL ($3OC_6$-HSL) and the *ainS*-dependent *N*-octanoyl-L-HSL (C_8-HSL). We used random transposon mutagenesis with MudI1681 (*lacZ*) to identify non-*lux* genes controlled by $3OC_6$-HSL in *V. fischeri*. Plasmid pPD104, containing MudI1681, was mobilized by conjugation from *Escherichia coli* into the acyl-HSL signal-deficient strain *V. fischeri* MJ-215 (Δ*luxI*, *ainS*⁻). Transconjugants were selected for resistance to neomycin (encoded by MudI1681), and strains with putative *lacZ* fusions to acyl-HSL signal-regulated promoters were identified by their response to cell-free media containing X-gal that had been "conditioned" by the wild-type strain. Of 2880 strains screened, three had sharply elevated levels of β-galactosidase activity and had lost the vector, as indicated by sensitivity to chloramphenicol (vector-encoded resistance). Like MJ-215, the mutants were dark but produced light when grown in proximity to the acyl-HSL donor strain MJ-203 (Δ*luxA*), indicating that the transposon had not inserted into a gene necessary for light production. Levels of β-galactosidase activity in the fusion strains responded to growth in proximity to MJ-216 (*ainS*⁻) but not MJ-211 (Δ*luxI*), indicating sensitivity to $3OC_6$-HSL but not C_8-HSL. The insertion point of MudI1681 in one of the strains was determined to be the *acfA* gene, a locus previously identified as responsive to $3OC_6$-HSL (see Chapter 4). In the course of isolating this locus, the region of the chromosome that encodes both the

anaerobic response regulatory protein, ArcA, and the enzyme that catalyzes the last step in menaquinone synthesis, MenG, was cloned from *V. fischeri* and sequenced. The gene and protein designations are based on sequence similarity to proteins of known function in *E. coli*. Additional experiments are needed to determine if transcription of *arcA* is controlled by quorum regulation. The remaining $3OC_6$-HSL responsive fusion strain remains uncharacterized.

INTRODUCTION

In a previous study we used 2-D PAGE to identify five proteins whose production is dependent on the acyl-homoserine lactone (acyl-HSL) intercellular signal, N-3-oxohexanoyl-L-HSL ($3OC_6$-HSL), and the transcriptional regulator, LuxR (See Chapter 3). These newly identified quorum-regulated proteins (QsrP, RibB, AcfA, Orf8, and QSR protein #7) are distinct from those encoded by the previously characterized *lux* operon (*luxICDABEG*). To our surprise, we detected only three of the seven Lux proteins in this previous study. LuxA, LuxB, and LuxE were easily resolved by 2-D PAGE analysis, but LuxI, LuxC, LuxD, and LuxG were not detected among the proteins from *V. fischeri* cells harvested when the luminescence system was fully induced. Presumably, they were lost during preparation of the sample, they were not resolved under the conditions of 2-D PAGE used, or their levels were below those detected with silver staining. In the same way, additional non-Lux quorum-regulated proteins may have eluded visualization by 2-D PAGE. To circumvent the limitations of protein visualization, we adopted a traditional genetic approach designed to monitor differential gene expression.

In the past, the transposon MudI1681 [1] has been used in *V. fischeri* to monitor gene expression [2] and to create motility mutants [3]. Therefore, we knew that the transposon transposed in an apparently random fashion in *V. fischeri* [3], and that the resulting transcriptional fusions to the *lacZYA* genes of the transposon could be used to monitor transcription levels. In the present study, we used MudI1681 to screen for promoters that are controlled by quorum regulation in *V. fischeri*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* Novablue and S17-1 were grown on LB medium [4] at 37°C with antibiotics as appropriate (ampicillin, 100 μg/ml; neomycin 200 μg/ml). Strains of *V. fischeri* were maintained on LBS agar [2] with the appropriate antibiotics (naladixic acid, 20 μg/ml; and neomycin, 200 μg/ml). Conditions for isolation and growth of the *V. fischeri* MudI1681 transconjugants has been described previously [3].

Screen for acyl-HSL-responsive genes. Plasmid pPD104, which contains MudI1681, was mobilized into *V. fischeri* strain MJ-215 by conjugation, and transconjugants were selected for their ability to grow on neomycin. 2880 of these colonies were each used to inoculate two 150 μl cultures in 96 well microtiter plates: one culture contained LBS, neomycin and X-gal, and in the second culture, half of the LBS was replaced with "conditioned" LBS, which served as a source of $3OC_6$-HSL and C_8-HSL. Cultures were incubated 18 hrs without aeration. 50 μl of artificial seawater [5] containing 135μg/ml X-gal was then added to the two sets of cultures, which were visibly screened for blue color after 10 min. Cultures grown with conditioned LBS that exhibited more or less β-galactosidase activity than their corresponding signal-deficient culture were subjected to an additional two rounds of screening. Conditioned LBS was made by growing *V. fischeri* strain MJ-100, which is wild-type for $3OC_6$-HSL and C_8-HSL production, to early stationary phase (A_{660}=1.2) in LBS and removing the cells by centrifugation and passage through a 0.2 micron filter.

Isolation of *arcA* from *V. fischeri*. The intact gene for *arcA* was isolated from a plasmid-borne library of MJ-1 chromosomal DNA in *E. coli* [5] using PCR analysis

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as a screen. Plasmids from 2000 colonies representing the library were initially pooled in groups of 50, and DNA from these 40 groups served as the template for individual PCR reactions using primers 47F (5'-GACGTAACTATCCGTCGCATCC) and 47R (5'-CGATTAAAGCAAGTGGTGAGTGG). Three of the reactions generated the expected 258 bp fragment, one of which was pursued further. Each plasmid from the pool of 50 for that reaction was then screened individually to identify a single positive clone. The sequence of the region containing *arcA* was obtained using a primer walk strategy. Standard conditions were used in PCR reactions.

Manipulation and sequencing of DNA. Cloning and subcloning of DNA followed standard techniques used in molecular biology [4]. DNA sequences were determined by the dideoxy-nucleotide chain termination method of Sanger et al. [6] using dye terminator labeling at the CRC DNA Sequencing Facility, University of Chicago.

RESULTS and DISCUSSION

Isolation of $3OC_6$-HSL-responsive fusion strains. As in the previous study where 2-D PAGE was used to identify 5 quorum regulated proteins, strain MJ-215 was used in the initial screening because it is deficient in the two acyl-HSL synthases made by *V. fischeri*. MJ-215, therefore, provides a signal-deficient background that can be manipulated by the addition of exogenous $3OC_6$-HSL and C_8-HSL to search for responsive promoters. Initially, we had intended to compare β-galactosidase activity of MJ-215::MudI1681 colonies on standard LBS agar media containing X-gal to that of colonies replica patched onto media "conditioned" by MJ-100. The conditioned media (Materials and Methods) would supply the cells with both $3OC_6$-HSL and C_8-HSL signal molecules. Screening on solid media was difficult, though, due to the high background level of β-galactosidase activity as indicated by blue colony color on X-gal. We thought that the presence of the chromogenic substrate in the media during growth of the colonies may have added to the high background. To circumvent this problem, we used replica cultures in liquid media and added X-gal after the cultures had reached stationary phase (Materials and Methods). Using this strategy, we isolated three *lacZ* fusion strains from 2880 colonies screened that consistently exhibited enhanced levels of β-galactosidase activity in conditioned media.

The elevated β-galactosidase activity in response to conditioned media could have resulted from the presence of one or both of the acyl-HSL signals or from a distinct factor that accumulates in the media as cells grow. Each possibility was interesting. To test if $3OC_6$-HSL or C_8-HSL was responsible, the three fusion strains were cross streaked on solid LBS containing X-gal with various acyl-HSL donor strains. The results are shown in

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Table 2. Induced β-galactosidase activity in response to cross streaking with MJ-203 and

MJ-216, and the lack of a response to MJ-211 and MJ-215, indicate that in all three fusion

strains, the expression of $lacZ$ is dependent on $3OC_6$-HSL, or a secondary signal that is

dependent on $3OC_6$-HSL for its activity, and not on C_8-HSL. Figure 1 shows a

representative example of the cross streaking results. Table 2 also shows that the

transposon had not inserted into a gene that is necessary for light production. Each of the

fusion strains was luminescent when cross streaked next to strain MJ-203, which can

donate both acyl-HSL signals involved in light production (Fig. 2). Therefore, none of the

insertions were in genes of the lux operon.

Cloning of the MudI1681-L fusion junctions. MudI1681 is about 16 kb in

size. Because of the large size of MudI1681, we decided to clone each end of the

insertions independently. Fusion junctions containing the distal half of MudI1681 (MuL),

relative to the $lacZ$ fusion, and downstream DNA were cloned into pBluescript with

digestion of chromosomal DNA from the fusion strains with $SaII$ or $BamHI$ and selection

for neomycin resistance. The fusion junctions of 25 of the resulting clones were sequenced

using MudI sequence as a primer. Each of the three strains had multiple insertions of

MudI1681. Three insertions were detected in MD029, four in MD030, and five in MD031

as determined by the number of unique sequences from each. Note that this is the

minimum number of insertions in each strain. Because each strain had multiple insertions,

further analysis was required to identify the $3OC_6$-HSL-responsive fusions.

The sequences from the fusion junction clones were compared with those in the

NCBI database at both the nucleotide and the translated amino acid level. Of the 12 distinct

sequences, only one from MD031 had significant similarity to a sequence in the database.

Sequence from clone pBS47 was very similar to that of ArcA from several species of

bacteria. In $E.\ coli$ ArcA (aerobic respiration control) is the response regulator of a two-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli		
Novablue	*endA hsdR17*(r_{K12}⁻m_{K12}⁺) *supE thi recA gyrA relA lac*[F' *proA*⁺*B*⁺ *lacI*^{q}*ZΔM15*::Tn*10*(Tc^r)]	Novagen
S17-1	RP4 *tra*⁺	[7]
V. fischeri		
MJ-100	Parent strain, Nx^r	[2]
MJ-203	MJ-100, Δ*luxA* (168-bp *Xho*I-*Nhe*I in-frame deletion	[8]
MJ-211	MJ-100, Δ*luxI* (~250-bp nonpolar deletion)	[8]
MJ-215	MJ-211, *ainS* (2-bp insertion)	[9]
MJ-216	MJ-100, *ainS*::*neo* (1.8-kb Nm^r cassette)	[10]
MD029	MJ-215 *acfA*::MudI 1681	This study
MD030	MJ-215::MudI 1681	This study
MD031	MJ-215::MudI 1681	This study
Plasmids		
pPD104	pSup102::MudI 1681, Cm^r, Nm^r	[3]
pBR322	Cloning vector, Ap^r	[11]
pBluescript SK+	Cloning vector, Ap^r	Stratagene
pBR029	pBR322 with an 8 kb *Sal*I fragment containing MudI 1681-R::MJ-215	This study

TABLE 2. Cross streaking of fusions strains with various acyl-HSL donor strains

Fusion strain	Resistance to		Light production in response to MJ-203 (3OC$_6$-HSL + C$_8$-HSL)	β-galactosidase activity in response to			
	Nm	Cm		MJ-203 (3OC$_6$-HSL + C$_8$-HSL)	MJ-211 (C$_8$-HSL)	MJ-216 (3OC$_6$-HSL)	MJ-215 Neither
MD029	R	S	+	+	-	+	-
MD030	R	S	+	+	-	+	-
MD031	R	S	+	+	-	+	-

Figure 1. MD029 cross streaked with the acyl-HSL donor strain MJ-203 on media containing X-gal. In close proximity to MJ-203 β-galactosidase activity is maximal, presumably in response to a diffusable compound released by MJ-203 but not by MD029.
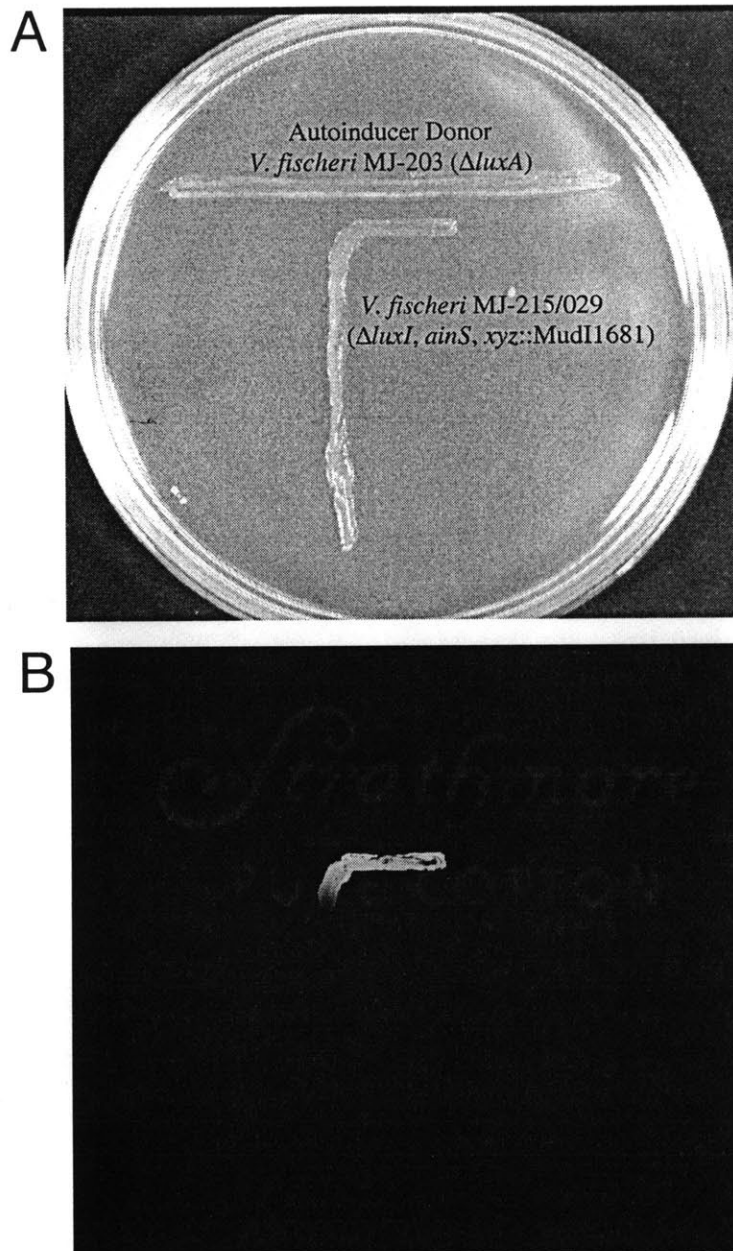
Figure 2. MD029 is capable of producing light when $3OC_6$-HSL and C_8-HSL are supplied exogenously. (A) MD029 cross streaked with the acyl-HSL donor strain MJ-203. (B) The same plate photographed in the dark, indicating that the genes necessary for light production are intact in MD029.

component system, which in combination with the cognate sensor histidine kinase, ArcB, acts as a transcriptional repressor of genes involved in aerobic metabolism and as a transcriptional activator of genes involved in anaerobic metabolism [12]. Because low oxygen levels have been reported to increase luminescence per cell [13], we decided to pursue the *arcA* gene of *V. fischeri* further.

**Isolation and sequence of the *arcA* gene from *V. fischeri*.** The intact gene for *arcA* was isolated from a plasmid-borne library of MJ-1 chromosomal DNA in *E. coli* using PCR analysis as a screen (Materials and Methods). The sequence of the gene and translation product are depicted in Figure 3, and the alignment of the ArcA deduced sequence from *V. fischeri* is aligned with ArcA from other species in Figure 4. The sequences of the ArcA proteins from *V. fischeri, E. coli,* and *Haemophilus influenzae* are very well conserved, which leads us to believe that the protein from *V. fischeri* probably serves a function analogous to that of the ArcA protein in *E. coli*.

Transcription of *arcA* in *E. coli* is positively regulated by the global regulator Fnr [14]. Regulation of transcription by Fnr is believed to involve binding of the protein to a target sequence, the Fnr box, in the promoter region of genes. The consensus sequence for the Fnr box is a 22 bp palindrome that includes the sequence TTGAT in one half and the complementary ATCAA in the other [14, 15]. In the promoter region of *arcA* from *V. fischeri* there are two putative Fnr boxes that match the consensus sequence well (Fig. 3). Multiple Fnr boxes in the promoter regions of target genes in *E. coli* have been reported previously [16]. Both the regulation of *arcA* and the structure of the ArcA protein appear to be very similar in *V. fischeri* and *E. coli*.

The *arcA* gene was isolated because we suspected that it may be regulated by quorum sensing. The hallmark of promoters that are controlled by LuxR and acyl-HSL signals is the *lux* box. In and around the promoter region of *arcA* we identified five

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  1 CAGCTAAGGTAGCAACTTGATCACCAAGTAACGCTTTTTGACGCGATCCATTTCCATCTATGAATAATAC
 96 <A  L  T  A  V  Q  D  G  L  L  A  K  Q  R  S  G  N  G  D  I  F  L  V

 71 TTTGCCCTTTCCATTCTGCTGGAGTACTTCTCTATCCAAAGAATTATCTTTATAACAACGAACCGTTACA
 73 K  G  K  G  N  Q  Q  L  V  E  R  D  L  S  N  D  K  Y  C  R  V  T  V

141 ATCTCGCCATAAAAACACGCCTTCCCGCCAAAGTTTTTAAAATCGATATCAACTANCTGCACTTTATCTT
 50 I  E  G  Y  F  C  A  K  G  G  F  N  K  F  D  I  D  V  V  Q  V  K  D  E

211 CAAACTGATCACAAATATCAGGTAATAAATCCATTTTATGTTCTCCTTGTTTGTAAAATCGACTATACAA
 26 F  Q  D  C  I  D  P  L  L  D  M  K  H  E  G  Q  K  Y  F  R  S  Y  L

281 TAACAACATTTTTCAAATTACTTAGTTAGTATTCGCATAGATATTTACAATTTCAATATAGATATGTTGC
  3 L  L  M
                                              |--Fnr binding site--|
351 CTTATTTATTACTACACTCTATTGTTATCGGTTGCTATTGATATAAATCAATAAAATGTATGGAAATAAA


421 AATCGCTTATTGTTAGAGTGGTGTGTTAAAGGATTAAAATCCTGCTTCTAATGGAGTTTCAGTAAGCAGCTC

                    |--Fnr binding site--|
491 ATCAAATTAGAGCTTTATTTTATTATTGATTTTATATTCAATAATAACCACCAAGGATGGCGTACCGTTT


561 AGGTTTCAATTCTTCTATATTACTTTTTATTAACTATTGAAGAATCTATTTTCCTATTTGTAAATTTTTT


631 ATTATAGAATTAAAATTTGTTAATTCTACTTTTGACTTTAACCAGTTAGTTAGGACCGCCCATGCAAACC

                 M  N  T  V  T  R  N  T  L  K  S  I  F  E  A  E   16
701 CCACAGATCCTAATCGTTGAAGATGAACACGGTAACTCGTAATACGTTAAAAAGCATTTTTGAAGCTGAA

     G  Y  N  V  F  E  A  S  D  G  E  E  M  H  K  V  L  S  E  Q  Q  L  N   39
771 GGCTACAATGTTTTTGAAGCTAGCGATGGCGAAGAAATGCACAAAGTGCTTTCTGAACAGCAGCTTAATC

     L  V  I  M  D  I  N  L  P  G  K  N  G  L  L  L  A  R  E  L  R  E  R  G  63
841 TTGTGATTATGGATATTAACCTGCCAGGTAAAAATGGCCTTCTTCTTGCTCGCGAACTTCGTGAACGAGG

     D  M  A  L  M  F  L  T  G  R  D  N  E  V  D  K  I  L  G  L  E  I  G   86
911 CGATATGGCATTAATGTTCCTAACAGGCCGTGATAATGAAGTTGATAAGATTTTAGGTTTAGAAATTGGC

     A  D  D  Y  I  T  K  P  F  N  P  R  E  L  T  I  R  A  R  N  L  L  T  109
981 GCTGATGATTACATCACAAAACCATTCAACCCACGTGAACTGACTATTCGTGCACGTAACTTATTAACTC

     R  S  M  N  Q  N  T  V  V  E  D  K  K  M  V  E  R  Y  V  F  N  G  W  T  133
1051 GTTCAATGAACCAAAACACAGTTGTTGAAGATAAGAAAATGGTTGAGCGCTATGTGTTCAACGGTTGGAC

     V  E  I  N  S  R  S  L  V  S  P  S  G  E  S  Y  K  L  P  R  S  E  F  156
1121 TGTAGAAATCAACAGCCGTTCTCTTGTTAGCCCAAGCGGTGAGAGCTACAAGCTTCCACGTTCTGAATTC

     R  A  L  L  H  F  C  E  N  P  G  K  I  Q  T  R  A  D  L  L  K  K  M  179
1191 CGTGCTCTGCTTCACTTCTGTGAAAACCCAGGCAAAATCCAGACTCGTGCAGATCTACTGAAAAAAATGA

     T  G  R  E  L  K  P  H  D  R  T  V  D  V  T  I  R  R  I  R  K  D  F  E  203
1261 CTGGTCGTGAGCTTAAGCCACACGATCGTACAGTAGACGTAACTATCCGTCGCATCCGTAAGGACTTTGA

     S  V  T  D  T  P  E  I  I  A  T  I  H  G  E  G  Y  R  F  C  G  D  L  226
1331 ATCTGTAACTGATACTCCAGAGATCATCGCTACAATTCACGGTGAAGGCTACCGCTTCTGTGGTGACCTA

     E  E  L  *                              stem and loop terminator  229
1401 GAAGAGCTATAAGAACCTCCTCCTTATATGACTTTTAAATAAAATGCCCAGTTATCACTAGCTGGGCATT>


1471 TTTTTATCTATATAACATAGC 1491
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Figure 3. Nucleotide sequence and deduced amino acid translation products of *arcA* and the 5'-end of the divergently transcribed *menG*. Putative Fnr binding sites and stem and loop transcriptional terminator are indicated. Five possible *lux* box-like sequences are underlined. An alternative translational start site is indicated in bold for *menG*.

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V. fisc ArcA    1   M N T - - - - - - - - - - V T R N T L K S I F E A E G Y N V   20
E. coli ArcA    1   M Q T P H I L I V E D E L V T R N T L K S I F E A E G Y D V   30
H. infl ArcA    1   M T T P K I L V V E D E I V T R N T L K G I F E A E G Y D V   30


V. fisc ArcA   21   F E A S D G E E M H K V L S E Q Q L N L V I M D I N L P G K   50
E. coli ArcA   31   F E A T D G A E M H Q I L S E Y D I N L V I M D I N L P G K   60
H. infl ArcA   31   F E A E N G V E M H H I L A N H N I N L V V M D I N L P G K   60


V. fisc ArcA   51   N G L L L A R E L R E R G D M A L M F L T G R D N E V D K I   80
E. coli ArcA   61   N G L L L A R E L R E Q A N V A L M F L T G R D N E V D K I   90
H. infl ArcA   61   N G L L L A R E L R E E L S L P L I F L T G R D N E V D K I   90


V. fisc ArcA   81   L G L E I G A D D Y I T K P F N P R E L T I R A R N L L T R   110
E. coli ArcA   91   L G L E I G A D D Y I T K P F N P R E L T I R A R N L L S R   120
H. infl ArcA   91   L G L E I G A D D Y L T K P F N P R E L T I R A R N L L H R   120


V. fisc ArcA  111   S M N Q N T V V E D K K M V E R Y V F N G W T V E I N S R S   140
E. coli ArcA  121   T M N L G T V S E E R R S V E S Y K F N G W E L D I N S R S   150
H. infl ArcA  121   A M P H Q E K - E N T F G R E F Y R F N G W K L D L N S H S   149


V. fisc ArcA  141   L V S P S G E S Y K L P R S E F R A L L H F C E N P G K I Q   170
E. coli ArcA  151   L I G P D G E Q Y K L P R S E F R A M L H F C E N P G K I Q   180
H. infl ArcA  150   L I T P E G Q E F K L P R S E F R A M L H F C E N P G K L Q   179


V. fisc ArcA  171   T R A D L L K K M T G R E L K P H D R T V D V T I R R I R K   200
E. coli ArcA  181   S R A E L L K K M T G R E L K P H D R T V D V T I R R I R K   210
H. infl ArcA  180   T R E E L L K K M T G R E L K P Q D R T V D V T I R R I R K   209


V. fisc ArcA  201   D F E S V T D T P E I I A T I H G E G Y R F C G D L E E L   229
E. coli ArcA  211   H F E S T P D T P E I I A T I H G E G Y R F C G D L E D   238
H. infl ArcA  210   H F E D H P N T P N I I M T I H G E G Y R F C G D I E   236
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Figure 4. Comparison of ArcA amino acid sequences from *V. fischeri, E. coli* [Drury L. S., 1985 #22], and *Haemophilus influenzae* [17]. The ArcA protein from *V. fischeri* is 83% identical and 91% similar in sequence to the ArcA protein of *E. coli*, and it is 74% identical and 85% similar to that from *H. influenzae*. In the figure identical amino acids are boxed and shaded dark, and similar amino acids are boxed and shaded lightly.

prospective LuxR binding sites (Fig. 3). Each of these putative *lux* boxes shares 11

nucleotides with the consensus sequence from *V. fischeri lux* boxes described in Chapter

4. Although the degree of similarity to the consensus sequence is lower than that for the

other quorum-regulated proteins identified, the large number of putative *lux* boxes in the

promoter region suggests that *arcA* may be regulated by LuxR and $3OC_6$-HSL. In general,

the degree of similarity between a particular *lux* box sequence and the consensus

palindrome may influence the extent or manner of a gene's regulation by quorum sensing in

V. fischeri. With respect to the presence of multiple *lux* box-like sequences in the promoter

of *arcA*, we have also observed multiple sequences that resemble a *lux* box in the promoter

region of *ribB* [18]. To investigate control of *arcA* by quorum sensing, the Mu-R fusion

junction could be cloned, or a similar fusion constructed, and mobilized into strain MJ215.

Comparison of β-galactosidase activity of this new strain grown with and without $3OC_6$-

HSL should resolve the issue.

Transcribed divergently from *arcA* in *V. fischeri* is a gene whose partial translation

product is very similar to that of *menG* from several species of bacteria (Figs. 3 and 5).

MenG in *E. coli* catalyzes the final step of menaquinone synthesis. Menaquinone serves as

a major quinone in the mediation of electron transfer between the protein components of

respiratory chains during anaerobic respiration [12]. The genes that encode ArcA and

MenG are located at 100 and 88.7 minutes, respectively, on the *E. coli* chromosome [19].

In *V. fischeri* the chromosomal architecture in this region has apparently diverged from that

of *E. coli*.

The MudI1681-R fusion junction from MD029 contains *acfA*. The

fusion joint containing the proximal half of MudI1681, containing the *lacZ* gene and

upstream *V. fischeri* DNA, was cloned from the MD029 chromosome into pBR322 to yield

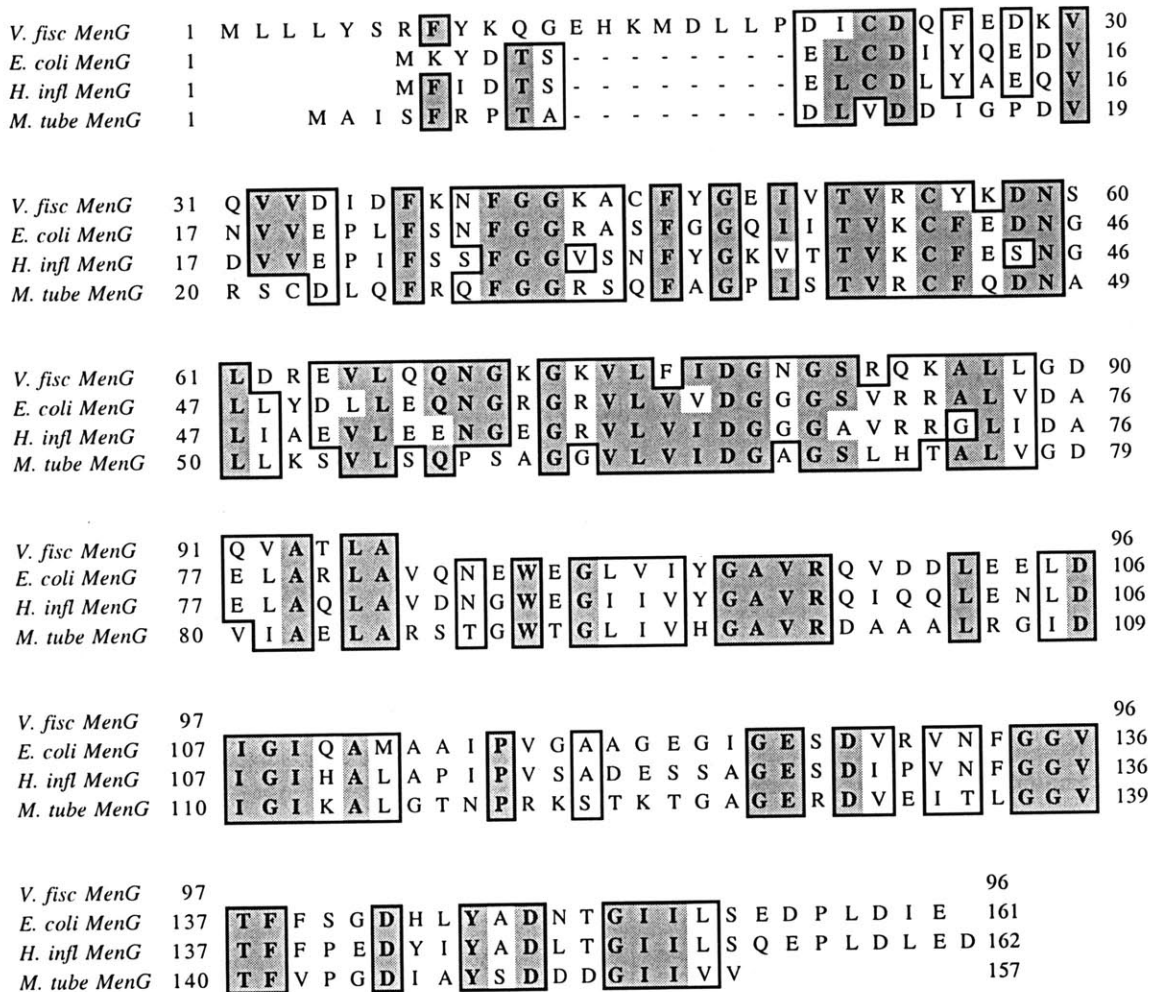pBR029. Chromosomal DNA from MD029 was digested with *Sal*I, ligated into the same

V. fisc MenG   1   M L L L Y S R F Y K Q G E H K M D L L P D I C D Q F E D K V   30
E. coli MenG   1   M K Y D T S - - - - - - - E L C D I Y Q E D V   16
H. infl MenG   1   M F I D T S - - - - - - - E L C D L Y A E Q V   16
M. tube MenG   1   M A I S F R P T A - - - - - - - D L V D D I G P D V   19

V. fisc MenG   31   Q V V D I D F K N F G G K A C F Y G E I V T V R C Y K D N S   60
E. coli MenG   17   N V V E P L F S N F G G R A S F G G Q I I T V K C F E D N G   46
H. infl MenG   17   D V V E P I F S S F G G V S N F Y G K V T T V K C F E S N G   46
M. tube MenG   20   R S C D L Q F R Q F G G R S Q F A G P I S T V R C F Q D N A   49

V. fisc MenG   61   L D R E V L Q Q N G K G K V L F I D G N G S R Q K A L L G D   90
E. coli MenG   47   L L Y D L L E Q N G R G R V L V D G G G S V R R G L I D A   76
H. infl MenG   47   L I A E V L E E N G E G R V L V I D G A G S L H T A L V G D   76
M. tube MenG   50   L L K S V L S Q P S A G G V L V I D G A G S L H T A L V G D   79

                                                                         96
V. fisc MenG   91   Q V A T L A   106
E. coli MenG   77   E L A R L A V Q N E W E G L V I Y G A V R Q V D D L E E L D   106
H. infl MenG   77   E L A Q L A V D N G W E G I I V Y G A V R Q I Q Q L E N L D   106
M. tube MenG   80   V I A E L A R S T G W T G L I V H G A V R D A A A L R G I D   109

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V. fisc MenG   97   136
E. coli MenG 107   I G I Q A M A A I P V G A A G E G I G E S D R V N F G G V   136
H. infl MenG 107   I G I H A L A P I P V S A D E S S A G E S D I P V N F G G V   136
M. tube MenG 110   I G I K A L G T N P R K S T K T G A G E R D V E I T L G G V   139

                                                              96
V. fisc MenG   97   161
E. coli MenG 137   T F F S G D H L Y A D N T G I I L S E D P L D I E   161
H. infl MenG 137   T F F P E D Y I Y A D L T G I I L S Q E P L D L E D   162
M. tube MenG 140   T F V P G D I A Y S D D D G I I V V   157

Figure 5. Comparison of the N-terminal 96 amino acids of MenG from *V. fischeri* to the sequences of MenG from *E. coli* [20], *H. influenzae* [17], and *Mycobacterium tuberculosis* [21]. In the figure identical amino acids are boxed and shaded dark, and similar amino acids are boxed and shaded lightly.

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site in the cloning vector, and used to transform *E. coli* strain Novablue. Two of the several thousand colonies screened appeared blue on LB agar with X-gal only after five days at 37°C and two days at room temperature. One of these colonies was recovered, but the other could not be regrown from the agar plate, which had dehydrated.

To test whether the cloned fragment contained the fusion joint that was responsive to 3OC$_6$-HSL, the 16-kb fragment was cloned into the *Sal*I site of pSup102 and conjugatively delivered into *V. fischeri* strains MJ-100 (wild-type for 3OC$_6$-HSL production) and MJ-215 (3OC$_6$-HSL-deficient). β-galactosidase activity in MJ-100 was higher than that in MJ-215, and the difference in activity could be complemented by exogenous addition of synthetic 3OC$_6$-HSL to the growth medium (data not shown). Stimulation of β-galactosidase activity in the mutant by addition of 3OC$_6$-HSL to levels in the parent strain indicated that the cloned DNA contained the 3OC$_6$-HSL-responsive *lacZ* fusion.

Subcloning and sequencing the area of interest revealed that MudI had inserted after nucleotide 461 of the coding region of the *acfA* gene in MD029. *acfA* was shown in Chapter 4 to encode one of the proteins identified by 2-D PAGE whose production is dependent on 3OC$_6$-HSL and LuxR. In *Vibrio cholerae* the *acfA* (accessory colonization factor) gene is necessary for efficient colonization of intestinal epithelium in an infant mouse model system [22].

By identifying a gene that is already known to be regulated by quorum sensing, we have demonstrated that transposon reporter fusions can be used to identify quorum-regulated genes in *V. fischeri*. Attempting to clone such gene fusions by screening for β-galactosidase activity in *E. coli* without the *trans* genes for LuxR and LuxI may seem foolish at first glance, given their presumed mode of transcriptional regulation. This had been a major concern of ours. The observation that identification of the relevant fusion

took seven days suggests that either the activity was very low in *E. coli* and the prolonged

incubation time was necessary for sufficient conversion of the chromogenic substrate, or *E.*

coli can transcribe *V. fischeri* quorum regulated genes in response to stress, such as

nutrient limitation and desiccation.

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Chapter 7. Future directions

Bioluminescence in *V. fischeri* is regulated in a cell-density dependent manner by quorum sensing. Quorum sensing is mediated by the transcriptional regulator LuxR and two acyl-HSL signal molecules, the activator molecule $3OC_6$-HSL and the inhibitor molecule C_8-HSL [1, 2]. In this study we have identified a riboflavin synthesis protein, a suspected pilus protein, and two proteins of unknown function whose production, along with that of the proteins necessary for light production, is regulated by quorum sensing. In addition, genes for two putative transcriptional regulators and a third gene of unknown function are also believed to be influenced by the LuxR regulatory circuit. Our results suggest that quorum sensing regulates a multi-locus regulon in *V. fischeri*.

Figure 1 combines the previously characterized regulation of the *lux* operon with the quorum sensing regulon of *V. fischeri* as discussed in the supporting chapters of this work. Many aspects of the diagram remain untested. In this section future studies that will further define the extent and regulation of the LuxR regulatory circuit are discussed.

A forward versus a reverse genetic approach to identify additional QSR proteins. The limits of 2-D PAGE to identify quorum-regulated proteins were obvious in the initial comparison of proteins made by MJ-100 and MJ-215. Only three of the seven Lux proteins were identified. Presumably, the other four Lux proteins were made, but they were not detected by our analysis. Loss of some proteins during sample preparation is possible. Certain proteins may not precipitate in 80% acetone, which is not typical in sample preparation for 2-D PAGE, but it was found to increase resolution dramatically and permit increased loading of the gels. The detection of proteins by the

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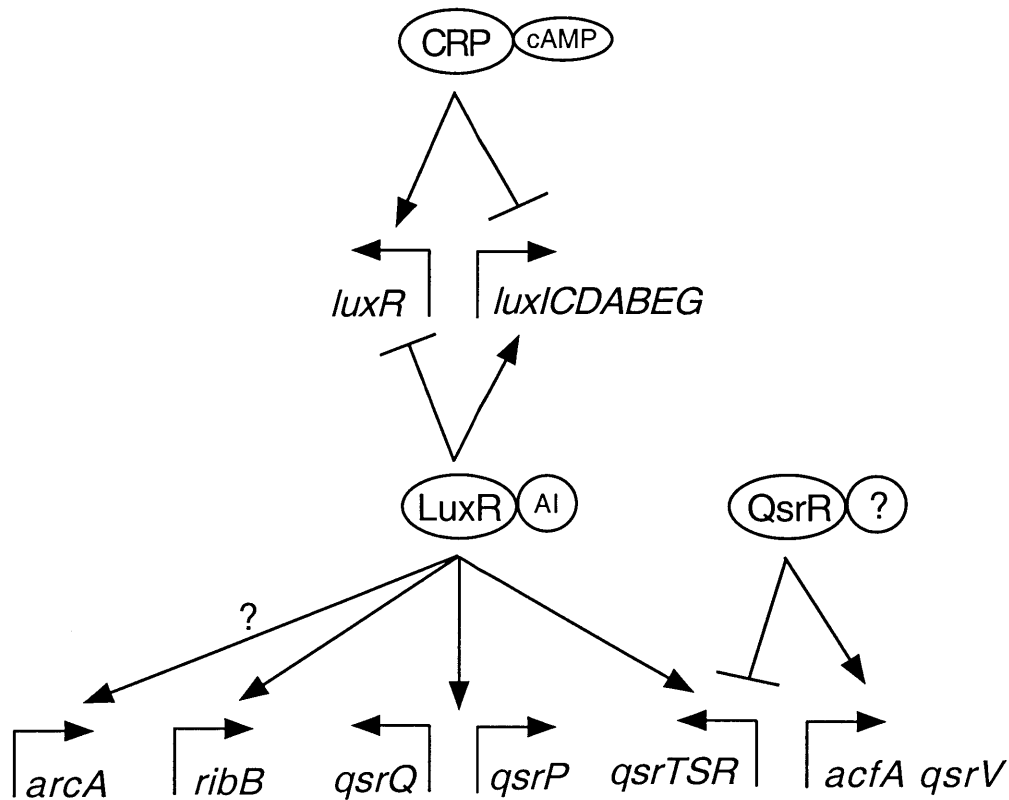
Figure 1. Model of quorum regulation in *Vibrio fischeri*.

silver staining procedure used here is among the most sensitive. Optimization of the

procedure for increased sensitivity may be possible, but its usefulness is doubtful given the

heavy loading of some gels that were used in this analysis (data not shown). In addition to

the limited sensitivity of silver-stained PAGE gels, and the possibility of loss during

sample preparation, the range of isoelectric points of proteins that can be visualized by

traditional 2-D PAGE is relatively narrow. According to the manufacturers of the

equipment we used in this study (Oxford Glycosystems) the high concentration of urea in

the isoelectric focusing first dimension gels has a buffering effect that prevents the pH

gradient from reaching basic values above about 7.5. Very basic proteins run off the end

of the gel and are lost from analysis. For analysis of basic proteins, nonequilibrium pH gel electrophoresis (NEPHGE) can be used in place of traditional 2-D PAGE as described originally by O'Farrell [3]. In NEPHGE proteins are not focused to their isoelectric point, but instead they move at varying rates through the gel according to charge. The increased range of NEPHGE may identify additional basic quorum-regulated proteins, but it probably will not account for the "missing" Lux proteins, because each has a predicted pI below 7.0 [4]. An expanded search for additional quorum regulated proteins probably requires a genetic approach.

2-D PAGE was used effectively here to identify quorum-regulated proteins, but the limited sensitivity and the indirect route to isolation of the relevant genes, the actual elements that are regulated by LuxR, highlights its inefficiency. 2-D PAGE has the great advantage over transposon mutagenesis of being able to identify proteins that are essential for the survival of the cell, but this is probably not an issue with LuxR regulated genes, because MJ-208 (ΔluxR) grows at a rate similar to that of the parent strain [5]. In this study we used MudI1681 to identify a gene (acfA) already thought to be regulated by quorum sensing and a second gene (arcA) that requires further testing with respect to regulation by quorum sensing. MudI1681 was chosen as the reporter vehicle because it was known from previous studies to transpose and have activity in V. fischeri [6, 7]. The choice of MudI1681, though, proved to be ill-advised for two reasons. First, the high background of β-galactosidase activity from all transconjugants made screening for activity in response to quorum regulation problematic. Second, because the transposase is encoded in the transposable element, multiple insertions were common. This made identification of the appropriate fusion junction difficult. Multiple insertions may have also contributed to the high background of β-galactosidase activity.

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Before choosing MudI1681, we experimented with some mini-Tn5 transposon derivatives [8] that have the potential to alleviate the problems with MudI1681 listed above. Mini-Tn5 Cm transposed inefficiently at a frequency of approximately 10^{-7} per recipient as did a chloramphenicol resistant derivative of mini-Tn5 phoA [9]. Optimization of Tn5 mutagenesis or use of another transposable element to saturate the chromosome of *V. fischeri* is probably the most efficient way to isolate additional quorum-regulated genes in this bacterium.

Transcriptional regulation of *arcA, qsrQ,* and *qsrR*. The promoter regions of *qsrR, qsrQ,* and, to a lesser extent, *arcA* suggest regulation by quorum sensing, but to demonstrate quorum-regulation, transcriptional fusions of each to a reporter gene, such as *lacZ*, should be constructed and tested. The constructs could be tested in either *E. coli* containing *luxR* or *V. fischeri* strain MJ-215 and assayed with and without autoinducer added exogenously. Demonstration of activity in the native organism is preferable, but working with plasmids is easier in *E. coli*. In Chapter 5 fusion of *qsrQ* to the gene for GFP suggested that *qsrQ* is regulated by quorum sensing. GFP fusions are not traditionally used for monitoring regulation of transcription, and the reported results were only qualitative in nature because regulation of luminescence and, presumably, flavonoids by quorum sensing in *V. fischeri* made quantitation of fluorescence difficult.

The genes for *qsrQ* and *qsrR* were identified based on the observation that the dyadic nature of the *lux* box from *V. fischeri* has the potential to regulate transcription in a bi-directional manner. Similar sequences have been identified in the promoter regions of quorum regulated genes from *P. aeruginosa* and *A. tumefaciens* [10, 11]. If the constructs described above do confirm bi-directional transcriptional activation by LuxR at target promoters, sequence upstream of genes known to be regulated by quorum sensing should be searched for divergent open reading frames in other organisms as well.

Pilus biogenesis and *acfA*. The finding that *acfA* from *V. fischeri* and *V. cholera* appears to encode a pilin suggests that pilus biogenesis in *V. fischeri* is regulated by quorum sensing. The N-terminal 20 amino acids of a 20 kD pilus protein isolated from *V. cholerae* are identical to the N-terminus of OmpW [12], which shares sequence similarity with AcfA. Pilins that act as adhesins and interact with receptors on eukaryotic cells would be expected to have diverse sequences that reflect the diversity of the molecules they interact with. Despite this expected variation, sequence similarity between pilins has been used to define classes of pili [13]. Because the two AcfA proteins and OmpW are not similar in sequence to pilins of classes that have been defined previously, they may represent a new class of pilins.

We plan to examine strains MJacfX and MJ-215 for altered pilus production at high population density. If differences are observed between the mutants and the parent strains, this would implicate *acfA/qsrV* and autoinducer in pilus biogenesis. To rigorously show that AcfA is a pilin, antibodies need to be raised against the purified protein, and the anti-AcfA serum should recognize pili on the surface of *V. fischeri*. Recent evidence that biogenesis of type 4 pili from *P. aeruginosa* requires *rhlI* [14] suggests that quorum sensing may regulate pilus formation in several species of bacteria that have quorum sensing systems. AcfA is not similar in sequence to the type 4 pilins, which have a highly conserved N-terminus.

Pili require many genes for their production and assembly. These can include structural pilins, adhesins, anchor proteins, periplasmic chaperones, members of dedicated secretion pathways amongst others [15]. New types of proteins involved in pilus biogenesis continue to be identified in model systems. The involvement of several proteins in pilus formation and ongoing identification of new types of pilus proteins suggests that the mutant phenotypes of *qsrP, qsrQ, qsrS,* and *qsrV* should be examined for altered pilus

formation. These genes that do not have a putative function may be instructive in identifying new proteins necessary for elaboration of pili.

LysR-type transcriptional regulators and quorum sensing. Perhaps the most intriguing observation contained here is the possible interaction between the regulation of gene expression by quorum sensing and two putative LTTRs. In Figure 1 LuxR is shown activating transcription of *qsrRST,* and QsrR is shown repressing its own transcription and activating transcription of *acfA.* This model is consistent with the action of many LTTRs [16] and sequence analysis of the promoter region shared by the two divergent operons, but it has not been demonstrated experimentally. Autoregulation by *qsrR* and regulation of *acfA* could be investigated initially by examining the activity of *qsrR::lacZ* and *acfA::lacZ* (already constructed in Chapter 6) transcriptional fusions in a *qsrR* mutant. A *qsrT* mutant would be helpful in determining its role in regulation of these and other genes. The promoter region of *acfA* contains an interrupted dyad and appropriately spaced -10 consensus sequence that suggests that the dyad is the activation binding site as identified in the promoter regions of other genes that are the target of LTTRs [16]. If the spacing between the activation and recognition sites for LTTRs is conserved in the *acfA* promoter region, then the *lux* box dyad is the likely recognition binding site. In this case, QsrR and/or QsrT could influence transcription of all genes with a *lux* box in the promoter region.

The vast majority of LTTRs require a small coinducer molecule for regulation of target genes [16]. The two autoinducers made by *V. fischeri* are likely candidates as coinducers. Redundancy of regulation by autoinducer sensing systems may reflect a fine tuning of population density sensing and response. Conversely, the coinducer may not be of bacterial origin. An environmental cue or host-derived signal may be involved in activating transcription via QsrR and QsrT. When the sequence of the LTTR operon was

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completed, the deduced translational product of QsrS was reminiscent of the precursor proteins used by Gram-positive bacteria and *Anabaena* as signal molecules. The structural genes for these short peptide signals code for precursor proteins that vary from about 20 to 60 amino acids. In the case of unmodified peptide signals, the structural gene for the precursor is usually linked to the gene for the protein that senses the signal peptide, usually a two-component signal transducer. The first step towards identifying the coinducer will be to identify genes that are regulated by QsrR and/or QsrT, such as *acfA,* and create a reporter construct whose activity is dependent on expression or repression of such a target gene. The engineered phenotype of the reporter can then be used to assay for the coinducer.

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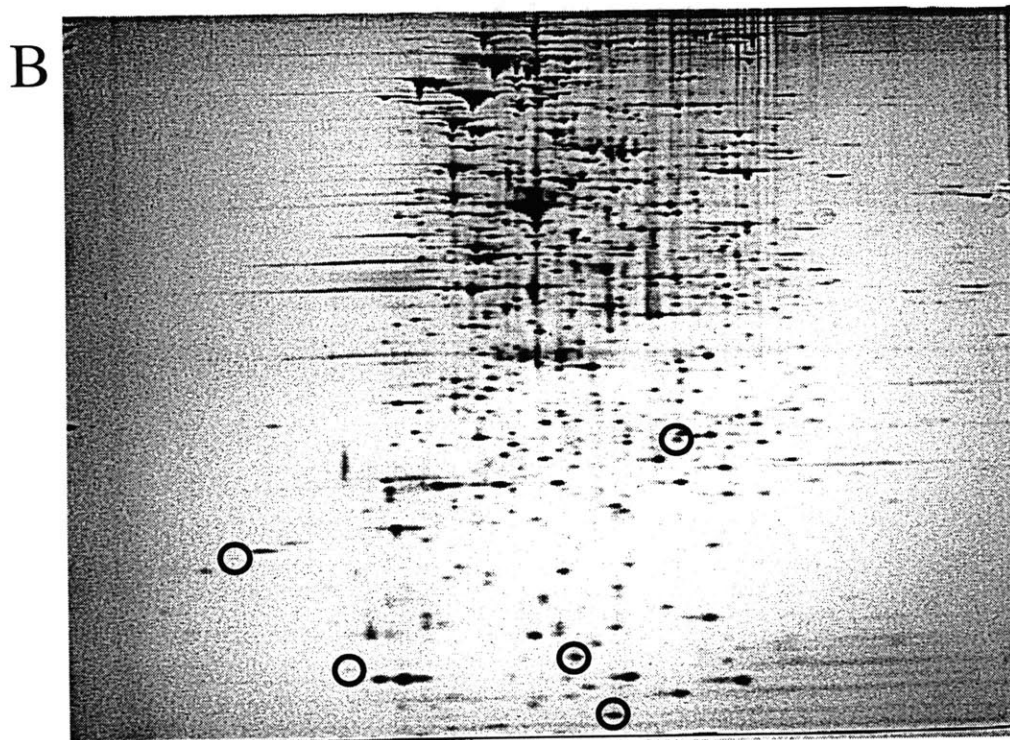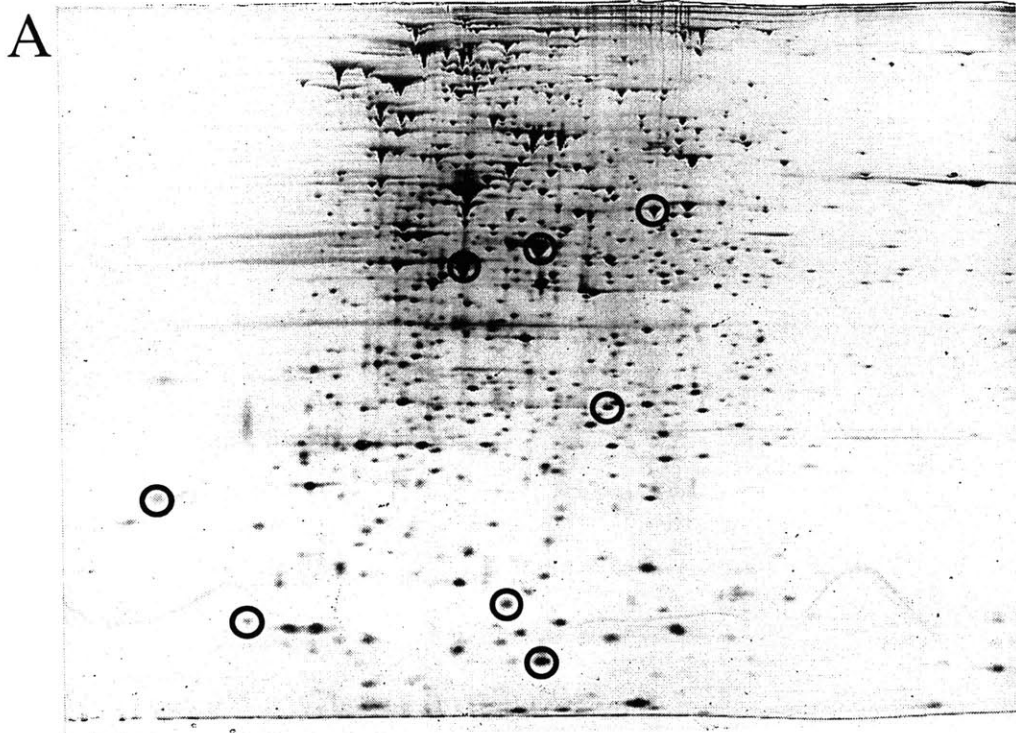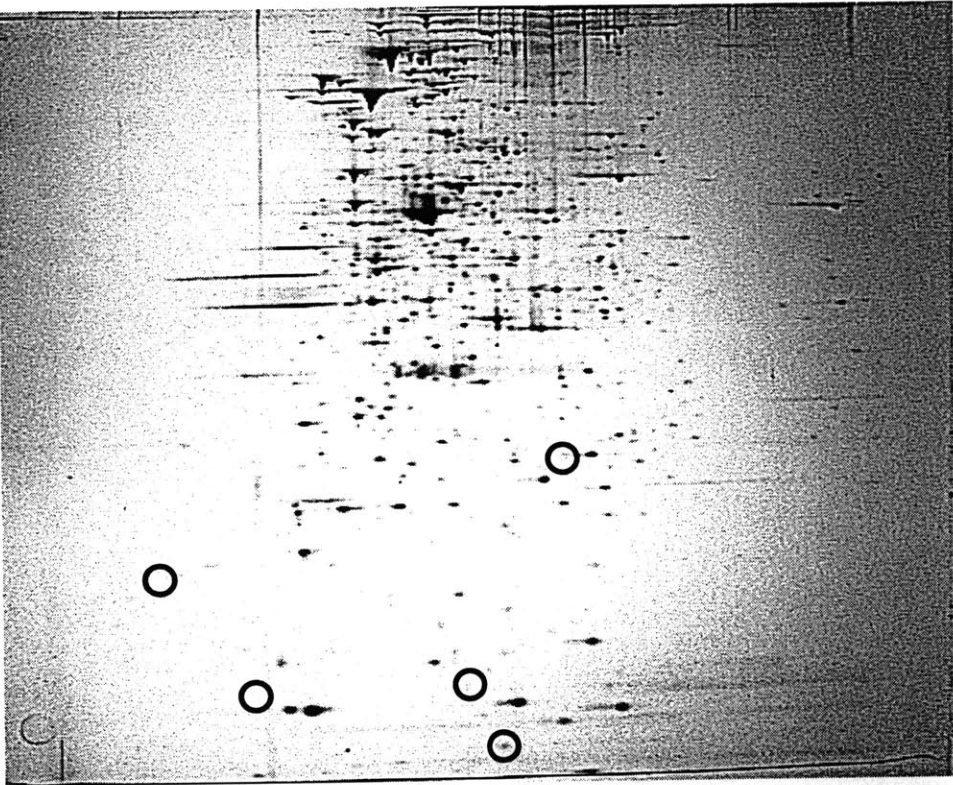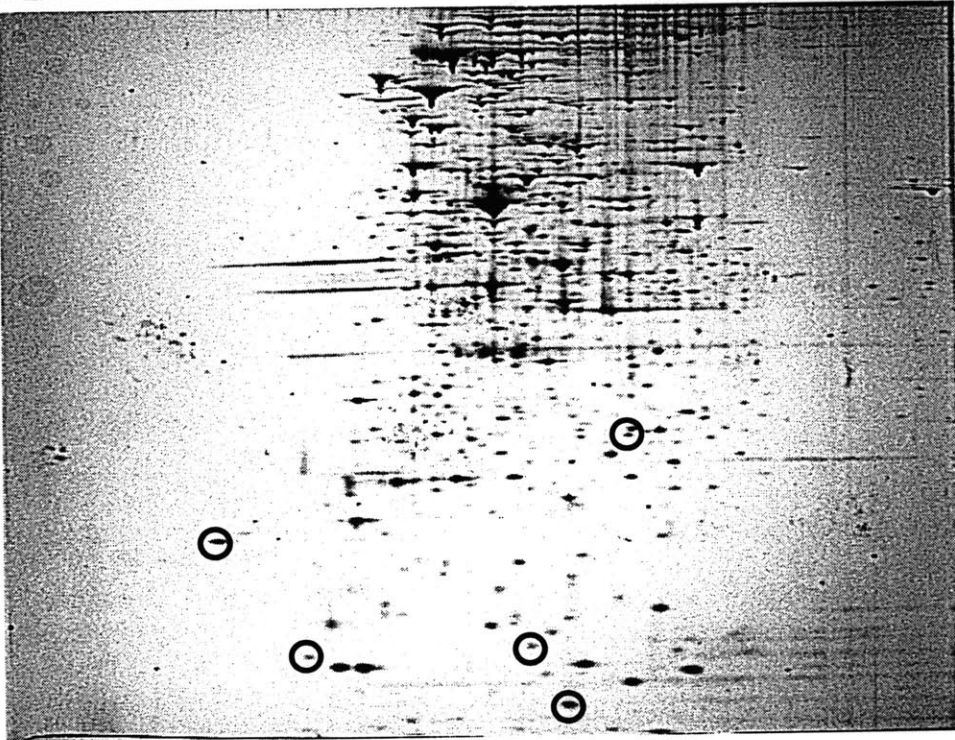**Appendix A.** Representative two-dimensional PAGE gels.

Figure A.1 MJ-100 grown to (A) stationary phase; (B) early stationary
phase; and (C) exponential phase. (D) MJ-216 grown to exponential phase
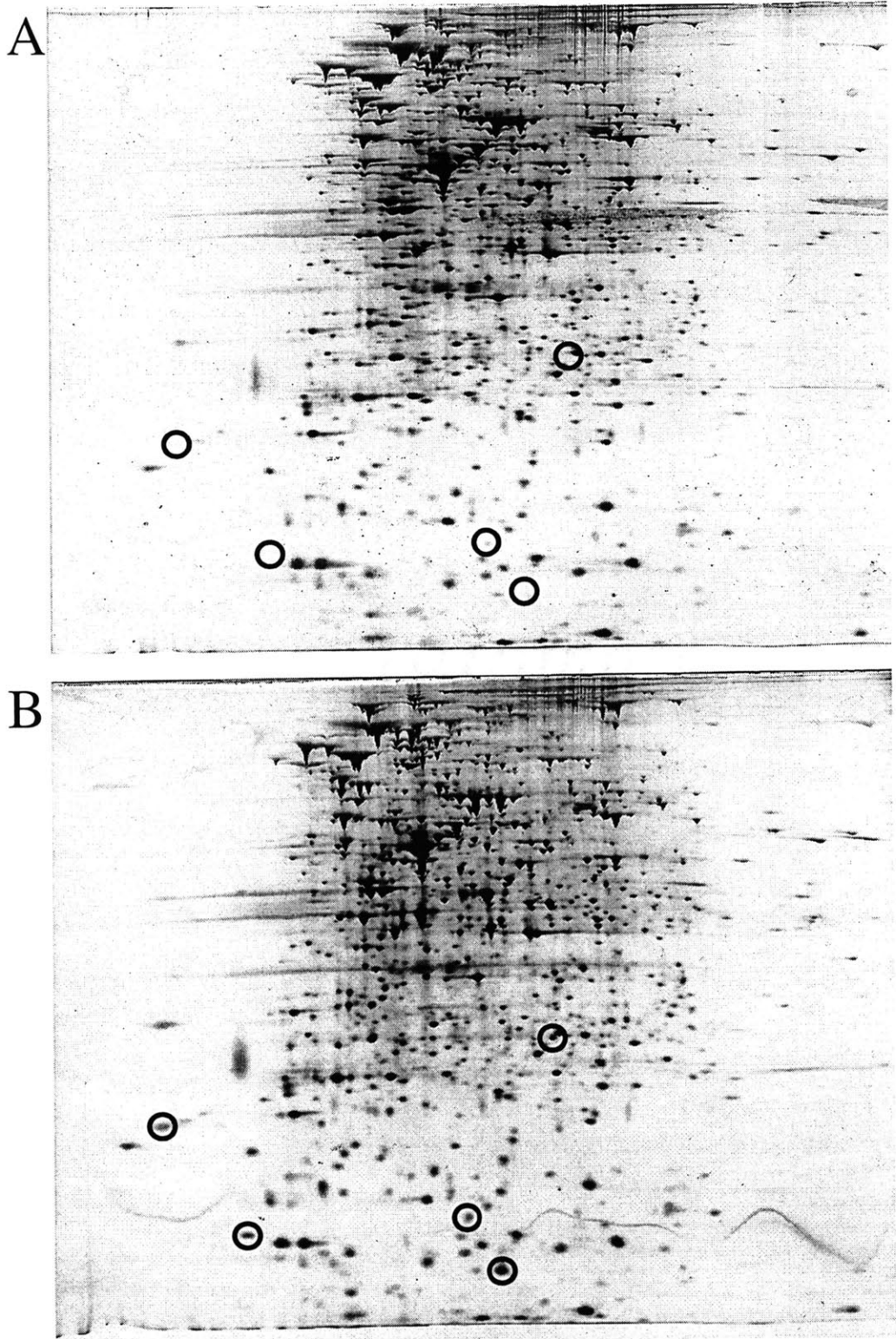(AI-2 deficient strain).

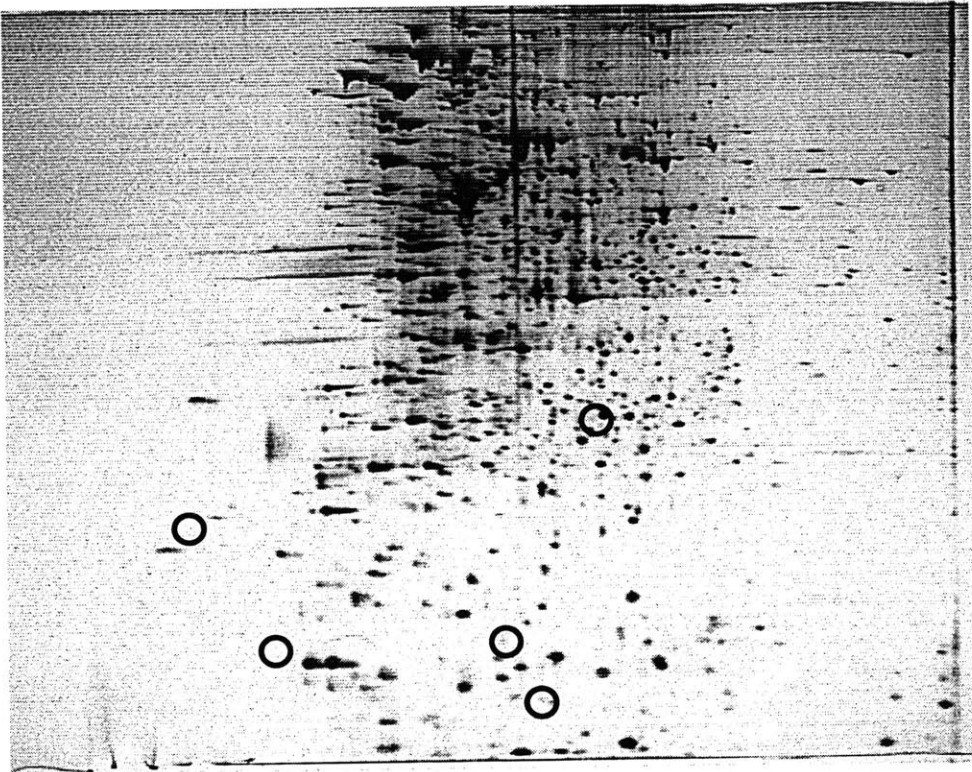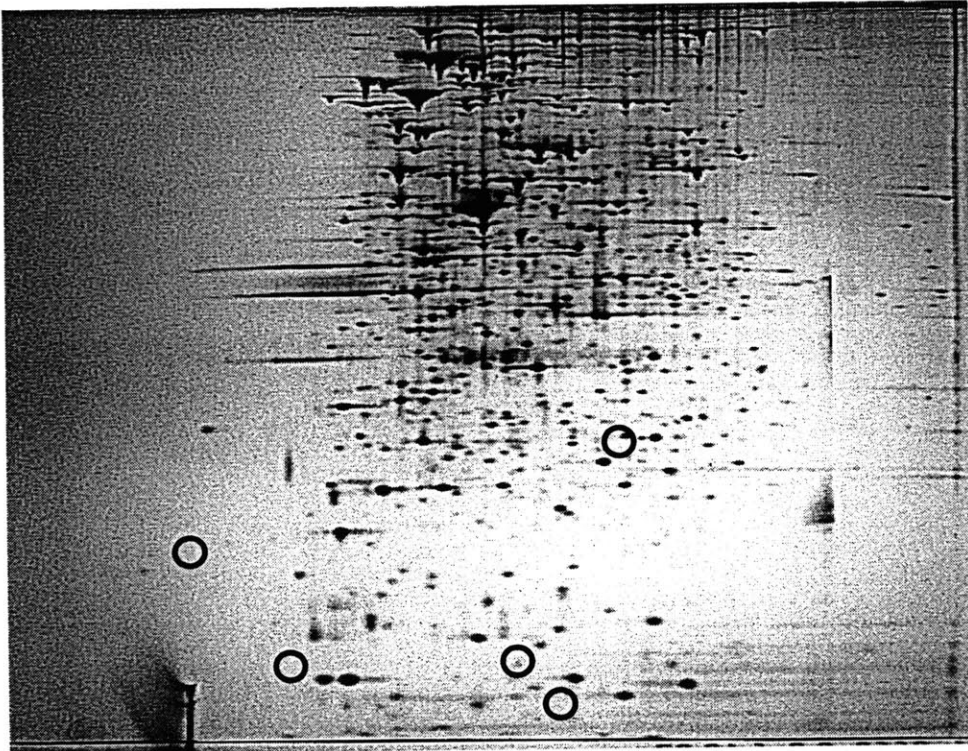Figure A.2 MJ215 (AI-1 and AI-2 deficient strain) grown (A) without autinducer; (B) with 100 nm AI-1; and (C) with 100 nm AI-2.

Fig. A.2 continued
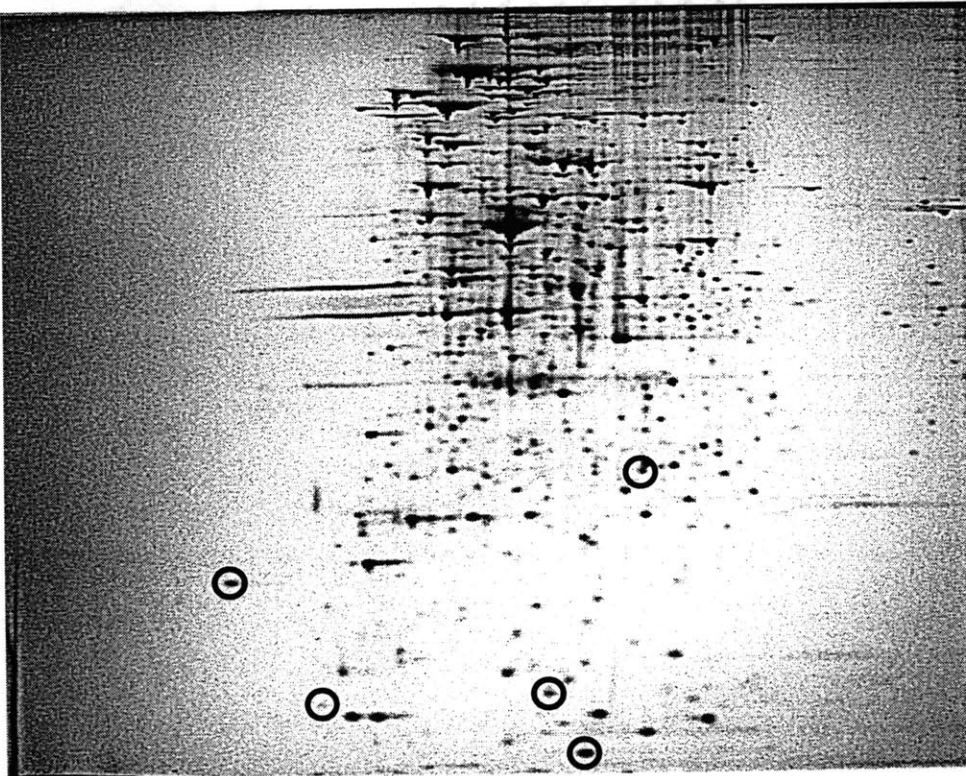
Figure A.3 Strains (A) MJ-211 (AI-1 deficient); (B) MJ-216
(AI-2 deficient); and (C) MJ-208 (LuxR deficient).

C
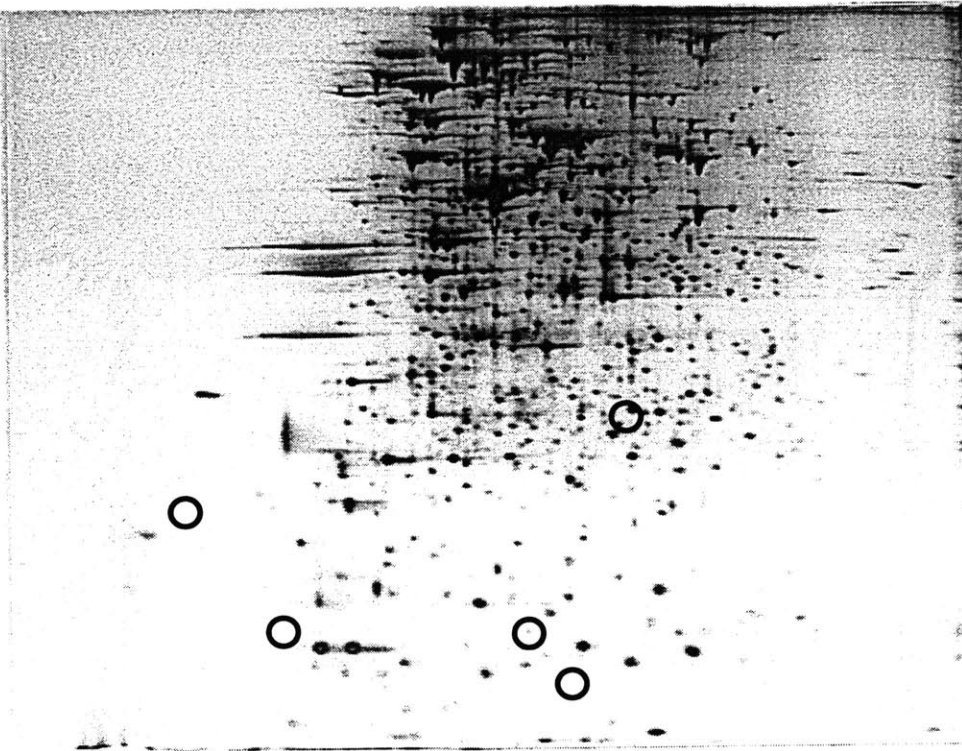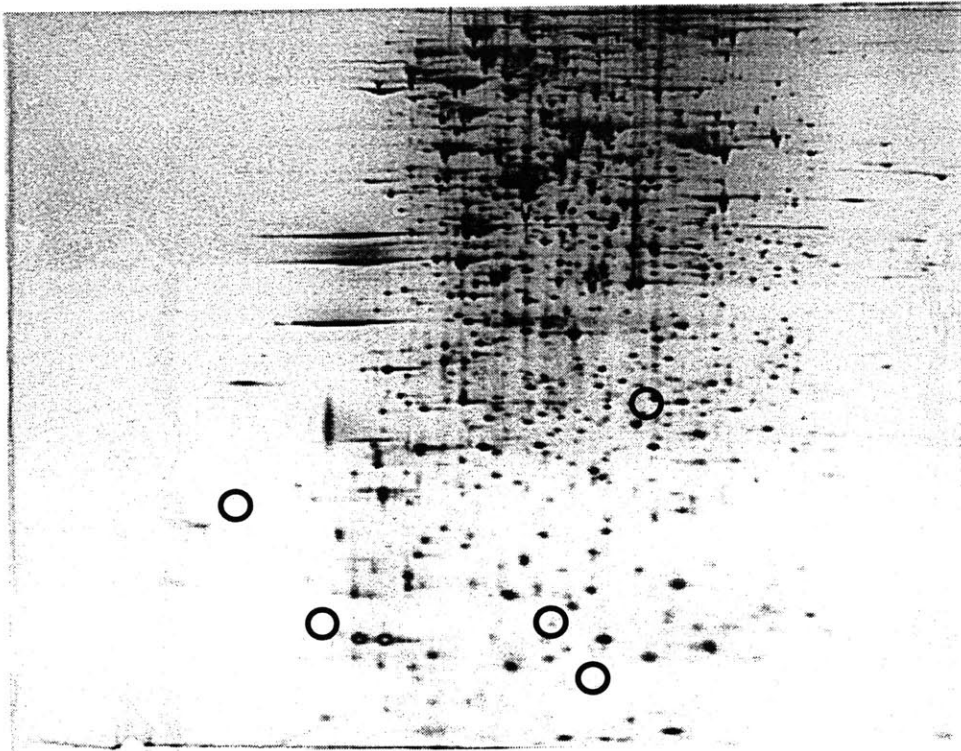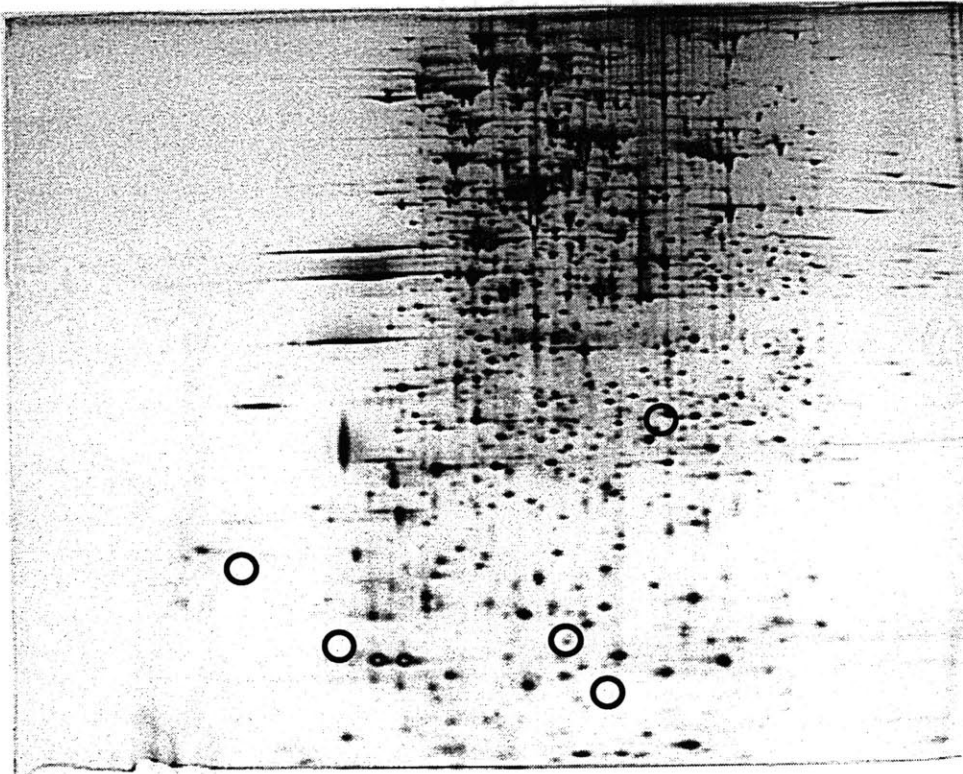
Figure A.4 Strain MJ-208 (LuxR deficient) grown with (A) 100 nm
AI-1; and (B) 100 nm AI-2.
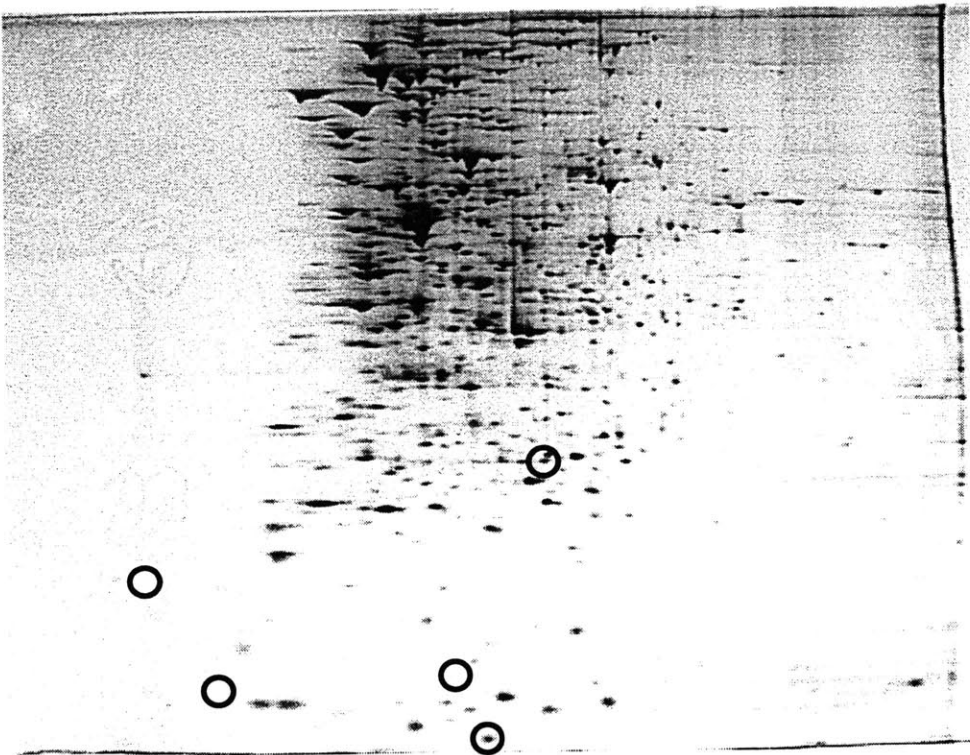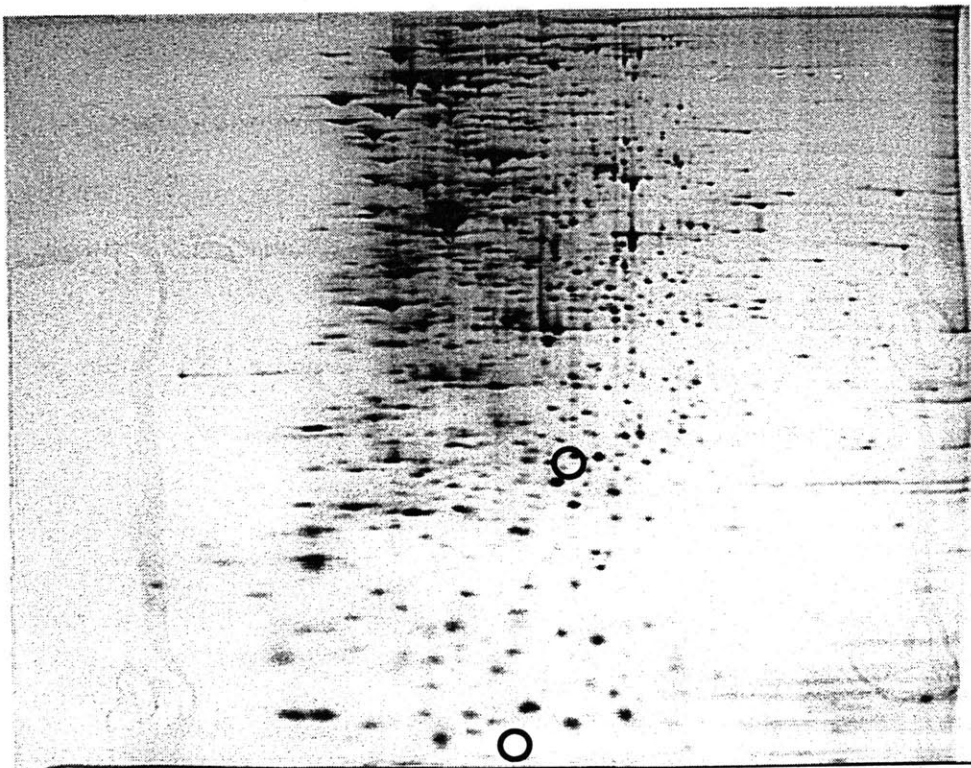
Figure A.5 Cell fractionation. Soluble fractions (cytoplasm
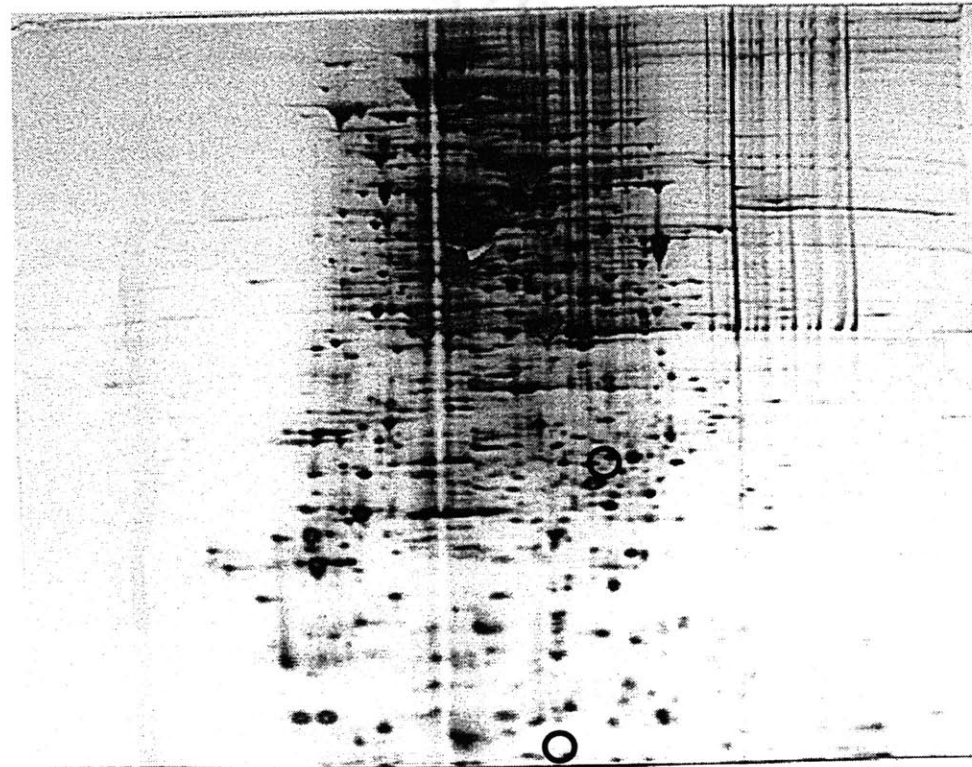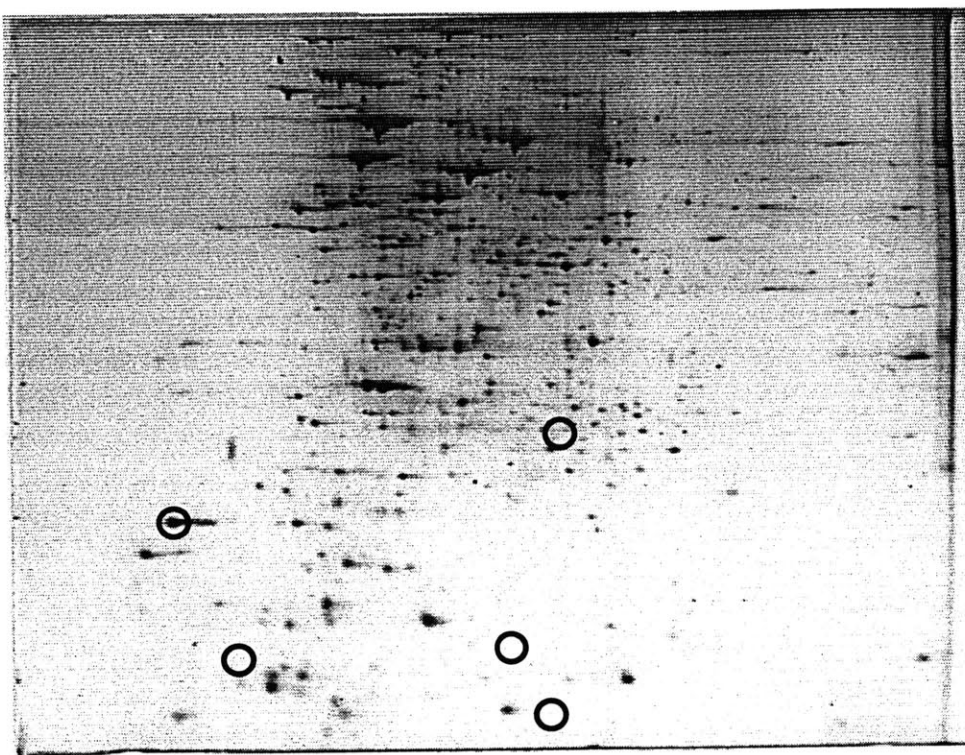and periplasm) of (A) strain MJ-100; and (B) strain MJ-215
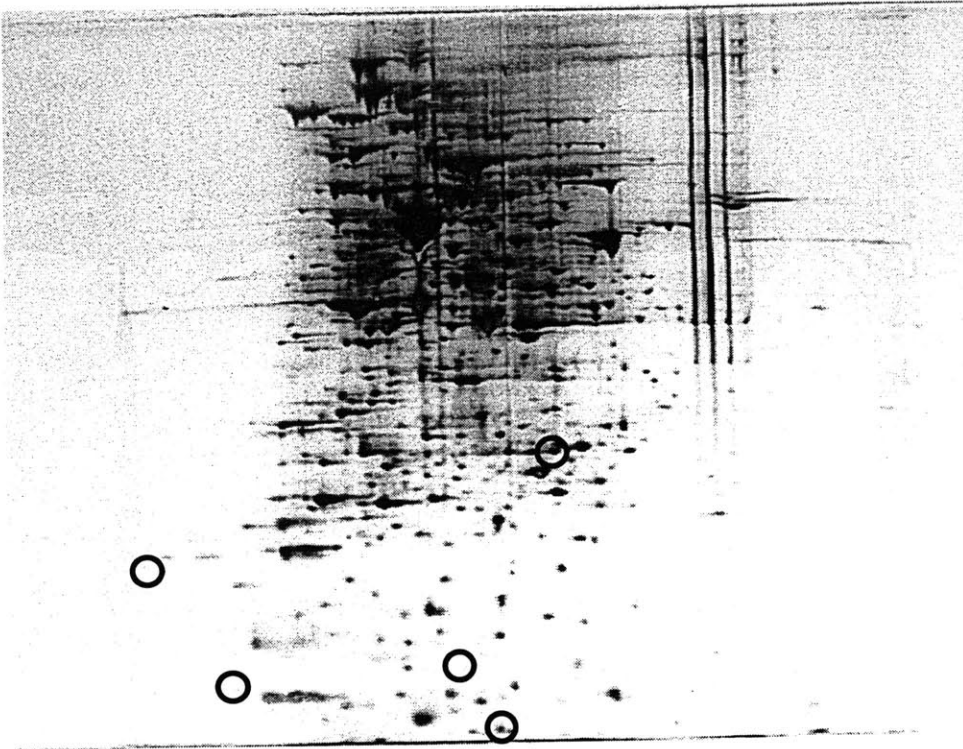
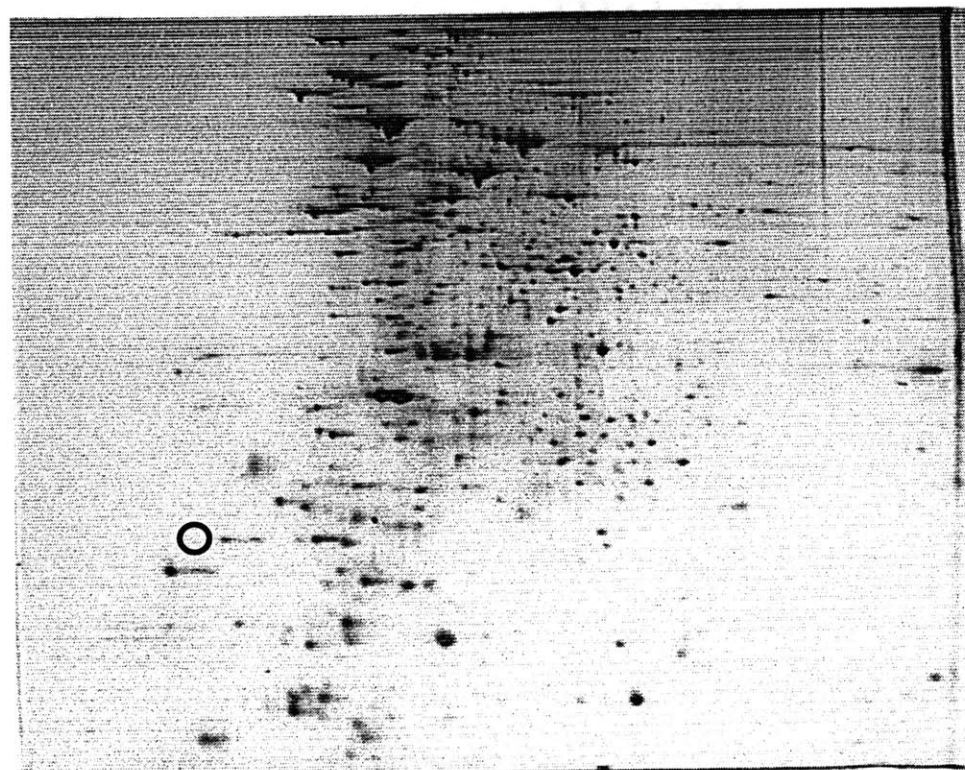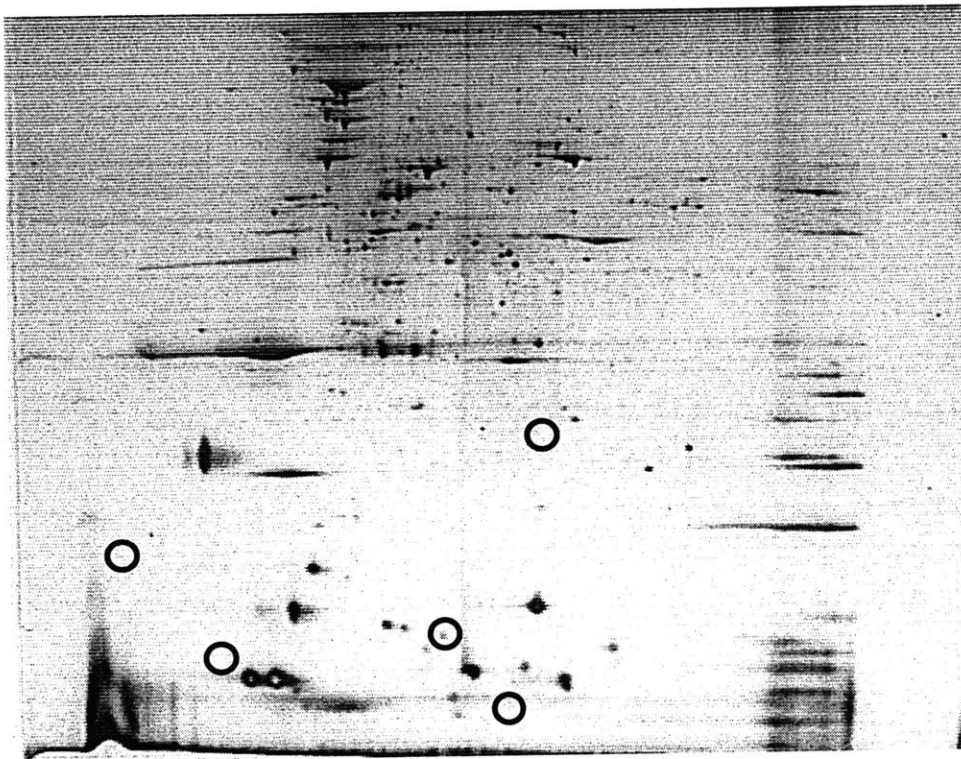Figure A6. Cell fractionation. Periplasmic fractions of (A) MJ-100; and (B) MJ-215.

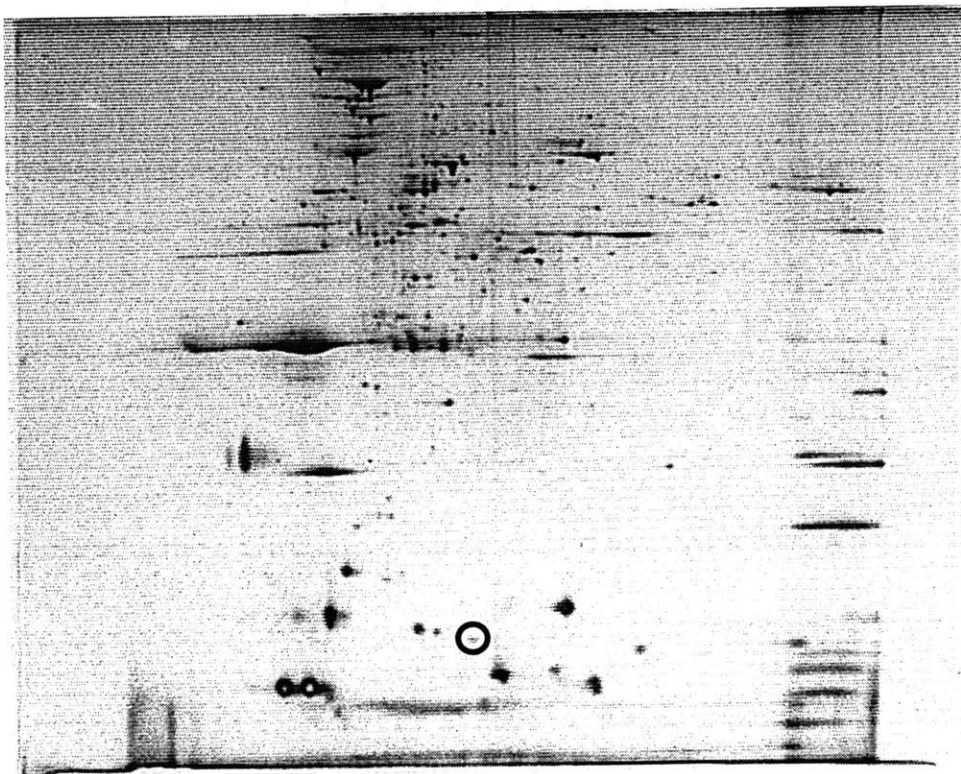Figure A7. Cell fractionation. Cytoplasmic membrane fractions of strains (A) MJ-100; and (B) MJ-215.

Figure A8. Cell fractionation. Outer membrane frations of strains
(A) MJ-100; and (B) MJ-215.

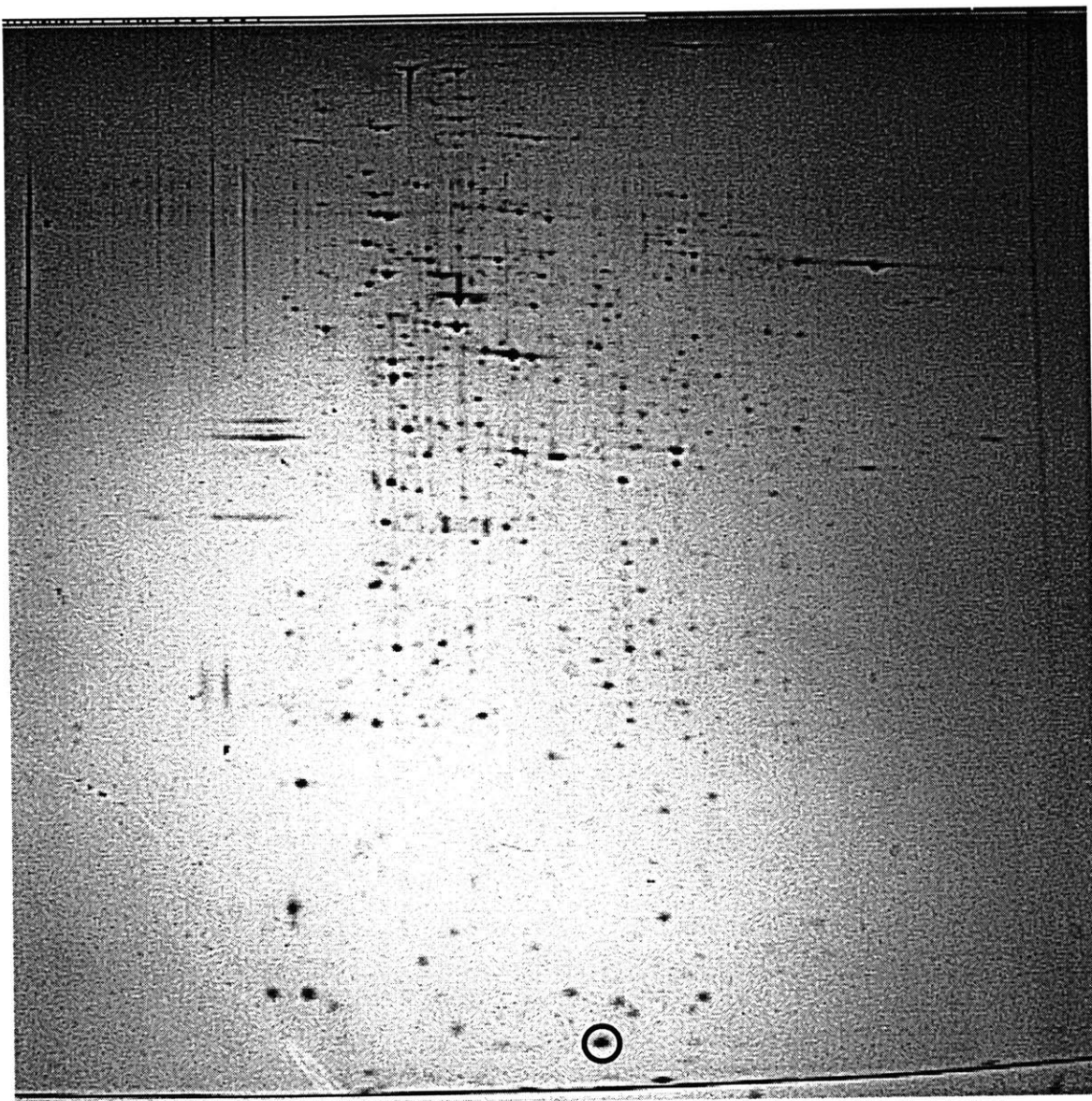Figure A9. 2-D PAGE of bacterial cells taken directly form the light organ of *Euprymna scolopes*. The circled protein is similar to QsrP in size and isoelectric point .

Appendix B. Characterization of a periplasmic 3':5'-cyclic nucleotide phosphodiesterase gene, *cpdP*, from the marine symbiotic bacterium *Vibrio fischeri*.

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Characterization of a Periplasmic 3':5'-Cyclic Nucleotide Phosphodiesterase Gene, *cpdP*, from the Marine Symbiotic Bacterium *Vibrio fischeri*†

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Vibrio fischeri, a marine bacterium that forms a bioluminescent symbiosis with certain fish and squids, exhibits the unusual attribute of growth on 3':5'-cyclic AMP (cAMP), apparently through the activity of a 3':5'-cyclic nucleotide phosphodiesterase (3':5'-CNP) with exceptionally high activity. The *V. fischeri* 3':5'-CNP is located in the periplasm, a novel cellular location for this enzyme in bacteria. To gain insight into the physiological function of this enzyme, we cloned the gene (designated *cpdP*) encoding it from *V. fischeri* MJ-1. This is the first bacterial 3':5'-CNP gene to be cloned. Sequencing and analysis of the 1.26-kb *cpdP* locus revealed a single open reading frame specifying a protein of 330 amino acid residues, including a 22-amino-acid leader peptide. The putative *cpdP* promoter contained a reasonable −10 promoter region (TATTAT) but contained no obvious −35 region; instead, a 12-bp inverted repeat (TTAAATATTTAA) occurred just upstream of this location. A possible rho-independent transcriptional terminator with a calculated free energy of −21.2 kcal · mol^{-1} (ca. −88.7 kJ · mol^{-1}) followed the CpdP protein coding sequence. The predicted subunit molecular weight of 33,636 for the mature CpdP protein (36,087 less 2,451 for the leader peptide) was consistent with the molecular weight of 34,000 estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The deduced amino acid sequence of the CpdP protein exhibited 30.3% identity with that of the low-affinity 3':5'-CNP (PDE1) of *Saccharomyces cerevisiae* and 33.6% identity with that of the extracellular 3':5'-CNP of *Dictyostelium discoideum*. The residue identities clustered in two regions, residues 100 to 146 and 238 to 269, which contained 30 of the 33 amino acids conserved in all three proteins, 4 of which were histidines. A gene replacement mutant of *V. fischeri* MJ-1 containing a 0.45-kb *Bgl*II deletion within the *cpdP* gene lacked periplasmic 3':5'-CNP activity and did not grow on cAMP, confirming for *V. fischeri* the relationship among *cpdP*, synthesis of the periplasmic 3':5'-CNP, and growth on cAMP. The mutant exhibited no obvious sensitivity to high extracellular concentrations of cAMP (5 and 10 mM), suggesting that the enzyme does not play a role in defense against extracellular cAMP.

3':5'-Cyclic nucleotide phosphodiesterase (EC 3.1.4.17; 3':5'-CNP), which converts 3':5'-cyclic nucleotides (e.g., 3':5'-cyclic AMP [cAMP] and 3':5'-cyclic GMP [cGMP]) to their corresponding 5'-nucleoside monophosphates, has been characterized extensively for eukaryotic organisms. The enzyme functions in a wide variety of signal-mediated cytoplasmic processes by modulating the levels of the secondary messenger 3':5'-cyclic nucleotides (5). Besides cytoplasmic functions in eukaryotes, an extracellular form of the enzyme in cellular slime molds, such as *Dictyostelium discoideum*, hydrolyzes cAMP, which is involved in morphogenetic and aggregational signalling between cells (50). The amino acid residue sequences have been obtained for several eukaryotic 3':5'-CNPs; nearly all contain a region of approximately 250 residues in which homology is present (15, 16, 29). The exceptions, which lack the 250-residue region, are the low-affinity 3':5'-CNP (PDE1) of *Saccharomyces cerevisiae* (40, 49) and the extracellular 3':5'-CNP of *D. discoideum* (31). The amino acid sequences of the *S. cerevisiae* (PDE1) and *D. discoideum* enzymes, however, exhibit substantial identity to each other, leading to the suggestion that they represent a second evolutionary lineage of eukaryotic 3':5'-CNPs (15, 40).

Many species of prokaryotes also possess 3':5'-CNP;

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however, in contrast to the situation with eukaryotes, the physiological significance of the bacterial enzyme is less well understood. In enteric and other gram-negative bacteria, the enzyme is cytoplasmic or associated with the cytoplasmic membrane (2, 12, 14, 26, 32). For *Salmonella typhimurium*, evidence suggests that the enzyme protects cells against high intracellular levels of cAMP (2, 9). The ability of the enzyme to hydrolyze cAMP would be consistent with it also modulating cellular levels of cAMP and thereby influencing cAMP-mediated gene transcription, but this role has been difficult to examine in detail because of the lack of genetically well-defined 3':5'-CNP mutants (2, 9). The *cpdA* gene (designation given by Beacham and Garrett [4]), which encodes this enzyme, has not been cloned. Parenthetically, it should be noted here that the gene (*cpdB*) for a different cyclic nucleotide phosphodiesterase, the periplasmic 2':3'-cyclic nucleotide phosphodiesterase:3'-nucleotidase (EC 3.1.4.16) of *Escherichia coli*, has been cloned (4, 32). Compared with the hydrolysis of cAMP by 3':5'-CNP, the excretion of cAMP and the regulation of its synthesis are thought to be more important ways by which bacteria control the cellular levels of this regulatory molecule (8, 10, 45, 58).

We recently described the novel ability of the common marine bacterium *Vibrio fischeri*, the species-specific bioluminescent symbiont of monocentrid fish and certain sepiolid squids (23, 43), to utilize cAMP as a sole source of carbon and energy for growth (25). This ability is correlated with

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
E. coli K-12		
AG-1	*thi-1*	11
S17-1	RP4 *tra*+	53
V. fischeri		
MJ-1	*cpdP*+ (parent strain)	44
MJ-100	MJ-1; spontaneous Nx^r strain	24
MJ-301	MJ-100; 0.45-kb *Bg*/II deletion; Δ*cpdP*::Nm^r	This study
Plasmids		
pSUP102	pACYC184; RP4 *mob*+ Cm^r Tc^r	53
pMER120	pSUP102 with a 12.0-kb *cpdP*+ *Sau*3AI partial-digest fragment of *V. fischeri* MJ-100 DNA; Cm^r	This study
pGEM-7Zf(+)	Ap^r	Promega
pMER083	pGEM-7Zf(+) with a 6.4-kb *Bam*HI-*Eco*RI fragment of pMER120; Ap^r *cpdP*+	This study
pMER037	pSUP102 with a 3.7-kb *Bam*HI-*Eco*RV fragment of pMER120; Cm^r *cpdP*+	This study
pGEM-5Zf(+)	Ap^r	Promega
pMER026	pGEM-5Zf(+) with a 2.3-kb *Bam*HI-*Nco*I fragment of pMER037; Ap^r *cpdP*+	This study
pMER023	pSUP102 with a 2.3-kb *Bam*HI-*Sph*I fragment of pMER026; Cm^r *cpdP*+	This study
pMER017	pMER026 digested with exonuclease III; Ap^r	This study
pMER013	pMER017 digested with exonuclease III; Ap^r	This study
pMER019	pMER023; 0.45-kb *Bg*/II deletion; Δ*cpdP* Cm^r	This study
pUC4K	*Bam*HI-excisable 1.3-kb Nm^r fragment	59
pMER213	pMER019 with a 1.3-kb *Bam*HI Nm^r fragment from pUC4K ligated to a *Bg*/II site; Cm^r	This study

[a] Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Nx^r, nalidixic acid resistant; Tc^r, tetracycline resistant.

and apparently depends on the synthesis by *V. fischeri* of a novel periplasmic 3':5'-CNP (25). The enzyme, which exhibits an exceptionally high specific activity (13, 25), has been proposed to play a role in the species specificity of the fish and squid symbioses by protecting *V. fischeri* cells from possible toxic effects of host-released cAMP and by specifically permitting *V. fischeri* cells to utilize host-released cAMP as a growth substrate (25). Alternatively, the enzyme may function in the scavenging of 3':5'-cyclic nucleotides in the marine environment (25). In this report, we describe the cloning and sequence analysis of the gene, *cpdP*, for the periplasmic 3':5'-CNP from *V. fischeri* MJ-1 and the construction of a *V. fischeri* Δ*cpdP* mutant by gene replacement procedures. This is the first bacterial 3':5'-CNP gene to be cloned.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The strains used in this study are derivatives of *E. coli* K-12 and *V. fischeri* MJ-1 and are listed in Table 1. For routine cultivation, the *E. coli* strains were grown on LB agar (52) and the *V. fischeri* strains were grown on LBS agar (20), with the appropriate antibiotics (ampicillin, 80 μg · ml^{-1}; chloramphenicol, 30 μg · ml^{-1}; kanamycin, 20 μg · ml^{-1}; nalidixic acid, 20 μg · ml^{-1}; neomycin, 200 μg · ml^{-1}) for selection procedures. For selection and screening of strains for growth on cAMP, an *E. coli* minimal medium (ECM) and a *V. fischeri* minimal medium (VFM) were used. ECM contained 50 mM NaCl, 1 mM MgSO$_4$, 1 mM CaCl$_2$, 5 mM NH$_4$Cl, 0.3 mM K$_2$HPO$_4$, 0.05 mM thiamine, 0.005% yeast extract (Difco Laboratories, Detroit, Mich.), 0.005% tryptone (Difco), and 50 mM Tris-HCl (pH 7.4). VFM contained 300 mM NaCl, 10 mM KCl, 50 mM MgSO$_4$, 10 mM CaCl$_2$, 5 mM NH$_4$Cl, 0.3 mM K$_2$HPO$_4$, 20 mg of ferric ammonium citrate liter^{-1}, and 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) (pH 7.5). ECM and VFM

were supplemented with appropriate carbon and energy sources (i.e., glucose or nucleotides) at 5 mM. For solid minimal media, Noble agar (Difco) was used at 15 g · liter^{-1}.

Construction of a *V. fischeri* MJ-1 gene library and isolation of the *cpdP* gene. A genomic library of DNA from *V. fischeri* MJ-1 was prepared essentially as described by Sambrook et al. (47) and Silhavy et al. (52). Purified chromosomal DNA was partially digested with *Sau*3AI and size fractionated on a 0.7% agarose-tris-acetate-EDTA gel, and the portion of the gel containing the 10- to 15-kb DNA was isolated. The DNA was electroeluted from the gel slice, purified by phenol extraction and ethanol precipitation, and then ligated to the *Bam*HI site of pSUP102 (53). The library was recovered by transformation of *E. coli* AG-1 (11), with selection on LB agar containing chloramphenicol. DNA containing the *V. fischeri cpdP* locus was isolated by plating *E. coli* AG-1 transformed with the MJ-1 gene library on ECM agar containing chloramphenicol and containing cAMP as the sole source of carbon, nitrogen, and phosphorus.

Subcloning and exonuclease III digestions. To define the *cpdP* locus, we used standard subcloning procedures (47), various plasmid vectors [pSUP102, pGEM-5Zf(+), and pGEM-7Zf(+)], and appropriate restriction endonuclease cleavage sites in the vectors and in the cloned *V. fischeri* MJ-1 DNA (Fig. 1). For unidirectional exonuclease III digestions, the Erase-a-Base kit (Promega Corp., Madison, Wis.) was used in accordance with the manufacturer's instructions.

Southern hybridizations. For Southern (55) hybridization analyses, the Genius nonradioactive DNA labelling and detection kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was used in accordance with the manufacturer's instructions. A 1.9-kb *cpdP* probe encompassing the *cpdP* gene plus flanking DNA was constructed from pMER037 by digestion with *Nco*I and *Nsi*I (Fig. 1). Hybridization of the probe was tested with *Nco*I-*Nsi*I digests of
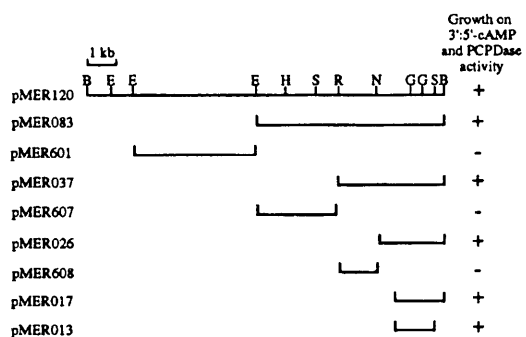
FIG. 1. Partial restriction map and localization of the *cpdP* region of the *V. fischeri* chromosome by subcloning and exonuclease III digestion. The ability (+) or inability (−) of *E. coli* AG-1 carrying a plasmid containing the indicated fragment of *V. fischeri* DNA to express periplasmic 3′:5′-CNP activity (PCPDase activity) and to grow on cAMP as a sole carbon and energy, nitrogen, and phosphorus source is indicated at the right. Unidirectional exonuclease III digestion from the left end of pMER026 gave rise to pMER017, and unidirectional exonuclease III digestion from the right end of pMER017 gave rise to pMER013. Restriction sites: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hind*III; N, *Nco*I; R, *Eco*RV; S, *Nsi*I.

genomic DNA from *V. fischeri* MJ-1 and *E. coli* AG-1. The probe hybridized to a single 1.9-kb DNA fragment from *V. fischeri* MJ-1 but did not hybridize to DNA from *E. coli* AG-1 under high- or low-stringency conditions.

DNA sequencing and sequence analysis. The nucleotide sequence of both strands of the cloned *V. fischeri* MJ-1 DNA in pMER013 [1.26 kb of MJ-1 DNA in pGEM-5Zf(+)] (Fig. 1) was determined by the dideoxy chain termination method of Sanger et al. (48) with Sequenase (version 2.0; United States Biochemical Corp., Cleveland, Ohio) and primers for the T7 and SP6 promoters of pGEM-5Zf(+) and for regions internal to the cloned DNA. DNA sequence analysis was performed with the MacDNASIS Pro 1.01 package (National Biosciences, Plymouth, Minn.) and the SEQ program (IntelliGenetics, Inc., Mountain View, Calif.). Analysis of the deduced amino acid residue sequence of the *V. fischeri* 3′:5′-CNP containing the leader peptide (pro-CpdP protein) was conducted with the FASTDB and GENALIGN programs provided in release 5.4 of the IntelliGenetics Suite. The Swiss-Prot protein sequence data base maintained by the University of Geneva, Geneva, Switzerland, was searched by use of the FASTDB program with the unitary similarity matrix, a gap penalty of one, and a k-tuple of two. Pairwise alignments were done with the same algorithm as the search. For multiple alignments, the GENALIGN program with the region method developed by Sobel and Martinez (54) was used.

NH₂-terminal sequence determination. Sequencing of the amino-terminal 20 amino acid residues of mature 3′:5′-CNP purified from the periplasm contents of *V. fischeri* MJ-1 (13) was performed on an Applied Biosystems pulsed liquid instrument (model 477A) with an on-line phenylthiohydantoin analyzer (model 120A). The protein solution was applied to a Polybrene-conditioned glass fiber disc in 30-μl aliquots, and approximately 50 pmol of protein was analyzed.

Construction of a Δ*cpdP* mutant of *V. fischeri*. To construct a *cpdP*-containing vector for mobilization into *V. fischeri*, we transferred the 2.3-kb *cpdP* DNA fragment from pMER026 [in pGEM-5Zf(+)] to pSUP102 (53) by using

*Bam*HI and *Sph*I, thereby forming pMER023 (Table 1). This vector was digested with *Bgl*II (two sites in the *cpdP* gene; Fig. 1) and religated, thereby creating a 0.45-kb deletion within the *cpdP* coding region (pMER019). *E. coli* AG-1 transformed with pMER019 did not grow on minimal medium containing cAMP as the sole carbon, nitrogen, and phosphorus source and produced no detectable periplasmic 3′:5′-CNP activity in assays with intact cells. To make the *cpdP* mutation selectable and thereby facilitate the isolation of a gene replacement mutant, we ligated the 1.3-kb neomycin resistance fragment of pUC4K (59), excised with *Bam*HI, to the *Bgl*II site of pMER019 to construct pMER213. *E. coli* S17-1 (53) was then transformed with pMER213 and mated to *V. fischeri* MJ-100 (Nxʳ) by a previously described procedure (24). *V. fischeri* transconjugants were isolated and purified on LBS agar plates containing nalidixic acid and neomycin. The transconjugants were then screened for loss of resistance to chloramphenicol as an indication of loss of the vector, with retention of neomycin resistance as an indication of replacement of the wild-type *cpdP* gene with the *cpdP* deletion. Several apparently identical chloramphenicol-sensitive, neomycin-resistant strains were isolated by this procedure and, when tested for periplasmic 3′:5′-CNP activity, were found to lack detectable activity. One of these mutants, designated MJ-301 (Δ*cpdP*::Nmʳ), was examined further.

For testing for complementation of the *cpdP* mutation in MJ-301 by the *cpdP* gene, pMER023 was conjugatively delivered from S17-1 to MJ-301, with transconjugant MJ-301(pMER023) being selected on LBS agar containing nalidixic acid and chloramphenicol. Growth was assayed for cells of MJ-301(pMER023) and of MJ-301 inoculated into VFM lacking NH₄Cl and K₂HPO₄ and with cAMP or cGMP as the sole carbon and energy, nitrogen, and phosphorus source.

Determination of 3′:5′-CNP activity. The procedure of Cheung (17) was used to assay for 3′:5′-CNP activity in intact cells and periplasmic extracts as described previously (25).

Chemicals. Antibiotics, cAMP, HEPES, and Tris were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence of the *V. fischeri* *cpdP* gene is L11527.

RESULTS

Cloning and expression of the *V. fischeri* cpdP gene in *E. coli*. The marine symbiotic bacterium *V. fischeri* exhibits the unusual attribute of growth on cAMP as a sole carbon and energy source. In describing this attribute, we demonstrated previously that *V. fischeri* cells contain a 3′:5′-CNP with very high activity and located in the periplasm, a novel cellular location for this enzyme in bacteria (25); we hypothesized that the activity of this enzyme enables cells to grow on extracellular cAMP (25). The periplasmic location of the enzyme and its possible involvement in the growth of *V. fischeri* cells on cAMP were unexpected; in bacteria, the enzyme is generally considered as functioning either to protect cells from the deleterious effects of high cytoplasmic levels of cAMP or to modulate the expression of cAMP-controlled genes by regulating cytoplasmic levels of cAMP (2, 9). Because the growth of *V. fischeri* on cAMP presents a new aspect of cAMP metabolism in prokaryotes, we undertook the cloning and analysis of the gene encoding periplasmic 3′:5′-CNP from *V. fischeri* MJ-1, with the initial

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intention of defining the relationship between the enzyme and growth on cAMP.

Previously, no bacterial 3':5'-CNP gene had been cloned. Consequently, given the periplasmic location of the enzyme and its possible involvement in the growth of *V. fischeri* cells on extracellular cAMP, our cloning strategy involved transforming cells of *E. coli* AG-1 with a gene library of *V. fischeri* MJ-1 chromosomal DNA constructed in a plasmid vector and plating the transformed cells on minimal medium plates containing cAMP as the sole carbon and energy source (see Materials and Methods). We envisioned that colonies would form from *E. coli* cells that were complemented for growth on cAMP by the *V. fischeri* DNA, i.e., cells that received and expressed the *V. fischeri* periplasmic 3':5'-CNP gene and exported the *V. fischeri* enzyme into the *E. coli* periplasm in an active form.

Initial attempts at isolating the 3':5'-CNP gene by this method, however, were not successful; no colonies arose on the ECM-cAMP agar plates. We had noted incidentally in other work, however, that *V. fischeri* can grow on cAMP as the sole source of carbon, nitrogen, and phosphorus and that, in the presence of NH_4Cl and K_2HPO_4, growth on cAMP was suppressed under some conditions (21). In accordance with these observations, we replated the transformed *E. coli* AG-1 cells on ECM-cAMP agar lacking NH_4Cl and K_2HPO_4. Several colonies arose in 2 days at 37°C. Plasmids of all 11 tested colonies were found by restriction endonuclease digestion to contain a similar 10- to 13-kb region of *V. fischeri* DNA. One clone, which was designated pMER120 and which contained approximately 12 kb of *V. fischeri* DNA, was chosen for further study.

Several criteria were used to confirm that the cloned *V. fischeri* DNA in pMER120 contained a gene(s) involved in periplasmic 3':5'-CNP activity and the growth of *E. coli* cells on cAMP. Specifically, cells of AG-1 containing pMER120 were tested and found to grow well in ECM-cAMP broth without NH_4Cl and K_2HPO_4, whereas cells of AG-1 containing the cloning plasmid (pSUP102) did not grow in this medium either with or without NH_4Cl and K_2HPO_4. Both AG-1 containing pMER120 and AG-1 containing pSUP102 grew in ECM containing glucose. When *E. coli* AG-1 cells were transformed with pMER120 and plated on selective medium, an equal number of colonies arose on ECM-cAMP agar plates (without NH_4Cl and K_2HPO_4) containing chloramphenicol and on LB agar plates containing chloramphenicol. Consistent with growth on cAMP, AG-1 cells containing pMER120 exhibited periplasmic 3':5'-CNP activity, as determined by enzyme assays with intact cells (see Materials and Methods), at levels similar to those in *V. fischeri*. *E. coli* AG-1 containing pSUP102, however, exhibited no detectable periplasmic 3':5'-CNP activity.

We designated the structural gene for the *V. fischeri* periplasmic 3':5'-CNP *cpdP*, with *P* being used as a mnemonic for periplasm. This designation distinguishes the *V. fischeri* gene from the *cpdA* gene (designation given by Beacham and Garrett [4]), which encodes the cytoplasmic 3':5'-CNP of *S. typhimurium* but which has not yet been cloned (2, 45), and from the cloned gene (*cpdB*) encoding the periplasmic 2':3'-cyclic nucleotide phosphodiesterase:3'-nucleotidase of *E. coli* (4, 34). To our knowledge, *cpdP* is the first bacterial 3':5'-CNP gene to be cloned.

Subcloning and deletion analysis of the *V. fischeri* cpdP locus. For definition of the portion of the 12 kb of *V. fischeri* DNA in pMER120 that specified the *cpdP* region, pMER120 was subjected to subcloning and exonuclease III deletion analysis (see Materials and Methods). First, restriction en-

donuclease cleavage sites found within the cloned DNA were used to cut it into smaller fragments, which were then ligated to appropriate sites in various vectors (Fig. 1). *E. coli* AG-1 was transformed with the resulting vectors, and the transformants were screened for their ability to grow on cAMP and synthesize periplasmic 3':5'-CNP (Fig. 1). This procedure localized the *cpdP* region to a 2.3-kb *Bam*HI-*Nco*I fragment of DNA (pMER026).

Next, exonuclease III digestion of the insert DNA in pMER026, unidirectionally from the left and then, with the resulting vector (pMER017), unidirectionally from the right, was used to resolve more finely the *cpdP* locus (Fig. 1). As described above, cells of *E. coli* AG-1 were transformed with the resulting vectors, and the transformants were screened for growth on cAMP and synthesis of periplasmic 3':5'-CNP. This process defined the *cpdP* locus to a 1.26-kb fragment of *V. fischeri* DNA (pMER013). In showing a one-to-one correlation between 3':5'-CNP activity and growth on cAMP (Fig. 1), the subcloning and exonuclease III digestion procedures demonstrated that the 1.26-kb *cpdP* locus of the *V. fischeri* chromosome is both necessary and sufficient for *E. coli* cells to grow on extracellular cAMP. Southern hybridization analysis demonstrated the absence of sequences similar to those of the *cpdP* locus in the genome of *E. coli* AG-1 (see Materials and Methods).

Nucleotide sequence of the *V. fischeri* cpdP gene. The DNA sequence of the 1.26 kb of *V. fischeri* DNA contained in pMER013 [pGEM-5Zf(+)] was determined bidirectionally with SP6 and T7 primers and with primers for regions internal to the cloned DNA (see Materials and Methods). Translation of the DNA sequence revealed one open reading frame, beginning at position 143 (ATG), ending at position 1133 (TAA), and specifying a protein of 330 amino acid residues (Fig. 2) and with a deduced molecular weight of 36,087. We conclude, therefore, that this locus contains a single gene, *cpdP*.

A putative promoter region upstream of the *cpdP* coding region was identified by visual inspection of the DNA sequence. Nucleotides 95 to 100 (TATTAT) conform reasonably well to typical −10 promoter sequences recognized by *E. coli* RNA polymerases (42), whereas a −35 promoter sequence apparently is absent (Fig. 2). Just upstream of the region at which a −35 hexanucleotide would be expected, however, a 12-bp inverted repeat (TTAAATATTTAA) was found (nucleotides 60 to 71). The *V. fischeri* MJ-1 luminescence (*lux*) operon promoter also lacks a canonical −35 region; a 20-bp inverted repeat adjacent to this site is the putative binding site for the LuxR protein, the *lux* transcriptional activator (see reference 36 for a review). A putative Shine-Dalgarno ribosome binding site (AAGG) occurs 6 bases upstream of the ATG translation initiation codon (51). This sequence and its location upstream of the ATG codon agree reasonably well with the preferred spacing and nucleotide sequence of ribosome binding sites in *E. coli* genes (27). A possible stem-loop structure consisting of a 12-base stem and an 8-base loop and followed by a poly-T region was identified 13 bp downstream of the TAA stop codon (Fig. 2). This structure could serve as a rho factor-independent transcriptional terminator; its calculated free energy is −21.2 kcal · mol^{-1} (ca. −88.7 kJ · mol^{-1}), a value that corresponds well to that of a demonstrated transcriptional terminator for the *V. fischeri* *lux* genes (−13 kcal · mol^{-1} [ca. −54.5 kJ · mol^{-1}]; 57) and to those of rho factor-independent transcriptional terminators for other bacterial genes (42, 56). A second possible stem-loop structure, with a calculated free energy of −15.2 kcal · mol^{-1} (ca. −62.8 kJ · mol^{-1}),

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1 AGTAGAACTATCAAATTCTGTGGAGCATACCGCTTATAATTACCTTCTTATCAATAGTCTTAAATATTTAATACATC

78 ATTCATTTATCTCATTGTATTATCTGATTGAGCACAAGGGTGTGCAGTATTATTTAAGGTTGGAT ATG TTT AAA
                 -10   SD   Met Phe Lys

Pos																	
152	AAT	AAA	TTA	GCA	GTG	TTA	TTT	ACG	TGT	TTG	TCT	GTA	TTT	TCT	TTT	TCG	GCT
	Asn	Lys	Leu	Ala	Val	Leu	Phe	Thr	Cys	Leu	Ser	Val	Phe	Ser	Phe	Ser	Ala
203	CAA	TCT |	GGG	AGT	TTT	GAT	ACC	GTT	ACG	TTA	GGG	AGT	AAA	GGT	GGG	ATT	CAA
	Gln	Ser ↑	Gly	Ser	Phe	Asp	Thr	Val	Thr	Leu	Gly	Ser	Lys	Gly	Gly	Ile	Gln
254	GAT	GGT	AAT	TTA	ACG	GCT	TTT	TTA	ATT	AAA	AGT	GAG	GCG	GAT	TCT	AAT	TTT
	Asp	Gly	Asn	Leu	Thr	Ala	Phe	Leu	Ile	Lys	Ser	Glu	Ala	Asp	Ser	Asn	Phe
305	GTT	ATG	CTT	GAT	GCA	GGT	TCT	GTT	GTT	AAT	GGA	TTA	ATT	GTT	TCA	GAG	CAG
	Val	Met	Leu	Asp	Ala	Gly	Ser	Val	Val	Asn	Gly	Leu	Ile	Val	Ser	Glu	Gln
356	AAA	GGA	GCG	TTT	AAA	GAT	ATT	ACC	GTC	CCA	GAT	AGT	TCG	CCT	TAC	ACC	AAA
	Lys	Gly	Ala	Phe	Lys	Asp	Ile	Thr	Val	Pro	Asp	Ser	Ser	Pro	Tyr	Thr	Lys
407	GTA	GGT	TAC	CTA	TTA	AAG	GAT	AGG	ATT	AAG	GGC	TAC	TTT	ATT	AGT	CAT	GCT
	Val	Gly	Tyr	Leu	Leu	Lys	Asp	Arg	Ile	Lys	Gly	Tyr	Phe	Ile	Ser	His	Ala
458	CAT	TTA	GAC	CAT	GTT	GCT	GGT	TTA	ATT	ATC	TCT	TCT	CCT	GAT	GAC	AGT	AAA
	His	Leu	Asp	His	Val	Ala	Gly	Leu	Ile	Ile	Ser	Ser	Pro	Asp	Asp	Ser	Lys
509	AAA	CCG	ATA	TAT	GGA	TTA	GCA	GCG	ACA	AAT	AAA	GAT	CTC	ATG	AAG	AAT	TAC
	Lys	Pro	Ile	Tyr	Gly	Leu	Ala	Ala	Thr	Asn	Lys	Asp	Leu	Met	Lys	Asn	Tyr
560	TTT	AAT	TGG	TCA	GCA	TGG	CCT	AAT	TTT	GGT	AAC	AAA	GGT	GAA	GGC	TTT	AAA
	Phe	Asn	Trp	Ser	Ala	Trp	Pro	Asn	Phe	Gly	Asn	Lys	Gly	Glu	Gly	Phe	Lys
611	CTG	AAC	AAA	TAC	AAT	TAT	GTG	GAT	CTT	CAA	CCG	GGC	GTT	TGG	AGT	CCG	GTA
	Leu	Asn	Lys	Tyr	Asn	Tyr	Val	Asp	Leu	Gln	Pro	Gly	Val	Trp	Ser	Pro	Val
662	GCT	GAA	ACA	ACA	ATG	AGT	GTG	GTG	TCA	TTG	CCT	TTG	TCT	CAT	TCT	GGT	GGG
	Ala	Glu	Thr	Thr	Met	Ser	Val	Val	Ser	Leu	Pro	Leu	Ser	His	Ser	Gly	Gly
713	CAA	TCT	ACG	GTA	TTT	ATT	CTA	AAA	GAC	AGT	GAA	GGG	GAT	GTA	TTT	GCT	TAT
	Gln	Ser	Thr	Val	Phe	Ile	Leu	Lys	Asp	Ser	Glu	Gly	Asp	Val	Phe	Ala	Tyr
764	TTT	GGT	GAT	ACA	GGA	CCT	GAT	GAA	GTA	GAG	AAA	AGC	TGG	GCA	ATG	AGG	ACT
	Phe	Gly	Asp	Thr	Gly	Pro	Asp	Glu	Val	Glu	Lys	Ser	Ser	Ala	Met	Arg	Thr
815	GCA	TGG	TCT	GTT	TTA	GCT	CCT	TTT	GTA	AAA	CAA	GGG	AAG	TTA	AAG	GGG	ATT
	Ala	Trp	Ser	Val	Leu	Ala	Pro	Phe	Val	Lys	Gln	Gly	Lys	Leu	Lys	Gly	Ile
866	ATT	ATT	GAA	GTT	TCT	TTT	ACC	AAC	GAA	ACC	CCA	GAT	AAA	TCT	TTG	TTT	GGA
	Ile	Ile	Glu	Val	Ser	Phe	Thr	Asn	Glu	Thr	Pro	Asp	Lys	Ser	Leu	Phe	Gly
917	CAC	TTA	ACG	CCA	AAC	TGG	TTA	GTT	AAA	GAA	TTA	AGT	GTA	CTT	GAA	GAC	ATG
	His	Leu	Thr	Pro	Asn	Trp	Leu	Val	Lys	Glu	Leu	Ser	Val	Leu	Glu	Asp	Met
968	AAT	GGC	AAA	GGC	TCT	TTA	AAA	GAT	CTA	AAT	GTA	GCC	ATA	AGC	CAC	ATC	AAA
	Asn	Gly	Lys	Gly	Ser	Leu	Lys	Asp	Leu	Asn	Val	Ala	Ile	Ser	His	Ile	Lys
1019	TAC	AGC	TTA	AAG	AAT	AGT	GAA	GAT	CCT	AAA	GTA	ATT	ATT	AAA	AAG	CAG	TTA
	Tyr	Ser	Leu	Lys	Asn	Ser	Glu	Asp	Pro	Lys	Val	Ile	Ile	Lys	Lys	Gln	Leu
1070	GTA	GAA	GTA	AAT	GAC	TTA	GGT	GTA	AAT	TTC	ATT	TTT	CCT	GAG	CAA	GGT	GAT
	Val	Glu	Val	Asn	Asp	Leu	Gly	Val	Asn	Phe	Ile	Phe	Pro	Glu	Gln	Gly	Asp

1121 TCA TTG CAG TTT TAA AAAAGTCATTGTAAAAGTGGATATGGTCAATACAAAGACCATATCCAC
      Ser Leu Gln Phe ***

1184 GGATTTGTTTTTATCTCTTAAATCGTCGTTTCTATCTTTTTAAGCACGGCGATCTCATTGGGTACATCAATGTT

FIG. 2. Nucleotide sequence and deduced amino acid translation of the *V. fischeri cpdP* gene. Possible Pribnow box (−10) and ribosome binding (SD) regions are underlined, as is a 12-bp inverted repeat adjacent to the location at which a −35 region would be expected. The arrow between amino acid residues 22 and 23 indicates the cleavage site for the CpdP leader peptidase. A possible rho-independent transcriptional terminator with a free energy of −21.2 kcal · mol^{-1} (ca. −88.7 kJ · mol^{-1}) and located at the end of the *cpdP* coding region is also underlined.

was identified within the coding sequence of the *cpdP* gene (Fig. 2, nucleotides 523 to 583); the physiological significance of this possible stem-loop structure was unclear. The moles percent G+C content of the 1.26 kb of *V. fischeri* DNA containing the *cpdP* gene is 35.2, a value somewhat lower than the moles percent G+C content of 40 for the *V. fischeri* genome (3).

Analysis of the *V. fischeri* CpdP protein leader peptide sequence. The CpdP protein is exported into the periplasm in *V. fischeri* (25) and in *E. coli* containing the *V. fischeri cpdP*

gene, so we anticipated that the protein would contain a leader peptide. Consistent with this idea, a hydropathy plot of the deduced amino acid sequence for the pro-CpdP protein (30, 61) revealed that the amino-terminal end was hydrophobic (data not shown). Furthermore, on the basis of the (−3, −1) rule of von Heijne (60) and other guidelines (19, 41, 60), the first 22 residues exhibited characteristics typical of a prokaryotic leader peptide, including a basic amino-terminal region, a central hydrophobic core, and a more polar carboxy-terminal region, with the cleavage site for the

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V. fischeri    1                              M F K N K L A V L F T C L S V F S F S A Q S G S F D T V T L G S K G G I
S. cerevisiae  1            M V V F E I T I L G A N G G P T E Y G T Q C F I L K P A R T E D          P E L I A
D. discoideum  1  M A L N K K L I S L L L L I F I I L N I V N S H Q Q E D C D D D D E D I G I S A E R S E R R S

37  Q D G                          N L T A F L I K S E A D S N F V M L D A G S V V N G L I V S E Q K G
38  V D G A G M Y Q L R E M L V Q G R N E N E G D D E L V P S F Y E H D R E P I E F F I D S K L N I Q K G L
48  V K N S N D G S N F Y N L N D Y Y T P E N W N H Y S G S F A T K D C R D A S Y I T I P L G T T G G L D E G N

74                                                      A F K D I T V P D S S P Y T K V
91  S K S F L                   Q S L K R Q G       E H F E S A N T   M K K T Y
102 L S S F L L T K K G S N L F I A L D A G T V W Q G V R R L T T F K Y F N T L F N I T Y P S W A V L P E Q R T

90  G Y L L K D R I K G Y F I S H A H L D H V A G L I I S S P
116     E V F Q G I T D Y Y I T H P H L D H I S G L V V N S P
156 S W F L K K H V M S Y F I G H S H L D H V G G L I L V S P E D Y L A K N W I D V Q P P I N N G I M G L I R K

119                D D S K K P I Y G L A A T N K D L M K N Y F N W S A W P   N F G N K G E G F K L
143       S I Y E Q E N S K K K T I W G L P H T I D V L Q K H V F N D L I W P   D L T A E R S R K L K L
210 L G F K P T D F T S S S I L Q K K T I M G L P S T I N S I S T N L F N N Q V W P N L P S F G R Y Q Y F S L A

158                N K Y N Y V D L Q P G V W S P V A E T T M S V V S L P L S H S G G Q S T   V F I L K D S E G D V
189 K C L N P K E V Q K C T I F P W D V I P F K V H H G I G V K   T G A P V Y S T F Y I F R D R K S K D C
264 S G I E Y P F T E L V P Y N A T T M S L V A N E F P F S V K V,K P F E L C H D N L I S T S F L F T D S I S G

205 F A Y F G D   T G P D E V E K S S A M R T A W S V L A P F V K Q G K L K G I I I E V S F T N E T P D K S L F
239 I I V C G D V E Q D R R E S E E S L L E E F W S Y V A E N I P L V H L K G I L V E C S C P L S S K P E Q L Y
318 E Q I A F F S D T G V P S S V A C D W E G K I Y A V W K Q I K I D K L K A I Y I E T S F P N N T P D S A M F

258 G H L     T P N W L V K E L S   V L E D M N G K G S L K D L N V   A I S H I K Y S L K N S E D P K V I
293 G H L     S P I Y L I N E L S N L N T L Y N S S K G L S G L N V I V T H V K S T P A K R D P   R L T I L E
372 G H L R P R D V M K L M D Q L L V Q S I Q T S P P M T N L K H V K L I I E H I K P Q V A E D P N G W T T Q R

305 I K K Q L V E V N D L G V N F I F P E Q G D S L Q F   330
343 E L R F L A E E R N L G D L R I S I A L E G H T L F   368
426 V I Y Q Q L K E A N N N G V R I I I P N Q G D P I C   451
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FIG. 3. Amino acid residue sequence comparison for the 3′:5′-CNPs of V. fischeri (periplasmic), S. cerevisiae (low-affinity; PDE1) (40), and D. discoideum (extracellular) (31). The boxes highlight amino acid residue identities. Regions of identity for the three sequences cluster in two areas, from residues 100 to 146 and residues 238 to 269 of the V. fischeri sequence.

leader peptidase predicted to be between residue 22 (S) and residue 23 (G) (Fig. 2). Cleavage between these two residues was confirmed by Edman degradation microsequence analysis of the first 20 amino-terminal residues of the mature protein, which was purified from periplasm contents of V. fischeri MJ-1 (13). The sequence, beginning with the amino-terminal residue, was found to be X-(S)-F-D-T-V-T-L-G-(S)-K-G-G-I-Q-(D)-G-N-L-T. Thus, the first 20 amino acid residues of the mature protein matched those of the deduced sequence following the proposed pro-CpdP leader peptide (residues 23 to 42; Fig. 2), except that a definite assignment could not be made for the first residue, X, because of the presence of contaminating free amino acids in the sample (G, S, A, M, and K were detected). Residues 2 (S), 10 (S), and 16 (D) were considered probable but tentative assignments because of the lower yields of these residues. The deduced monomeric molecular weight of the mature protein was calculated to be 33,636 (36,087 less 2,451 for the leader peptide), similar to the value of 34,000 estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the purified protein (13). The pI of the mature protein was calculated to be 5.49.

Comparison of the deduced amino acid sequence for the V. fischeri CpdP protein with those for other proteins. We compared the deduced amino acid sequence for the V. fischeri pro-CpdP protein with sequences available in the Swiss-Prot protein sequence data base (see Materials and

Methods). In this analysis, alignments were optimized and we considered statistically significant those sequences with identity scores more than 10 standard deviations above the mean score of random permutation. Only two proteins, both 3′:5′-CNPs, scored at this level; both had standard deviation scores at least twice that of the next most similar protein. One, the low-affinity 3′:5′-CNP (PDE1) of the yeast S. cerevisiae (40), exhibited 30.3% identity to the V. fischeri sequence, and the other, the precursor polypeptide of the extracellular 3′:5′-CNP of the slime mold D. discoideum (31), exhibited 33.6% amino acid residue identity. A multiple alignment of the three sequences is shown in Fig. 3. The gaps necessary for optimal alignment presumably reflect in part the phylogenetic differences among the three organisms as well as differences in the structural and functional aspects of the three enzymes. The substantial sequence similarity between the two eukaryotic 3′:5′-CNPs was described previously (40). We noted, moreover, that the amino acid residue identities for the three proteins clustered in two regions, residues 100 to 146 and residues 238 to 269 of the V. fischeri sequence (Fig. 3). These regions contained 30 of the 33 amino acids conserved in all three proteins. Furthermore, histidine, which is present at only six positions in the V. fischeri sequence, accounted for 4 of the 30 amino acids conserved in these two regions.

Altered phenotype of a V. fischeri ΔcpdP mutant. To rigorously define for V. fischeri the possible relationship

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TABLE 2. Growth of a *V. fischeri* periplasmic 3′:5′-CNP mutant
on various substrates

Substrate[a]	Growth of:	
	MJ-100 (*cpdP*⁺)	MJ-301 (Δ*cpdP*::Nm^r)
Glucose	+	+
cAMP[b]	+	−[c]
5′-AMP[b]	+	+
Adenosine	+	+
cGMP[b]	+	−[c]
5′-GMP[b]	+	+
Guanosine	+	+
2′:3′-cAMP[b]	+	+
3′-AMP[b]	+	+
ATP[b]	+	+
ADP[b]	+	+

[a] VFM containing 5 mM substrate.
[b] VFM without NH₄Cl and K₂HPO₄.
[c] The inability of MJ-301 to grow on cAMP and cGMP was complemented by the introduction of pMER023 (*cpdP*⁺).

among the *cpdP* gene, periplasmic 3′:5′-CNP activity, and growth of cells on cAMP, we next constructed and examined a Δ*cpdP* mutant, MJ-301 (Δ*cpdP*::Nm^r). To construct this mutant, we replaced by marker exchange procedures (24) the wild-type *cpdP* gene with a 0.45-kb *BglII cpdP* deletion. To facilitate the isolation of recombinant strains, we had subcloned into the *cpdP* deletion a gene encoding neomycin resistance (see Materials and Methods).

MJ-301, which produced no periplasmic 3′:5′-CNP activity, did not grow in VFM broth containing cAMP as the sole carbon and energy source (with or without NH₄Cl and K₂HPO₄), but it grew well in VFM broth containing glucose (Table 2). The introduction of pMER023 (*cpdP*⁺) into MJ-301 enabled this strain to grow on cAMP, thereby demonstrating that the mutation in *cpdP* could be genetically complemented. These results, in demonstrating that the *cpdP* gene is both necessary and sufficient for *V. fischeri* cells to grow on cAMP, confirm the relationship among the *cpdP* gene, the synthesis of periplasmic 3′:5′-CNP, and the ability of *V. fischeri* to grow on cAMP. Furthermore, they demonstrate that *cpdP* is not a gene essential for the survival and growth of *V. fischeri*.

In an earlier study (25), we had demonstrated that the partially purified enzyme attacked only cAMP and cGMP of the substrates tested. Besides not growing on cAMP, MJ-301 also did not grow on cGMP whereas, like MJ-1, it grew on all the other nucleotides tested as sole carbon and energy sources (Table 2). The lack of growth of MJ-301 on cAMP and cGMP is consistent with our earlier data on the enzyme and affirms our previous conclusions that the enzyme exhibits specificity for 3′:5′-cyclic nucleotide substrates and does not function as a nonspecific phosphatase. As with cAMP, MJ-301 regained the ability to grow on cGMP when it contained pMER023 (*cpdP*⁺) (Table 2).

The periplasmic 3′:5′-CNP of *V. fischeri* could have additional functions besides permitting cells to grow on 3′:5′-cyclic nucleotides, such as defense against extracellular cAMP (25). We therefore tested the effects of high concentrations of extracellular cAMP on the growth characteristics and luminescence of *V. fischeri* MJ-301. Many cellular growth activities in enteric bacteria are controlled by cAMP (8), and cAMP is a primary activator of *lux* gene transcription in *V. fischeri* (see reference 36 for a review). We reasoned, therefore, that if extracellular cAMP were toxic

for *V. fischeri* and if the periplasmic 3′:5′-CNP functioned to protect *V. fischeri* from that toxic effect by degrading the cAMP, then cells of MJ-301, which lack the enzyme, would exhibit altered growth and luminescence characteristics when exposed to high concentrations of cAMP in the medium. However, regardless of the presence or amount of cAMP added to the medium (LBS broth with no added cAMP, 5 mM cAMP, or 10 mM cAMP), MJ-301 grew at a rate and to a final cell density like MJ-1 and produced light and regulated light production like MJ-1. Similar results were obtained with cells tested in VFM broth. The lack of obvious effects on growth or luminescence suggests that extracellular cAMP is not toxic for *V. fischeri*. Consequently, it is unlikely, at least under the conditions considered here, that the periplasmic 3′:5′-CNP of *V. fischeri* is necessary for defense against extracellular cAMP.

DISCUSSION

The synthesis of a periplasmic 3′:5′-CNP accounts for the unusual ability of *V. fischeri*, a common marine bacterium, to grow on cAMP as a sole source of carbon and energy, nitrogen, and phosphorus. The novel periplasmic location of the enzyme and its exceptionally high specific activity (25) suggested that the enzyme might be involved in the growth of *V. fischeri* cells on cAMP. This possible relationship provided a strategy by which the gene (*cpdP*) for this enzyme was successfully cloned in *E. coli*. Mutagenesis of the gene and use of gene replacement procedures to construct a *cpdP* mutant of *V. fischeri* permitted the relationship between the enzyme and growth on cAMP to be confirmed.

Cloning of the *cpdP* gene through selection for growth of *E. coli* cells on cAMP also confirmed an earlier conclusion that for growth on 3′:5′-cyclic nucleotides, most enteric bacteria would require only one new enzyme, periplasmic 3′:5′-CNP (25). *E. coli* and many other enteric bacteria synthesize a periplasmic (membrane-associated) 5′-nucleotidase that permits cells to dephosphorylate 5′-AMP from the environment to adenosine (e.g., 6, 7, 37, 46, 62), which can then be transported across the cytoplasmic membrane and catabolized for growth (39, 62). Exceptions to this single new enzyme idea would be certain strains of *S. typhimurium* that lack 5′-nucleotidase activity (38).

Isolation of the *cpdP* gene through selection for growth on cAMP was successful when the selection medium lacked nitrogen and phosphorus. We had noted that the presence of nitrogen and phosphorus can suppress the growth of *V. fischeri* on cAMP under some conditions (21); this suppression might be more severe in *E. coli*. The growth suppression, however, apparently is not due to inhibition of the activity of 3′:5′-CNP by phosphate or nitrogen, since the presence of neither 0.25 mM KH₂PO₄ nor 15 mM NH₄Cl decreases the activity of the purified enzyme (13). Other possibilities include repression of the synthesis of 3′:5′-CNP or effects on 5′-nucleotidase and the adenosine uptake system. Regardless of this issue, *E. coli* containing the *cpdP* gene, like *V. fischeri*, can utilize 3′:5′-cyclic nucleotides as its sole source of carbon and energy, nitrogen, and phosphorus for growth.

The rapid growth on cAMP of *E. coli* containing the *V. fischeri cpdP* gene, besides indicating that the *cpdP* gene is expressed properly and that the CpdP protein functions appropriately in the environment of the *E. coli* periplasm, suggests that the *E. coli* protein secretory apparatus recognizes and properly processes the *V. fischeri* pro-CpdP leader peptide. Analysis of the pro-CpdP leader peptide sequence

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(Fig. 2) supports this suggestion in that the sequence exhibits features typical of leader peptides of *E. coli* and other bacteria (19, 41, 60). The description given here is, to our knowledge, the first report of an exported protein and a leader peptide in *V. fischeri*.

Beacham and coworkers (4, 34) cloned from *E. coli* and sequenced a different periplasmic cyclic nucleotide phosphodiesterase gene, *cpdB*, which encodes a 2':3'-cyclic nucleotide phosphodiesterase:3'-nucleotidase. The periplasmic *V. fischeri* 3':5'-CNP differs from that enzyme in substrate specificity (25) and in other biochemical attributes (13). However, *V. fischeri* does synthesize a periplasmic 2':3'-cyclic nucleotide phosphodiesterase:3'-nucleotidase, since it grows readily on 2':3'-cAMP (25; Table 2). That Δ*cpdP* mutant MJ-301, like parent strain MJ-1, grows on 2':3'-cAMP and on 3'-AMP but, in contrast to MJ-1, has lost the ability to grow on 3':5'-cyclic nucleotides (Table 2) further strengthens our conclusion that periplasmic 2':3'-cyclic nucleotide phosphodiesterase:3'-nucleotidase and periplasmic 3':5'-CNP are distinct enzymes encoded by separate genes. With regard to cytoplasmic 3':5'-CNP (the *cpdA* gene product), the lack of hybridization of a 1.9-kb *cpdP*-based probe to sequences in the *E. coli* genome (see Materials and Methods) is consistent with *cpdP* and *cpdA* being distinct genes.

The *cpdP* promoter region lacks a canonical −35 hexanucleotide but contains adjacent to the site at which a −35 hexanucleotide would be expected a 12-bp inverted repeat that might serve as the binding site for a regulatory protein involved in activating *cpdP* transcription. If a transcriptional activator protein is involved, this protein probably is present in *E. coli* as well, since *E. coli* containing the *V. fischeri cpdP* gene expressed periplasmic 3':5'-CNP at levels similar to those in *V. fischeri*. We presently know very little about how the *cpdP* gene is regulated. Its expression might be influenced by the composition of the medium, since the specific activity of the enzyme is 2- to 10-fold higher for cells grown in minimal medium than in complete medium and since cells grown in minimal medium exhibit up to a 5-fold variation in specific activity, depending on the carbon and energy source (25). No evidence for induction or repression of the enzyme, however, has been obtained. *V. fischeri* cells at different stages of growth in batch cultures exhibit similar 3':5'-CNP specific activities (22, 25). Furthermore, cells grown on glucose in minimal medium exhibit no lag in growth when transferred to minimal medium containing cAMP as the sole carbon and energy source, whereas they do exhibit a 1- to 2-h lag when transferred to ribose (21). These observations suggest that glucose does not repress the synthesis of 3':5'-CNP. In *V. fischeri*, the regulatory region for the *lux* gene system contains a canonical cAMP receptor protein (CRP) binding site and a 20-bp inverted repeat that is thought to be the binding site for the LuxR protein, the transcriptional activator of the *lux* operon (see reference 36 for a review). In addition, cAMP controls the expression of the *cpdB* genes of *E. coli* and *S. typhimurium* and the *D. discoideum* extracellular 3':5'-CNP gene (31, 33). The *cpdP* regulatory region, however, contains no sequences similar to the CRP or LuxR protein binding site. Consequently, it is likely that neither CRP nor the LuxR protein is involved in regulation of the *cpdP* gene. The apparent absence of glucose repression of the enzyme is consistent with the lack of a CRP binding site in the *cpdP* regulatory region.

The deduced amino acid sequence of the *cpdP* gene exhibited substantial identity with the low-affinity 3':5'-CNP (PDE1) of *S. cerevisiae* and the extracellular 3':5'-CNP of *D.*

discoideum (30.3 and 33.6%, respectively). The clustering of residue identities in two regions (residues 100 to 146 and residues 238 to 269 of the *V. fischeri* sequence; Fig. 3) suggests that these regions may have functional importance for the enzyme. A hydropathy plot (30, 60) of the protein reveals that the region containing residues 100 to 146 is strongly hydrophilic, consistent with this region being able to interact with charged molecules, such as cAMP and cGMP. The two regions together contain 30 of the 33 residues conserved in all three proteins. Moreover, a single residue, histidine, accounts for 4 of the 30 residues conserved in all three proteins (3 in the first *V. fischeri* region and 1 in the second). Histidine represents only 2% of the residues in proteins of other prokaryotes (18), and it is similarly uncommon in the *V. fischeri* sequence (6 of 330 residues; Fig. 2). The scarcity of histidine residues is contrasted by their importance for catalysis in a recently described eukaryotic 3':5'-CNP (29) and by their involvement in the binding of metal ions, such as zinc, in metalloproteins (1, 15, 28). With respect to metal ion binding by histidine, the presence of the chelating agent EDTA decreases the activity of the *V. fischeri* periplasmic 3':5'-CNP (25). Also, the low-affinity 3':5'-CNP (PDE1) of *S. cerevisiae* contains two zinc atoms per peptide monomer (35). For these reasons, we surmise that the two conserved regions contribute to the catalytic site, substrate binding, or conformation of the enzyme.

The similarity between the *S. cerevisiae* (PDE1) and *D. discoideum* 3':5'-CNP was noted previously by Nikawa et al. (40), who proposed that these two proteins represent an evolutionary lineage distinct from other eukaryotic 3':5'-CNPs. In contrast to the *S. cerevisiae* (PDE1) and *D. discoideum* enzymes, all other eukaryotic 3':5'-CNPs share a conserved domain of 250 amino acid residues (15, 16, 40). The *V. fischeri* enzyme lacks this domain. On the basis of the absence of this domain, the level of residue identity with the *S. cerevisiae* (PDE1) and *D. discoideum* enzymes, and other aspects discussed above, the *V. fischeri* 3':5'-CNP appears to be a member of the *S. cerevisiae* (PDE1) and *D. discoideum* lineage. The presence of a third member in this lineage, in this case a prokaryote, strengthens the proposal by Nikawa et al. (40) for two distinct lineages of 3':5'-CNPs.

The *V. fischeri* 3':5'-CNP permits cells to utilize 3':5'-cyclic nucleotides as growth substrates. The notion has been put forward (25) that the enzyme plays a role in species specificity of the bioluminescent symbiosis of *V. fischeri* with monocentrid fish and sepiolid squids (23, 43). On the basis of this notion, cells of the developing animal light organ release cAMP in response to the presence of colonizing bacteria, and *V. fischeri* cells selectively colonize the light organ in the presence of other bacteria through the activity of the periplasmic 3':5'-CNP, which protects *V. fischeri* cells from the toxic effects of host-released cAMP and permits them to utilize this compound as a growth substrate (25). In the present study, however, we obtained no evidence supporting a role for the enzyme in defense against extracellular cAMP. MJ-301 (Δ*cpdP*::Nm^r) growth and luminescence, which are controlled by cAMP, exhibited no obvious sensitivity to high extracellular levels of cAMP. Nonetheless, the enzyme could be important nutritionally in the symbiosis by permitting *V. fischeri* cells to inhibit by competition other bacteria unable to utilize cAMP. With the recent development of an experimental sepiolid squid symbiosis (43) and with the construction of a *cpdP* mutant of *V. fischeri*, as described here, these issues can now be addressed experimentally. Alternatively, it is possible that the enzyme is

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involved in recovery of the carbon, nitrogen, and phosphorus released from the cell as cAMP, a process that could indicate that the synthesis and degradation of cAMP are separated into different cellular compartments in *V. fischeri*. Moreover, recent studies (22) indicate that certain other marine and terrestrial enteric bacteria produce a periplasmic 3′:5′-CNP at levels similar to that in *V. fischeri* and that natural marine water samples contain substantial 3′:5′-CNP activity (22). A broad distribution of bacteria that possess periplasmic 3′:5′-CNP suggests that the enzyme is an adaptation for scavenging 3′:5′-cyclic nucleotides from the environment. Given its periplasmic location and exceptionally high specific activity (13), the *V. fischeri* 3′:5′-CNP may be well suited to one or more of these functions.

ACKNOWLEDGMENTS

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