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PURIFICATION, CLONING , AND FUNCTIONAL STUDIES OF A NEW TRANSCRIPTIONAL FACTOR LUXT FROM *VIBRIO HARVEYI*

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FORWARD

The present thesis includes the text of original papers submitted for publication (Chapters 2, 3,4, in press or submitted). In compliance with the guidelines for thesis preparations provided by the Faculty of Graduate Studies and Research, I also include a general abstract, a full introduction and literature review (Chapter 1), and a general overall discussion (Chapter 5) as well as connecting texts as prefaces before each Chapter.

ABSTRACT

A *luxO* DNA binding protein (LuxT) was purified to homogeneity from *V. harveyi* after five major chromatography steps including a highly effective DNA affinity chromatography step and reverse phase HPLC. The sequences of three tryptic peptides obtained on digestion of the purified protein did not match any sequences in the protein data bank indicating that LuxT is a new *V. harveyi* protein. Inverse PCR was conducted sequentially to obtain the complete gene (*luxT*) coding for a protein of 153 amino acids which shares homology with the AcrR/TetR family of transcriptional regulators. Gene disruption of *luxT* in *V. harveyi* increased *luxO* expression and affected the cell density dependent induction of luminescence showing that LuxT is a repressor of *luxO*. As LuxT also affected the survival of the *V. harveyi* cells at high salt concentration and close homologues were present in other bacterial species suggested that the LuxT regulatory protein appears to be a general rather than a *lux*-specific regulator.

The *rpoS* gene in *V. harveyi* has been cloned in this work and shown to code for a protein with high homology to the RpoS proteins in other species. The null mutant of RpoS has been constructed and the effect of *rpoS* deletion on stress resistance as well as the cell density dependent luminescence in *V. harveyi* were examined.

RÉSUMÉ

LuxT, une protéine se liant au promoteur *luxO*, a été purifiée jusqu'à homogénéité à partir de *V. harveyi* après 5 étapes majeures de chromatographie incluant une étape hautement efficace de chromatographie d'affinité de l'ADN et un passage en phase inverse sur HPLC. Les séquences de trois peptides tryptiques obtenuent par la digestion de la protéine purifiée ne correspondaient à aucune séquence de la banque de données des protéines, ce qui signifie que LuxT est une nouvelle protéine *V. harveyi*. Un PCR inversé a été effectué de manière sequentielle pour obtenir le gène complet (*lux*T) encodant pour une protéine de 153 acides aminés qui possède une homologie avec la famille de régulateurs transcriptionnels AcrR/TetR. La disruption du gène LuxT dans *V. harveyi* a fait augmenter l'expression de *luxO* et a affecté l'induction de luminescence dépendante de la densité cellulaire, démontrant que LuxT est un répresseur de *luxO*. Le fait que LuxT affecte aussi la survie des cellules *V. harveyi* en concentration saline élevée et que d'autres homologues étaient présents dans d'autres souches bactériennes, suggèrent que la protéine régulatrice LuxT ressemble à un régulateur général plutôt qu'à un régulateur spécifique à *lux*.

Le gène *rpoS* dans *V. harveyi* cloné dans le présent travail démontre qu'il code pour une protéine hautement homologue à la protéine RpoS de d'autres souches. Le mutant de délétion de RpoS a été construit et l'effet de la supression de *rpoS* sur la résistance au stress ainsi que la luminescence dépendante de la densité cellulaire dans *V. harveyi* ont été examinés.

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PUBLICATIONS

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Lin, Y.H., Miyamoto, C., and Meighen, E.A. (2000) Cloning, gene replacement and phenotype characterization of *luxT* , a transcriptional factor of *luxO* from *V. harveyi*. Submitted to *Biochim. Biophys. Acta.*

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ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

I. A new regulatory protein (LuxT) found in cell extracts of *V. harveyi* that bound to a region of DNA upstream from the *V. harveyi luxO* promoter was purified to homogeneity.

II. The *luxT* gene was cloned from *V. harveyi* and the functional role of LuxT studied by constructing a null mutant followed by phenotype characterization. It was demonstrated that LuxT is a transcriptional regulator that negatively regulates the expression of *luxO* gene and modulates the light emission in the early stages of cell growth. Greater resistance to high osmolarity of the LuxT deletion mutant compared to wild type cells was also demonstrated suggesting a pleiotropic role was played by LuxT in *V. harveyi*.

III. The r*poS* gene from *V. harveyi* was cloned and a null mutant constructed. Phenotype studies of the *rpoS* disrupted mutant suggested that RpoS is not involved in controlling luminescence and autoinducer production. The responses of *V. harveyi rpoS* deletion mutant to various environmental stresses compared to wild type cells were also examined.

LIST OF FIGURES

CHAPTER 3

CHAPTER 4

CHAPTER 5

LIST OF TABLE

LIST OF ABBREVIATION

AB	autoinducer bioassay
ATP	adenosine triphosphate
bp	base pair(s)
BSA	bovium serum albumin
cpm	counts perminute
CM	carboxymethyl
CoA	coenzyme A
DTT	dithiothreitol
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
dsDNA	double-strand DNA
HPLC	high performance liquid chromatography
KDa	kilodaltons
LB	Luria-Bertani
PAGE	polyacrylamide gel electrophroresis
PMSF	phenylmethanesulfonyl fluoride
TBE	Tris-borate EDTA.
Tris	tris(hydroxymethyl)amino methane

CHAPTER 1

GENERAL INTRODUCTION

References for chapters 1 through 5 can be found under " GENERAL REFERENCES".

1.1 Historical perspectives on bioluminescence

Bioluminescence is a natural phenomenon in which visible light is generated by an organism as a result of chemical reactions. This phenomenon was recorded over 2500 years ago by the ancient Chinese and Greeks, including Aristotle in the 4th century B.C. The science of bioluminescence, due to its inherent beauty and ease of detection, has fascinated scientists for hundreds of years. In the 17th century, Robert Boyle observed the luminescence of rotten fish, presumably caused by luminescent marine bacteria growing in a saprophytic mode. By showing that light from the dead fish was extinguished in vacuum, the absolute requirement of oxygen for bioluminescence was demonstrated for the first time (Boyle 1668). Two centuries later, French physiologist Raphael Dubois (1887) demonstrated that the luminescence system required luciferase and luciferin, by performing classic *in vitro* experiments with hot and cold water extracts from the luminous mollusc *Pholus dactylus*. A general model for the bioluminescence reaction involving the oxidation of a substrate (luciferin) by the enzyme catalyst (luciferase) has been established. Since the 19th century, the structures of a number of luciferases and luciferins from different organisms have been identified. With the onset of modern biochemistry and molecular biology, many luciferase genes from bacteria, fireflies, clickbeetles, jellyfish, and crustaceans have been cloned and expressed (Meighen, 1991).

The ease and simplicity of light detection has led to the widespread use of luciferase as a sensor for specific metabolites within the cell, as well as a reporter for gene expression and regulation. Recently, accessory proteins in bioluminescence systems,

especially green fluorescent protein (GFP) and aequorin, which are involved in bioluminescence in the jellyfish *Aequorea victoria* have generated great interest among scientists. Both proteins have been cloned, and the recombinant proteins have assumed important roles in an array of biotechnological applications, ranging from monitoring protein dynamics and gene transcription to sensitive labels in analytical assays (Kendall and Badminton, 1998). There is now also an expanding body of knowledge exploring the complicated regulation of bioluminescence in marine bacteria. Of particular interest is the cell-density dependent induction of the luminescence system, which has led to a more advanced understanding of how bacteria, luminous or not, can sense the cell density and regulate specific genes by chemical communication (Hastings and Greenberg, 1999). The mechanistic aspects of cell-cell communication will be reviewed later.

1.2 Bioluminescence

Bioluminescent organisms are widely distributed in nature and compromise a remarkably diverse number of species. Among the light-emitting species are bacteria, dinoflagellates, fungi, fish, insects, shrimp, and squid. This group of organisms includes terrestrial, freshwater, and marine species (Harvey 1952) from almost 50% of the different phyla in the animal and plant kingdoms, although light-emitting species have not yet been found in viruses, higher plants, and vertebrates. In addition to the absence of strong evolutionary relationships between many of the light emission systems, significant differences exist between the bioluminescence reactions as well as between the structures of the luciferases and luciferins from different organisms. Aside from light emission, only

the requirement for oxygen has been clearly recognized as a common feature of these luminescence systems.

1.3 Bioluminescent bacteria

Almost all luminous bacteria have been classified into four genera: *Vibrio*, *Photobacterium*, *Shewanella*, and *Photorhabdus* (Xenorhabdus) (Baumann *et al.*, 1983; Akhurst, 1993). They are the most abundant and widely distributed of the light-emitting organisms and are found in marine, fresh water and terrestrial environments. Their most common habitats are as free-living species in the ocean, as saprophytes growing in dead fish or meat, as gut symbionts in the digestive tracts of marine fish, as parasites in crustacea and insects, and as light organ symbionts in teleost fish and squid (Hastings, 1986). The bacteria are all gram-negative motile rods and can function as facultative anaerobes (Baumann *et al.*, 1983) .

The most thoroughly investigated light-emitting bacteria are *Vibrio fischeri, Vibrio harveyi, Photobacterium phosphoreum, P. leiognathi* and *Photorhabdus luminescens*. *V. fischeri* and *P. leiognathi* are mainly found as symbionts in fish living in shallow and temperate water, while symbiotic *P. phosphoreum* are found in deep water fish. *V. harveyi*, isolated as free-living in marine environments, have not been found to be specific light organ symbionts. *Ph. luminescens* is only found infecting terrestrial organisms. This bacteria primarily acts in symbiosis with nematodes in a parasitic infection of caterpillars and has been isolated from human wounds (Farmer *et al.*, 1989).

1.4 Bioluminescence reaction in bacteria

The light-emission reaction in bacteria involves the oxidation of reduced riboflavin phosphate ($FMNH_2$) and a long-chain fatty aldehyde, resulting in the emission of blue-green light. Because the structures of the substrates are relatively simple, the term luciferin has not been applied to the substrates catalyzed by bacterial luciferase. The reaction is as follows:

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + H_2O + RCOOH + light$$

The reaction is highly specific for $FMNH_2$. Modification of the flavin ring or removal of the phosphate group decreases the activity significantly (Meighen and Mackenzie, 1973).

The natural aldehyde for the bioluminescence reaction is believed to be tetradecanal mainly on the basis of the identification of the compound in lipid extracts (Shimomura *et al.*, 1974). However, differences in aldehyde specificity do exist among different bacterial luciferases. For example, the relative luminescence responses of *V. harveyi* and *Ph. luminescens* luciferases are high with nonanal and decanal at saturating concentrations, while higher light intensities could be obtained with dodecanal for luciferase from *P. phosphoreum*, *P leiognathi* and *V. fischeri* (Meighen, 1991).

The mechanism of the bioluminescence reaction catalyzed by luciferase as well as the enzyme intermediates have been studied extensively (Hastings *et al.*, 1985), primarily because of the slow turnover rate of the enzyme. The reduced flavin ($FMNH_2$) binds to the enzyme and reacts with O_2 to form a 4a-peroxyflavin. This complex interacts with aldehyde to form a highly stable intermediate which decays slowly, resulting in the emission of light, as well as the oxidation of the aldehyde.

The synthesis of long chain aldehyde for the bioluminescence reaction is catalyzed by a multienzyme fatty acid reductase complex which has been purified from *P. phosphoreum* and shown to contain NADPH-dependent acylprotein (and acyl-CoA) reductase (r), acyl transferase (t) and ATP-dependent synthetase subunits (Riendeau *et al.*, 1982; Rodriguez *et al.*, 1983). The three different polypeptides, r, s, and t, with molecular masses of 54, 42, and 33 kDa, respectively, interact to form a large molecular aggregate of approximately 500 kDa with a central core composed of four reductase subunits. Each reductase subunit interacts with a synthetase subunit which in turn interacts weakly with a transferase subunit, resulting in the formation of a complex with the structure $r_4s_4t_{2-4}$. Since the subunit interactions are relatively weak and the protein complex easily dissociates, purification of the intact complex is relatively difficult and needs to be conducted at high protein concentrations. However, the three components can be resolved from each other, purified separately, and then complemented to reconstitute the functional complex.

The transferase subunit diverts fatty acids from the fatty acid biosynthetic pathway into the luminescence system. It catalyzes the transfer of activated fatty acyl groups to water or other oxygen and thiol acceptors, with the acyl group forming a covalent bond with the enzyme during the reaction:

$$RCOX + HOH\ (HSR') \rightarrow RCOOH\ (RCOSR') + XH$$

The acyl donor can be acyl-ACP (acyl carrier protein), acyl coenzyme A, or acyl-p-nitrophenol, with the enzyme showing maximal activity for acyl groups with chain lengths of fourteen carbons (Ferri and Meighen, 1991). The transferase was found to be

related to eukaryotic serine esterases, which diverts short-chain fatty acids from the fatty acid biosynthetic pathway.

The synthetase subunit activates the fatty acid with ATP and forms an acyl-AMP bound intermediate (Wall *et al.*, 1986). The site of acylation on the synthetase was determined to be a cysteine residue near the carboxyl terminal, where the enzyme bound acyl group can interact with the reductase subunit to effect the transfer of the acyl group between the two proteins (Soly and Meighen, 1991). The reductase subunit then catalyses the NADPH-dependent reduction of the activated fatty acid with the overall reaction catalysed by the synthetase and reductase subunits being:

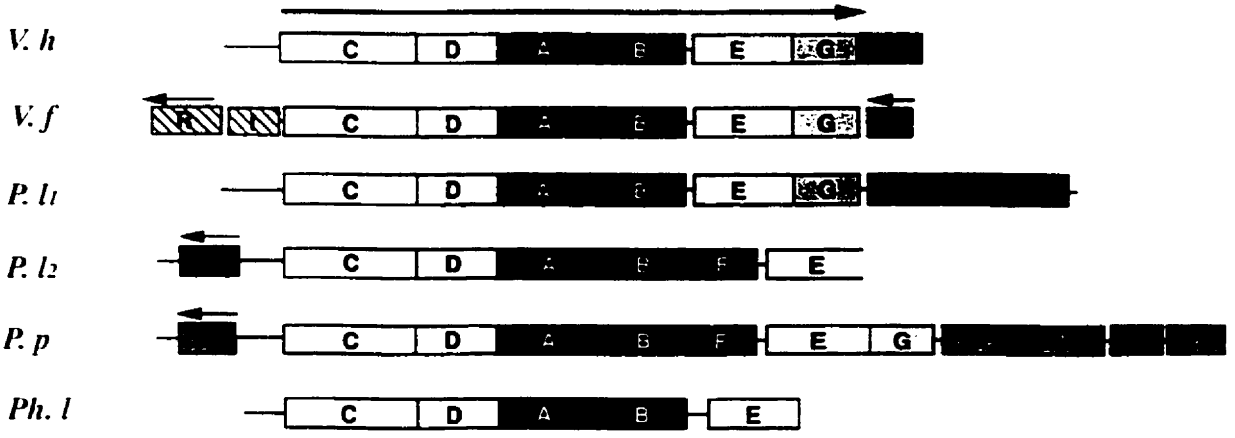
$$RCOOH + ATP + NADPH \rightarrow NADP + AMP + PPi + RCHO$$

The aldehyde product is used as a substrate for luciferase and converted back to its fatty acid form.

1.5 The *lux* structural genes

The *lux* structural genes have been cloned from different bacteria. Figure 1 illustrates the arrangements of the *lux* operon from *V. harveyi, V. fischeri, P. leoignathi, P. phosphoreum,* and *Ph. luminescens*. Aside from the *luxAB* genes encoding for the luciferase α and β subunits respectively, only three genes, *luxC, D,* and *E*, encodes for the reductase, transferase, and synthetase subunits respectively have been associated with all *lux* systems in luminescent bacteria. Other *lux* genes have also been found in certain species. In the marine *Photobacterium* and *Vibrio* genera, the *luxE* gene is immediately followed by the *luxG* gene (Swartzman *et al.*, 1990 a, b) which codes for a

Fig.1. Organization of the *lux* operons of bioluminescent bacteria. *Lux* operons in order from the top are: *V. harveyi* (*V. h*), *V. fischeri* (*V. f*), two strains of *P. leoignathi*(*P. l1* and *P. l2*), *P. phosphoreum* (*P. p*), and *Ph. luminescens* (*Ph. l*). Transcription (indicated by arrows) is from left to right unless indicated otherwise. Single letters refer to the genes in the *lux* operons. Also shown are the closely linked *rib* genes.


V. h
C
D
A
B
E
G
V. f
C
D
A
B
E
G
P. l1
C
D
A
B
E
P. l2
C
D
A
B
F
E
P. p
C
D
A
B
F
E
G
Ph. l
C
D
A
B
E

protein related in sequence to enzymes involved in electron transport and flavin reduction(Andrews *et al.*, 1992). There is another gene in *V. harveyi*, *luxH*, which is located immediately after *luxG* (Swartzman *et al.*, 1990) and bears a strong similarity to *ribB* in *Escherichia coli*, coding for dihydroxy-4-butanone phosphate (DHBP) synthetase, an enzyme involved in the riboflavin biosynthetic pathway. Interestingly, the unlinked *ribB* gene in *V. fischeri* appears to be under the same control as the *lux* genes (Callahan and Dunlap, 2000). In *P. phosphoreum* and *P. leiognathi*, the *ribEBHA* genes encode riboflavin synthetase, DHBP synthetase, lumazine synthetase, and GTP cyclohydrolase II activities, respectively are immediately downstream of the *lux* operon (Lee *et al.*, 1994; Fig. 1). In *V. fischeri*, a convergent gene (Lee *et al.*, 1993) encodes a protein related to RibG in *Bacillus subtilis*, while in *Ph. luminescens*, *rib* genes are not linked to *lux* genes.

Another gene, *luxF*, which encodes the nonfluorescent flavoprotein containing a flavin adduct covalently linked with tetradecanoic acid, has been found to be located between *luxB* and *luxE* genes in some *Photobacterium* species (Mancini *et al.*, 1988; Baldwin *et al.*, 1989). LuxF is a nonfluorescent flavoprotein containing a flavin adduct convalently linked with tetradecanoic acid, as demonstrated by X-ray crystallography (Moore *et al.*, 1993). The function of *luxF* is unknown. Interestingly, only those *Photobacterium* strains with *luxF* have another gene *luxL*, located 600 bp upstream of *luxC* and transcribed in the opposite direction. (Lee *et al.*, 1991). The *luxL* gene encodes the lumazine protein that binds lumazine and/or riboflavin and modulates the spectrum and efficiency of light emission in these species (Lee *et al.*, 1990). Another gene, the *luxY*

gene, related in sequence to *luxL* but not linked to the *lux* operon, has also been discovered in certain strains of *V. fischeri*. LuxY results in a shift of light emission to longer wavelengths rather than to the shorter wavelengths found with LuxL (Eckstein *et al.*, 1990).

1.6 Cell density dependent induction and regulation in bacterial systems

In the 1960s, bacterial bioluminescence attracted increasing interest among microbiologists and biochemists. Plating seawater samples taken from the oceans around the world revealed the presence of bioluminescence bacteria, but at a rather low level of abundance, not more than a few cells per milliliter. A question often raised was what function the light emission might have in free-living planktonic bioluminescent bacteria. It was even more curious that luminescent populations found free in seawater would produce nowhere near enough light to have physiological or ecological significance. The density of the bacteria seems to be critical for light emission.

New insights about cell to cell communication and gene regulation in many bacteria came from the initial observation that newly inoculated cultures of a marine bacteria such as *V. fischeri*, for example, remained dim during early growth until the cells reached a certain density, then the luminescence rapidly increased (Nealson *et al.*, 1970). The lag and subsequent sharp rise of luminescence were attributed to transcriptional regulation and referred to as autoinduction. The autoinduction was attributed to chemicals (autoinducers) synthesized by the cells and accumulated in the media as the cells grow. (Eberhard, 1972). By sensing the level of autoinducers, the cells can determine their

density and initiate the synthesis of luciferase and other enzymes involved in bioluminescence. Autoinduction demonstrates characteristics of cell development and differentiation response to different environments observed in other prokaryotic cells.

As the bioluminescence system was not expressed until the autoinducer(s) accumulated to a certain level, it would be expected to only find light emission at high bacteria concentrations. Isolation and characterization of bacteria from the light organs of fish answered this question. It was found that the luminescent bacteria were concentrated at about 10^{10} per ml, so the autoinducer(s) could accumulate and the fish could use the light for their own purposes.

The autoinduction of bacterial luminescence has been studied in detail. With the isolation of autoinduction genes and identification of the excreted autoinducers as acylhomoserine lactones (acyl-HSL) in *V. fischeri* (Eberhard *et al.*, 1981) and *V. harveyi* (Cao and Meighen, 1989) as well as in nonluminescent bacteria (Fuqua *et al.*, 1994; 1996), it is clear at this stage of scientific inquiry that bioluminescence is induced through cell-to-cell communication. It is also evident that this cell-to-cell signaling is a common phenomenon in bacteria and is referred to as quorum sensing (Fuqua *et al.*, 1994). Despite the widely held view that bacteria are primitive, unicellular organisms that struggle for individual survival, they actually possess extraordinary sophistication in communicating with one another, both among their own species and with others, and sometimes with higher organisms, as well. The following is a short review of some well-studied cell-to-cell communication systems, including the bioluminescence systems in *V. fischeri* and *V. harveyi*.

1.6.1. Regulatory proteins involved in the quorum sensing system related to the *V. fischeri* bioluminescence system

The proteins involved in the quorum-sensing system in *V. fischeri*, the LuxR/LuxI pair, are responsible for regulating bioluminescence. Syntheses of the homologues of this pair of proteins have been shown to occur in over thirty species of gram-negative bacteria (Fuqua *et al.*,1996).

1.6.1.1 LuxI and LuxR constitute the main components of the luminescent regulatory system in *V. fischeri*

Two key components are responsible for the quorum-sensing system of *V. fischeri*: an enzyme that makes the autoinducer, N-3-oxohexanoyl-L-homoserine lactone (AI-1), and a transcriptional factor that can detect the autoinducer and respond to it by activating specific genes (Engebrecht *et al.*, 1983; Engebrecht and Silverman, 1984). The genes that encode these two proteins are *luxI* and *luxR* respectively. The *luxI* gene along with *luxCDABEG* was assigned as the right *lux* operon of *V. fischeri*, while *luxR*, located immediately upstream and transcribed in the divergent direction, was designated as the left operon. *E. coli* recombinants containing both the right and left *lux* operons are able to generate light at the same levels and in the same cell-density-dependent manner as *V. fischeri* (Engebrecht *et al.*, 1983; Engebrecht and Silverman, 1984). As the bacteria multiply within a confined space, the enzyme LuxI produces the autoinducer (AI-1), which diffuses freely into and out of the bacteria cells and eventually reaches a critical

concentration and activates LuxR by binding to the autoinducer at its N-terminal end and turns on the *luxICDABE* operon (Choi and Greenberg, 1991).

In addition to stimulating the right operon through a positive feedback loop, the autoinducer-LuxR complex has also been shown to regulate the *luxR* gene itself, both positively and negatively (Shadel and Baldwin, 1991) depending on the presence or absence of a negative cis-acting element located in the right operon. Stimulation of *luxR* expression by the autoinducer-LuxR complex could only be observed if the right operon was removed or a low concentration of the LuxR protein was present. In contrast, negative regulation could only be observed when the concentration of LuxR was high and DNA from the right promoter extending downstream from *luxI* and *luxC* into *luxD* was present. Deletion analysis of the *luxICDABEG* operon demonstrated that a negatively-acting element is located in the *luxD* gene at a position 2.0 kilobases from the *lux* operator (Shadel and Baldwin, 1992). The nucleotide sequence of this *luxD* element is similar to that of the *lux* operator (with 11 of 20 base pairs being identical) and can function as a LuxR-binding site when it replaces the *lux* operator in the control region. It was proposed that the *luxD* element functions as a low affinity binding site for LuxR and that occupation of this site is required to achieve transcriptional negative autoregulation of *luxR*. Both the positive and negative regulation require the DNA region located between the right and left operons.

1.6.1.2 AinS codes for an enzyme involved in the second autoinducer system

There is evidence that *V. fischeri* cells respond to more than one autoinducer. A gene, *ainS*, has been cloned (Gilson *et al.*, 1995) and held to be responsible and necessary for the synthesis of a second autoinducer, N-octanoyl-L-homoserine lactone (AI-2) (Kuo *et al.*, 1994), which can activate *lux* operon transcription via LuxR in the absence of AI-1. A mutant defective in *ainS* exhibited accelerated luminescence induction compared with that of the parental strain, indicating that AI-2 functions in *V. fischeri* to delay luminescence induction. Increasing amounts of AI-1 overcame the inhibitory effect of AI-2, and the same activation of luminescence required 25- to 45-fold more AI-2 than AI-1. It was concluded that AI-2 inhibits luminescence and apparently functions as a modulator in *V. fischeri* to suppress or delay induction at low and intermediate population densities (Kuo *et al.*, 1996). AinS was found to share some homology with LuxM in *V. harveyi*, which is responsible for synthesizing autoinducer, N-(3-hydroxybutanoyl)-L-homoserine lactone in the latter species. The sequence similarity between AinS and LuxM along with a recent cloning in *V. fischeri* of a protein similar in sequence and properties to a *V. harveyi* regulatory protein, LuxO (Miyamoto *et al.*, 2000), seems to suggest that the two apparently different luminescent regulatory systems actually share some common regulatory proteins. A third *V. fischeri* autoinducer, N-hexanoyl-L-homoserine lactone was also identified (Kuo *et al.*, 1994) .

1.6.1.3 Catabolic repression and the role of cAMP/CRP in the regulation of luminescence in *V. fischeri*

Catabolic repression of bioluminescence was observed in both *V. fischeri* (Ruby and Nealson, 1976) as well as *V. harveyi* (Nealson *et al.*, 1972). The catabolic effect in *V. fischeri* is transient, with the addition of glucose to a *V. fischeri* batch culture resulting in a temporary decrease in light emission, a repression that was not reversed by cAMP. Upon growth in phosphate limited chemostat cultures, light production in *V. fischeri* was repressed permanently by glucose and was restored with the addition of cAMP or autoinducer, AI-1 (Friedrich and Greenberg, 1983). Batch cultures of *V. harveyi*, however, were permanently repressed by glucose and light could only be restored by addition of cAMP to the growth medium.

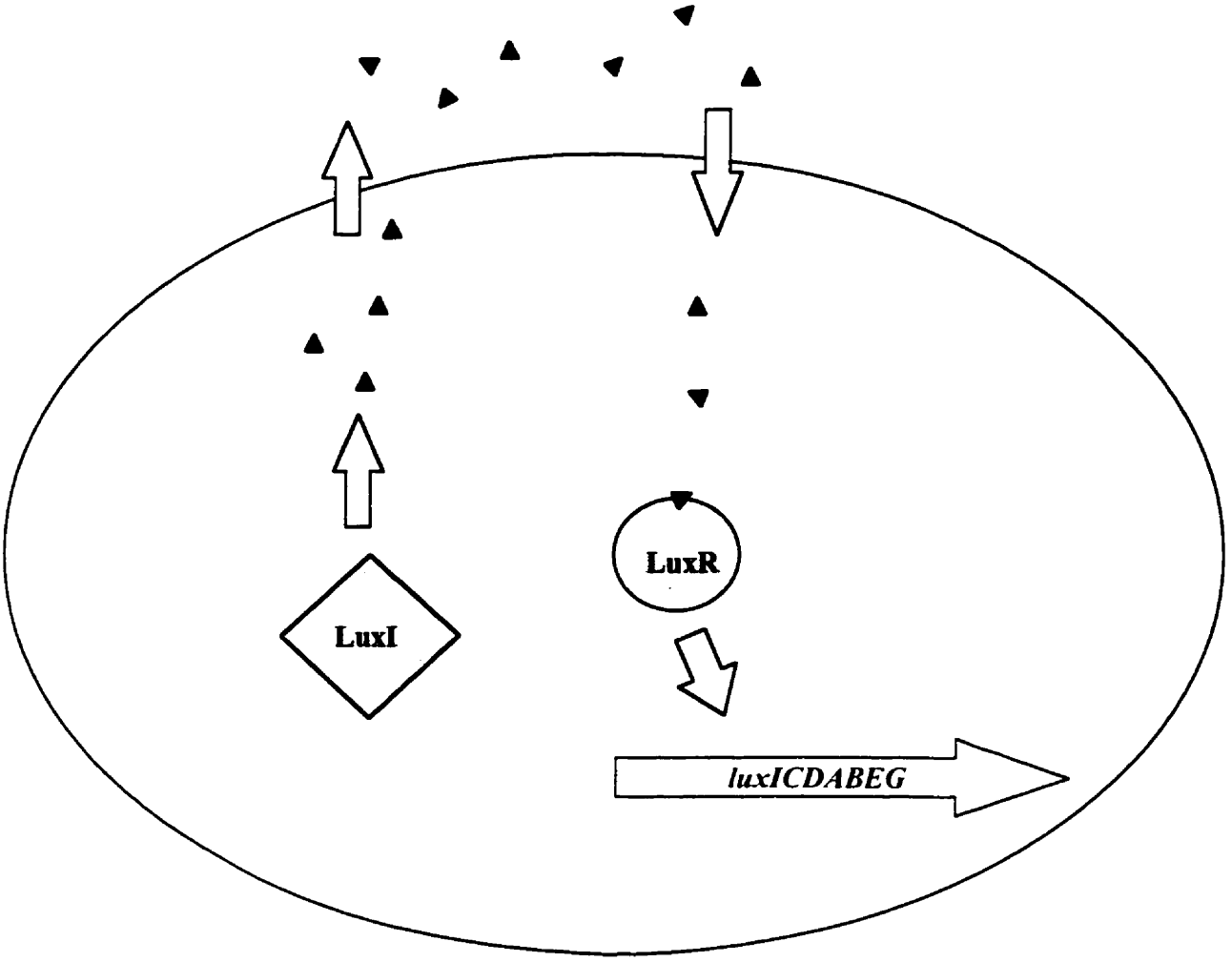
Evidence for the involvement of CRP in the *V. fischeri* bioluminescence regulatory system has been shown by examining the *lux* gene regulation in *E. coli* cAMP and CRP mutants (Dunlap and Greenberg, 1988; Dunlap and Ray, 1989). It was shown that cAMP-CRP activates expression of the left operon containing *luxR*. The production of LuxR, along with the autoinducer, then activates expression of the right operon and induces luminescence. As cAMP-CRP may also inhibit expression of the right operon to some degree and because of the apparent repression of the left operon by autoinducer-LuxR, regulation of the expression of the promoters of the right and the left operon may be indicative of a form of transcription regulation involving a balance between a global regulator (cAMP-CRP) and a local regulator (LuxR).

1.6.2 LuxI/LuxR system is the prototype of the quorum-sensing systems in many gram-negative bacteria

Since the 1990s, a growing number of Gram-negative bacteria have been discovered to have LuxR and/or LuxI homologues (Fuqua *et al.*, 1996). Most of the time, the LuxR homologues function in concert with their respective LuxI homologues and control various physiological functions in bacteria. For examples, the LasR/LasI, RhlI/RhlR systems were shown to control multiple physiological functions in *Pseudomonas aeruginosa* including (i) production of virulence factors (Passador *et al.*,1993), (ii) the twitching motility, a flagellum-independent mode of surface translocation which requires functional type four pili (Glessner *et al.*, 1999), (iii) and the formation of biofilms, structured microbial communities which shelter the bacteria from antibiotics, detergents, and the host's immune system (de Kievit and Iglewski, 1999; Parsek and Greenberg, 1999). TraR and TraI proteins are responsible for conjugate transfer of Ti plasmids in *Agrobacterium tumefaciens* (Hwang *et al.*, 1994). ExpR and ExpI are known to synthesize antibiotics and plant cell-wall degrading exoenzymes in *Erwinia carotovora* (Pirhonen *et al.*, 1993). Although there are highly conservative regions, the total level of homology is generally quite low, around 18-25% identity for LuxR homologues and 28-35% identity for LuxI homologues. Most of the LuxI family of proteins are found to produce acyl-homoserine autoinducers to regulate cell-cell communication. The bioluminescence regulatory system in *V. fischeri* as a prototype of the LuxI/LuxR quorum-sensing systems is illustrated in Figure 2.

1.6.3 Biosynthesis of autoinducers

Fig.2. The bioluminescence regulatory system in *V. fischeri* as a prototype of LuxI/LuxR quorum sensing systems. The autoinducer produced by LuxI (or LuxI homologues), diffuses freely across the membrane and can activate LuxR (or LuxR homologues) and turn on the *lux* operon (or the respective target operons).


LuxR
LuxI
luxICDABEG

Eberhard *et al.* (1991) hypothesized that the homoserine groups of acyl-homoserine lactones might have been derived from S-adenosyl-methionine (SAM) and that the fatty acyl chain originated from acyl-ACP or from acyl-CoA. N-(3-oxohexanoyl) homoserine lactone was synthesized in vitro using crude *V. fischeri* cell extracts as a source of the LuxI protein together with SAM and 3-oxohexanoyl-CoA as substrates. Later evidence, however, suggested that LuxI type proteins synthesize autoinducers with acyl-ACP and SAM as the substrates. Using purified recombinant LuxI, experiments with TraI (homologue from *A. tumefaciens*) and RhlI (homologue from *Pseudomonas aeruginosa*) have shown that N-acyl HSL AIs are synthesized "in vitro" from SAM and acyl-ACPs rather than acyl-CoAs (Schaefer *et al.*, 1996; More *et al.*, 1996; Parsek *et al.*, 1999). All of these proteins produce the appropriate acyl homoserine lactones. The acyl-CoAs cannot substitute for the acyl-ACPs in these reactions. The findings of Eberhard *et al.* (1991) would then be assumed to be due to an enzymatic activity in the *V. fischeri* extract which converted the added acyl-CoA into an acyl-ACP. *In vitro* data were later obtained to provide further evidence that the acyl chains in autoinducers synthesized by LuxI-family synthases are derived from acyl-ACP substrates rather than acyl-CoA. Synthesis of N-(3-oxooctanoyl) homoserine lactone by TraI was unaffected in an *E. coli fad D* mutant blocked in β-oxidative fatty acid degradation. Also, conditions known to induce the *fad* regulon did not increase autoinducer synthesis. In contrast, specific inhibitors of the fatty acid synthesis pathway blocked autoinducer synthesis even in a strain dependent on β-oxidative fatty acid degradation for growth (Val and Cronan, 1998). Evidence suggests that the synthesis of autoinducer is also through the fatty acid

biosynthesis pathway in *V. harveyi*. Blockage of fatty acid biosynthesis by the addition of fatty acids and/or the antibiotic cerulenin to the cells prevented synthesis of the N-(3-hydroxylbotanoly)homoserine lactone in *V. harveyi* (Cao and Meighen; 1993).

1.6.4. Regulatory proteins and the mechanism of autoinduction of the *V. harveyi* bioluminescence system

The regulatory system for control of luminescence in *V. harveyi* differs substantially from that for *V. fischeri* despite the employment of an acyl-homoserine lactone as one of its autoinducers. The activator of the *lux* operon, LuxR* (Showalter *et al.*, 1990; Swartzman *et al.*, 1992; Swartzman and Meighen, 1993), shares no homology to the LuxR of *V. fischeri*. Moreover, instead of the LuxI/LuxR system in *V. fischeri*, the mechanism for the signal transduction in *V. harveyi* is a two-component system generally composed of two key proteins. The first protein (sensor or transmitter) is usually a transmembrane protein with cytoplasmic and extracytoplasmic domains. The extracytoplasmic domain (sensor) senses the environment and transfers the signal through the transmembrane domain to the cytoplasmic domain (transmitter), which has kinase activity. The second protein is located in the cytoplasm and contains an amino-terminal domain (receiver), which can be phosphorylated by the transmitter, and a carboxy-terminal region (regulator), which regulates gene expression by binding to DNA. Two parallel two-component systems have been found to regulate the luminescence in *V. harveyi*, with system one responding to a acyl-homoserine lactone (AI-2) and system two to a yet unidentified organic molecule (AI-2) (Bassler, 1999). The following is a short

review of proteins and mechanisms involved in the *V. harveyi* luminescence regulatory system.

1.6.4.1. LuxR* is an activator of the *lux* operon

Transposon mutagenesis of the genome in *V. harveyi* demonstrated that mutation of a locus other than the *luxCDABE* gene resulted in loss of light. This locus contained *luxR**, which coded for a 24 kDa regulatory protein (Showalter *et al.*, 1990). Complementation of *E. coli* containing the *lux* operon of *V. harveyi* with *luxR** resulted in a 10000 fold stimulation in luminescence (Swartzman *et al.*, 1992). LuxR* has been purified to homogeneity by assaying its binding activity to the promoter of the *lux* operon (Swartzman and Meighen, 1993). The cell-density-dependent induction of light and the dependence on the autoinducer could not be reconstituted and the light levels in *E. coli* were still 10- to 100-fold lower than the native strain. As mentioned previously, *luxR** in *V. harveyi* has no relation to the *luxR* gene in *V. fischeri*.

The *luxR** gene is also subject to autoregulation by its own protein (Chatterjee *et al.*, 1996). Mobility shift assays have shown that LuxR* binds to the *luxR** promoter. This promoter could be readily expressed in a transcriptional fusion with the chloramphenicol acetyl transferase (*cat*) gene in *E. coli* and *V. harveyi* without the presence of LuxR*. This is not the case for the *lux* operon, which needs the LuxR* protein as an activator. It was shown that LuxR* could work as a repressor for the *luxR** gene by interfering with the RNA polymerase. Evidence has also been provided that

autoinducer AI-1 could stimulate the expression of the *luxR** gene (Miyamoto *et al.*, 1996).

In addition to being the key transcriptional factor in the luminescence system, LuxR* was also found to control the synthesis of poly-(3-hydroxybutyrate) in *V. harveyi* (Sun *et al.*, 1994; Miyamoto *et al.*, 1998). These results have extended the role of homoserine lactones and LuxR in metabolic regulation to include the control of synthesis of potential energy reserves in *V. harveyi* in addition to regulating the bioluminescence.

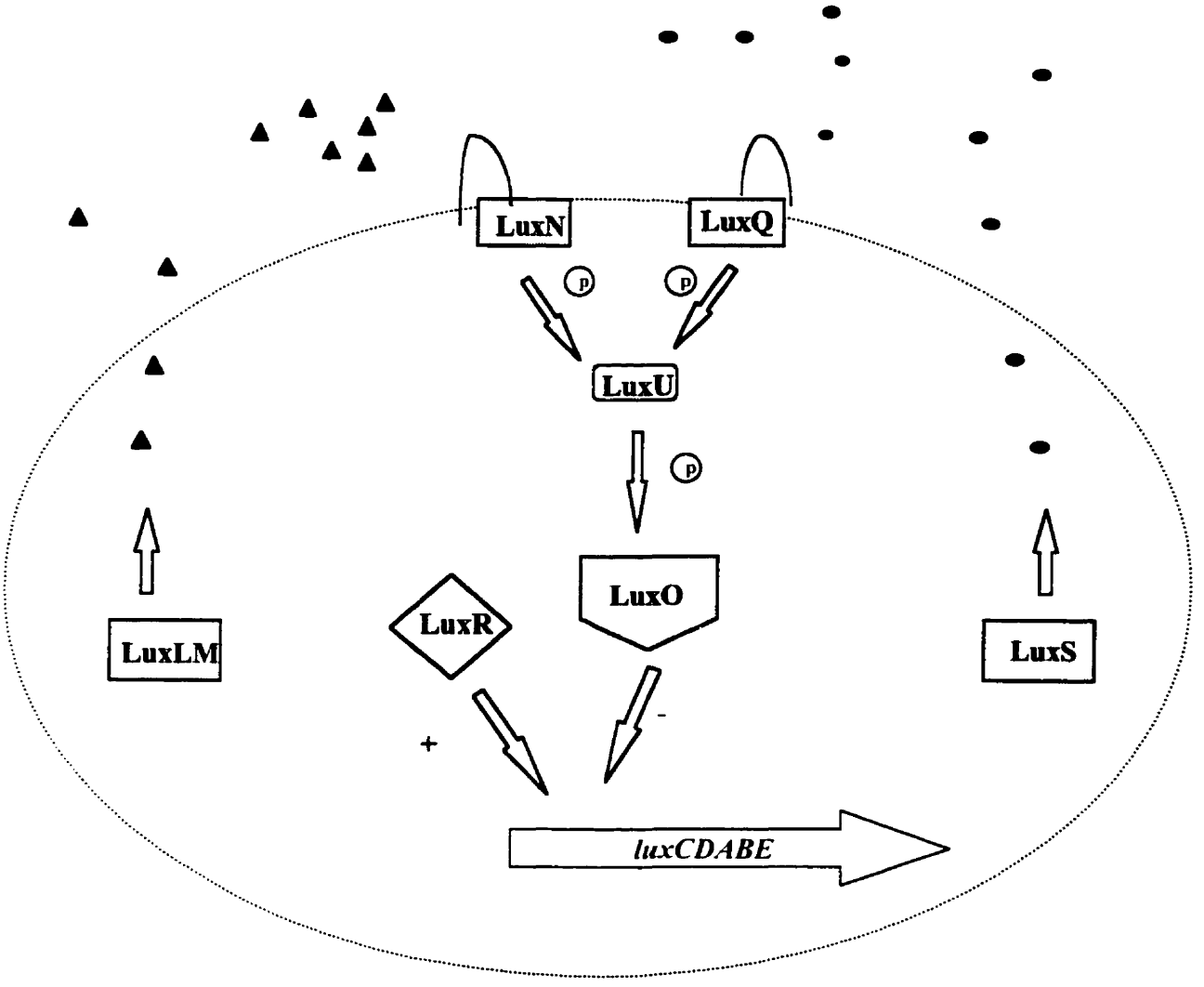
1.6.4.2 The parallel autoinducer-response systems in *V. harveyi*

Transposon mutagenesis studies on several genes which could complement a spontaneous dim mutant have led to the establishment of an initial model for the luminescent regulatory system in *V. harveyi*. Two autoinducers, N-(3-hydroxybutanoyl)-L- homoserine lactone (AI-1) and the as yet to be identified chemical (AI-2), produced by LuxLM (Bassler *et al.*, 1993) and LuxS respectively (Surette *et al.*, 1999) are recognized by their cognate sensors, LuxN and LuxQ, which are transmembrane proteins (Bassler *et al.*, 1993; 1994a; Freeman *et al.*, 2000). Under low cell-density conditions, without autoinducers, LuxN and LuxQ are kinases which can undergo autophosphorylation and intramolecular phosphotransfer from their respective sensor kinase domains to the response regulator domains. The transfer of the phosphoryl group will then continue from the sensors via a shared intermediate phosphotransfer protein, LuxU (Freeman and Bassler, 1999a), to LuxO, the proposed repressor protein of the *lux* operon in its phosphorylated form (Bassler *et al.*, 1994b; Freeman and Bassler, 1999b). In contrast,

under the condition of high cell density, when autoinducer concentrations are high, LuxN and LuxQ respond to these signals, dephosphorylate themselves and cause the backward flow of the phosphoryl group from LuxO, which results in its inactivation upon dephosphorylation. Consequently, the *lux* operon is derepressed, and the luminescent structure genes turned on, causing the bacteria to emit light. The initially proposed model for the two component quorum sensing system for luminescence in *V. harveyi* is illustrated in Figure 3. It was recently proposed, however, that the LuxO, along with an alternative sigma factor, σ^{54}, affects cell-density-dependent *lux* expression indirectly by activation of a negative regulator of the luminescence system (Lilley and Bassler, 2000).

Two-component systems such as the one regulating luminescence in *V. harveyi* (sensor kinase: LuxN and LuxQ; response regulator: LuxO) can be found in mediating adaptive responses in many different bacteria and include a variety of diverse functions, such as control of chemotactic behavior, global control of metabolic activities, and regulation of cell differentiation (Stock *et al.*, 1989). The LuxI/LuxR quorum-sensing system in *V. fischeri*, whose homologous system also exists in many nonluminescent bacteria for various physiological functions, is not present in *V. harveyi* despite the similar structure of the signal molecule, acyl-homoserine lactone. However, as mentioned previously, the discovery of the presence of a LuxO protein homologue in *V. fischeri* (Miyamoto *et al.*, 2000) and the similarity between LuxM and AinS (Gilson *et al.*, 1995) seem to suggest that the *V. harveyi lux* regulatory system may be the common regulatory system in these luminescent bacteria.

Fig.3. The proposed model for the two-component quorum-sensing system for luminescence in *V. harveyi* (Bassler, 1999). Autoinducers produced by LuxLM and LuxS and excreted into the media are recognized by their cognate transmembrane sensors, LuxN, and LuxQ, respectively. The autoinducer signal will cause the dephosphorylation of the regulator molecules leading to the loss of phosphate from the sensors as well as LuxU and LuxO. As LuxO is a negative regulator of luminescence in its phosphorylated form, dephosphorylation of LuxO will result in an increase in light emission.


LuxN
LuxQ
P
P
LuxU
P
LuxO
LuxR
LuxLM
LuxS
+
-
luxCDABE

1.6.4.3 LuxS is present in many bacteria other than *V. harveyi*

It is interesting to note that while the *luxLM* gene and the corresponding autoinducer, N-(3-hydroxybutanoyl)-L-homoserine lactone (AI-1) seem unique to *V. harveyi*, the parallel autoinducer-producing (yet unidentified AI-2) gene, *luxS*, can be found in many Gram negative as well as Gram positive bacteria, including *Escherichia coli, Salmonella typhimurium, Salmonella typhi, Salmonella paratyphi, Haemophilus influenzae, Helicobacter pylori, Bacillus subtilis, Borrelia burgdorferi, Neisseria meningitidis, Neisseria gonorrhoeae, Yersinia pestis, Campylobacter jejuni, Vibrio cholerae, Deinococcus radiodurans, Mycobacterium tuberculosis, Enterococcus faecalis, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus mutans, Staphylococcus aureus, Clostridium perfringens, Clostridium difficile, Shewanella putrefaciens, Klebsiella pneumoniae,* and *Pasteurella multocida* (Surette *et al.*, 1999). The discovery of the existence of *luxS* in these strains was either obtained by direct cloning or database analysis of finished and unfinished bacterial genomes. Some species have been tested for their ability to produce AI-2, which is not an acyl-homoserine lactone. Currently, the only target operon shown to be regulated by LuxS besides controlling luminescence in *V. harveyi* is the LEE (Locus of Enterocyte Effacement) genes in *E. coli* which encode factors critical for the bacteria's successful infection into animal cells (Sperandio *et al.*, 1999). Evidence exists, however, indicating that pathogenicity is regulated by AI-2 in *S. typhimurium* and *V. cholerae* (Bassler, 1999).

It was suggested that AI-1, being unique to *V. harveyi*, is the signal for intra-species communication while AI-2 might be the signal for inter-species communication.

This hypothesis is based on an experiment showing that the *V. harveyi* mutant that cannot sense AI-2, but only pick up AI-1, can respond only to the conditioned media from *V. harveyi* and a few other bacteria, while the mutants which cannot sense the AI-1, but can detect AI-2, are able to respond to a broad range of bacteria (Bassler *et al.*, 1997).

1.6.4.4 The role of CRP/cAMP in the regulation of bioluminescence in *V. harveyi*

In addition to the observation of catabolic repression in *V. harveyi*, a *crp* gene has been cloned and a CRP null mutant of *V. harveyi* constructed to study the relationship between CRP and the regulation of bioluminescence. The elimination of luminescence in the *V. harveyi crp*$^-$ mutant suggests that CRP is essential for the emission of light. It is not clear, however, that CRP, a global regulator, affects the luminescence through control of the *lux* and/or *luxR** operons, or indirectly via other mechanisms. A consensus CRP binding site is located 455 bp upstream of *luxC* and a DNA fragment containing this region was shown to bind to CRP in a cAMP-dependent manner in a mobility shift assay implying that CRP might control luminescence by binding to the promoter of the *lux* operon. (Jadip Chatterjee, personal communication).

1.6.4.5. Regulation of the *lux* operon by multiple transcriptional factors

In addition to LuxR and CRP, a transcriptional factor homologous to MetR of *E. coli* has been found to bind to the *lux* operon promoter (Jadip Chatterjee, personal communication) and has been proposed to control light expression with its ligand, homocysteine. Addition of homocysteine to the media has been shown to delay the

induction of luminescence, suggesting that MetR might function as a repressor of the *lux* operon. Recent data (Lilley and Bassler, 2000) suggested that the action of LuxO affects luminescence by activating a repressor of the *lux* operon. Assuming this factor is not MetR, it would appear that multiple-regulators (LuxR, CRP, MetR, and the unknown repressor) are needed to control expression of the *lux* operon.

1.6.5. Cell-cell communication in *E. coli* and *S. typhymurium*

Using a *V. harveyi* mutant which could only respond to the second autoinducer (AI-2) media of both *E. coli* and *S. typhymurium* were shown to contain this particular autoinducer (Surette and Bassler 1998). In addition, the discovery of *luxS* in both bacteria (Surette *et al.*, 1999) suggested the existence of quorum-sensing systems similar to *V. harveyi*'s second system. As previously mentioned, the substance produced by *luxS* induces the expression of *E. coli*'s LEE genes which produce a "molecular syringe" causing the injection of bacterial factors critical for successful infection of animal-host cells (Sperandio *et al.*, 1999).

In addition to the quorum sensing system homologous to the *luxS* system of *V. harveyi*, additional quorum sensing systems might also exist in *E. coli*. An *E. coli* genomic library has been screened for genes regulated by extracellular molecules. Sixteen genes were identified and preliminary characterization of the extracellular molecules (there are more than one) controlling these genes revealed that they are not the same as AI-2 synthesized by *V. harveyi* (Baca-DeLancey *et al.*, 1999). These results provided evidence

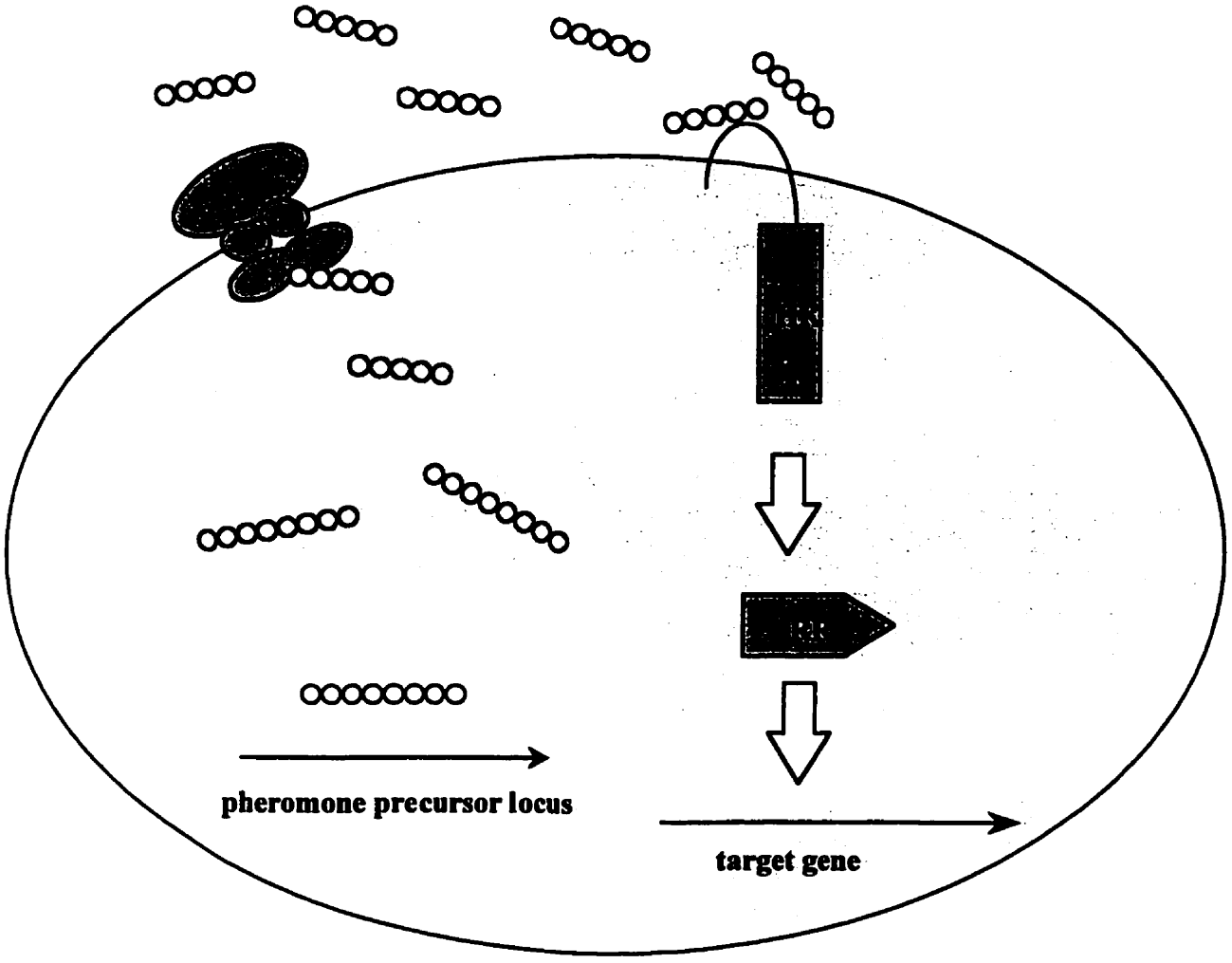
that multiple quorum-sensing systems are present in *E. coli* and control various physiological functions.

1.6.6 Cell-cell communication in Gram-positive bacteria

A variety of processes are known to be regulated in a cell-density-dependent or growth-phase-dependent manner in Gram-positive bacteria (Dunny and Leonard, 1997). For example, the regulation of virulence response in *Staphylococcus aureus*; the induction of competence for transformation in *Bacillus subtilis* and *Streptococcus pneumoniae*; the induction of conjugation in *Enterococcus faecalis*; and the production of antimicrobial peptides, known as bacteriocins by several Gram-positive bacteria, including lactic acid bacteria. The mechanism of cell-cell communication is the two-component phosphorelay cascade, which is also employed by *V. harveyi*. However, Gram-positive bacteria do not employ acyl-homoserine lactones as autoinducers. Instead, all autoinducers for the Gram-positive bacteria are small peptides posttranslationally processed from larger precursor peptides. In most cases, the signal peptides actively secreted by ABC (ATP binding cassette) exporter proteins are sensed by two-component membrane-sensor kinases which will undergo phosphorylation and transfer the phosphoryl groups to the cytoplasmic respond regulators of the target genes. A model of the quorum-sensing system for Gram-positive bacteria is shown in Figure 4.

1.7. Alternative sigma factors might play a role in luminescence

Fig.4. A general model of the quorum sensing system for Gram positive bacteria. The peptide precursors undergoe posttranslational modification and are exported to the media by protein transporters. The signal is tranduced to the target gene through a two-component system indicated by SK (sensor kinase) and RR (response regulator) .


pheromone precursor locus
target gene

In addition to the most common sigma factor, σ^{70}, both Gram-negative and Gram-positive eubacteria employ alternative sigma factors that confer different promoter specificities on the core form of RNA polymerase (Helmann and Chamberlin, 1988). Some of the alternative sigma factors allow transcription of genes whose products contribute to common physiological responses. For example, sigma 32 of enteric bacteria allows the transcription of genes whose products are needed for protection from heat shock and certain other stresses (Grossman *et al.*, 1984). Both Sigma F of enteric bacteria (Arnosti and Chamberlin, 1989) and sigma 28 of *Bacillus subtilis* (Helmann *et al.*, 1988a, 1998b) confer the same promoter-specificity on core polymerase and allow for the transcription of genes required for motility and chemotaxis. The gene product of sigma S, or sigma 38, is responsible for the expression of many genes for the bacteria to survive in the stationary phase and other stress conditions (Hengge-Aronis, 1993; Loewen and Hengge-Aronis, 1994). Examples of genes regulated by sigma S are: genes that confer the resistance to H_2O_2 for many bacteria; virulence genes in *E. coli* and *Salmonella* strains, *Vibrio cholerae*, and *Pseudomonas aeruginosa*; genes producing the enzymes responsible for the synthesis of trehalose (O-α-D-glucosyl(1-1)-α-D-glucoside) involved in osmoprotection in a variety of bacteria species; *glgS*, a gene which synthesizes glycogen, a storage compound which can be the carbon source for stationary-phase cells of *E. coli*. Sigma N, or sigma 54, is needed for transcription of genes whose products have diverse physiological roles (Kustu *et al.*, 1989). Examples of such genes are (i) genes involved in assimilation of nitrogen in many enteric bacteria; (ii) genes on the TOL (toluene) plasmid of *Pseudomonas putida* that encode proteins required for catabolism of toluene and

xylene; (iii) genes producing transport components for dicarboxylic acids; (iv) genes synthesizing two of the components of a formate-degradative pathway in *E. coli*; (v) genes encoding hydrogenase responsible for the oxidation of molecular hydrogen in *Alcaligenes* eutrophus and *Pseudomonas facilis*; (v) genes synthesizing the hook and filment proteins of *Caulobacter* flagella and (vi) genes coding for pilins in *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae* that allow these organisms to adhere to human epithelial cells.

Sigma 54 has recently been connected to luminescence regulation by sequence analysis of the the LuxO protein which shows significant homology to a large number of sigma 54-dependent regulators, including several prototype regulators of this family, such as, NtrC, nitrogen assimilation regulatory proteins, and NIF-specific proteins in various bacteria. It was suggested that the action of LuxO was sigma 54-dependent when a *luxO* homologous gene was cloned along with other sigma 54-dependent regulators from *V. cholerae* using degenerate primers designed according to a highly conserved catalytic domain required for the activation of transcription by 54-holoenzyme (Klose *et al.*, 1998). Recently, the *rpoN* gene in *V. harveyi* has been cloned and a null mutant constructed (Lilley and Bassler, 2000). The *rpoN* deletion mutant does not express luminescence in a density- dependent manner, instead, it constitutively emits light which is indistinguishable from the phenotype of the LuxO null mutant. This result not only demonstrated that sigma 54 is required for the LuxO activity of the density dependent regulation of Lux expression in *V. harveyi*, but also suggests that LuxO which has been previously proposed as a repressor of *lux* operon is actually an activator controlling

luminescence indirectly through a negative regulator of the *lux* operon in the early stages of cell growth.

The other sigma factor that might be implicated in luminescence is sigma S. This connection derives from the experiment showing that homoserine lactone affects the level of sigma S in *E. coli* cells (Huisman and Kolter, 1994). Although it was then found that homoserine lactone and acyl-homoserine lactone share different metabolic pathways, mutants blocked in homoserine lactone biosynthesis can make acyl-homoserine lactone but are unable to respond to it unless they are provided with HS or HSL. This suggests that in addition to sensing the extracellular concentration of acylated homoserine lactone, the autoinduction circuitry also senses intracellular homoserine lactone (Zambrano and Kolter, 1996). The possibility that sigma S might be involved in luminescence also comes from the studies of the LasI/LasR and RhlI/RhlR quorum-sensing systems in *Pseudomonas aeruginosa* (Latifi *et al.*, 1996). It was found that the *rpoS-lacZ* fusion is regulated directly by RhlR and its cognate autoinducer, N-butanoyl-L-homoserine lactone. In *R. solanacearum*, however, the acyl-HSL-dependent autoinduction system was shown to be controlled by the sigma S factor (Flavier *et al.*, 1998). Studies on strain WCS358 of *Pseudomonas putida* have shown that *rpoS* is not involved in the production of three acyl-homoserine lactones in this bacteria (Kojic *et al.*, 1999).

1.8 Contents of this thesis

This thesis will focus on the regulation of the luminescence system in *V. harveyi* with particular emphasis on the transcription regulation of the *luxO* promoter. Chapter 2

will describe the purification to homogeneity of a new DNA-binding protein binding to the promoter of *luxO* which we have referred to as LuxT. The *luxT* gene was then cloned and a null mutant constructed for functional studies, with the data being presented in Chapter 3. Chapter 4 investigated the role of the *rpoS* gene and whether or not it plays a role in luminescence. A general discussion is presented in Chapter 5.

CHAPTER 2

Purification and characterization of a *luxO* promoter binding protein LuxT from *Vibrio harveyi*

PREFACE

The following chapter has been published as presented: Lin YH, Miyamoto C, and Meighen EA (2000) *Protein Expr. Purif.* In press.

CONTRIBUTION BY OTHER WORKERS

Carol Miyamoto conducted the initial mobility shift of the DNA fragment containing the *luxO* promoter region with the *V. harveyi* crude extract.

ABSTRACT

Bioluminescence in the marine bacterium *Vibrio harveyi* is cell density dependent and is regulated by small molecules (autoinducers) excreted by the bacteria. The autoinducer signals are relayed to a central regulator, LuxO, which acts in its phosphorylated form as a repressor of the *lux* operon at the early stages of cell growth. We report in these studies the purification to homogeneity of a *luxO* DNA binding protein (LuxT) from *V. harveyi* after five major chromatography steps including a highly effective DNA affinity chromatography step and reverse phase HPLC. Regeneration of binding activity was accomplished after HPLC and SDS PAGE by renaturation of LuxT from guanidine chloride. It was also demonstrated that the functional LuxT was a dimer of 17 kDa that bound tightly (Kd = 2 nM) to the *luxO* promoter. The sequences of three tryptic peptides obtained on digestion of the purified protein did not match any sequences in the protein data bank indicating that LuxT is a new *V. harveyi lux* regulatory protein.

INTRODUCTION

The bioluminescence reaction in *Vibrio harveyi* is catalyzed by luciferase which utilizes $FMNH_2$, O_2, and a fatty aldehyde as substrates (Hastings *et al.*, 1985). The *luxCDABEGH* operon contains the critical genes required for light emission; *luxAB* encoding the α and β subunits of luciferase and *luxCDE* encoding the fatty acid reductase subunits responsible for synthesis of the fatty aldehyde (Engebrecht and Silverman, 1984; Meighen, 1994). The role of the *luxGH* genes is still unknown although the gene products have been implicated in the metabolism and/or synthesis of flavins (Andrew *et al.*, 1992; Richter *et al.*, 1992; Lee and Meighen, 1992).

Luminescence in *V. harveyi* is controlled by a quorum sensing mechanism. Signal molecules (autoinducers), synthesized and excreted by the cell, accumulate in the media and are sensed by the cells resulting in induction of light. An integrated sensor system has been proposed to respond to two distinct autoinducers, AI-1 and AI-2 (Bassler *et al.*, 1993; 1994a). One of the autoinducers, AI-1, has been identified as N-(3-hydroxybutanoyl)-homoserine lactone (Cao and Meighen, 1989) with the *luxLM* genes required for its synthesis (Bassler *et al.*, 1993). The second autoinducer is not identified yet although the *luxS* gene has been cloned and is responsible for production of this second signal (Surette *et al.*, 1999). The two autoinducers are proposed to interact with their respective transmembrane two-component sensor kinases, LuxN and LuxQ (Bassler *et al.*, 1993; 1994a). At low concentrations of autoinducer in the early stages of cellular growth, the LuxN and LuxQ sensors undergo an autophosphorylation on a histidine residue followed by an intramolecular transfer of the phosphoryl group to an aspartate

residue. The two signals are then integrated by transfer of the phosphoryl group to LuxO (Bassler *et al.*, 1994b) via a small phosphorelay protein, LuxU (Freeman and Bassler, 1999a). Transposon insertion in the *luxO* locus in the *V. harveyi* genome causes constitutive expression of light (Bassler *et al.*, 1994b) showing that LuxO functions as a repressor of luminescence. The LuxO response regulator is proposed to cause repression in its phosphorylated form by blocking the induction of luminescence at the early stages of cellular growth. As the cells grow and autoinducers accumulate in the media, LuxN and LuxQ sense their respective autoinducers inhibiting their kinase and preferentially becoming phosphatases which in turn cause the dephosphorylation of P-LuxO resulting in its inactivation and the derepression of the *lux* operon (Freeman and Bassler, 1999b; Freeman *et al.*, 2000). In addition, a transcriptional activator, LuxR, binds to the *lux* operon and is required for its expression (Showalter et al., 1990; Swartzman *et al*, 1992; Swartzman and Meighen, 1993).

In the present studies, we report the purification to homogeneity of a DNA binding protein (LuxT) which binds tightly to the promoter region of the *luxO* gene. As only low levels of the protein were present in the cell, multiple chromatographic steps including a separation on a highly specific DNA affinity column containing the *luxO* promoter DNA were necessary to purify the protein. A single band of 14-17 kDa was obtained on SDS gel electrophoresis which could be renatured resulting in the restoration of the binding function. The purified protein was shown to bind very tightly to a binding site between 117 and 149 bp upstream of the *luxO* initiation codon.

MATERIALS AND METHODS

Bacterial strains and cell growth

V. harveyi BB392 was grown in LB medium at 27 °C and harvested at A_{660}=1.5.

Mobility shift assay

The DNA binding assay was performed according to Swartzman and Meighen (1993). The reaction mixtures (20 µl) containing approximately 1 ng of a 5'-^{32}P labelled DNA fragment amplified from PCR (1000 cpm) and the indicated amount of protein extract was incubated in 10 mM Hepes, pH 7.9, 100 mM KCl, 2 mM DTT, 0.4 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA, 10% glycerol with 3 µg of poly(dI-dC) for 30 min at room temperature. The incubation mixtures were resolved by electrophoresis on a 5% polyacrylamide gel in 0.5 x TBE .

Protein Purification

(i) Preparation of the cell extract: The DNA binding activity was purified from 20 liters of *V. harveyi* cells (28 g wet weight), Clear lysate was obtained by sonication of the cells followed by centrifugation. The lysate was then precipitated with 25% to 65% saturated ammonium sulfate, collected by centrifugation, and then dialyzed overnight against buffer A (10 mM Tris-HCl, pH 7.9, 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 5% glycerol).
(ii) DEAE -Sepharose chromatography: The sample was loaded onto a 250 ml DEAE - Sepharose column and washed with buffer A until A_{280} was under 0.05. Protein was eluted using a 500 ml gradient of buffer A from 0.1 M KCl to 1.0 M KCl. Fractions with DNA binding activity were pooled and dialyzed against 2 liters of buffer A.

(iii) CM-Sepharose chromatography: The dialysate was loaded onto a 100 ml CM-Sepharose column, and the column washed with buffer A until A_{280} was under 0.05. A 250-ml gradient of 0.1 M to 1.0 M KCl in buffer A was applied and the fractions with activity were pooled and dialyzed against 2 liters of buffer A.

(iv) dsDNA cellulose column: The dialysate was loaded on a 50 ml dsDNA cellulose column (Sigma), the column washed with 300 ml of buffer A before eluting with a 100 ml gradient from 0.1 M to 1.0 M KCl in buffer A and the activity collected and dialyzed against 2 liters of buffer A.

(v) Affinity DNA chromatography: The procedure of Kadonaga and Tjian (1986) was followed to generate a DNA affinity column. Two complementary 28-base oligonucleotides (with complementary protruding ends) designed and synthesized according to the binding region defined by mobility shift assays (see Fig. 3) were annealed to each other, 5'phosphorylated and then ligated to give oligomers of the basic oligodeoxynucleotide unit. The ligated DNA was then coupled to CNBr-activated Sepharose CL-2B (Pharmacia) and the resin (10 ml) packed into a column. Samples from the previous step were loaded onto the affinity column and eluted with 20 ml of a 0.1 M KCl to 1.0 M KCl gradient in buffer A.

(vi) Reverse phase HPLC: The samples from the DNA affinity column were mixed with an equal volume of 0.12% trifluroacetic acid (TFA) and subjected to reverse phase HPLC on a Vydac C4 column (The Nest Group, Inc., Southboro, MA, 0.21x 20 cm) in the 1090 HPLC system from Hewlett-Packard Co., Palo Alto, CA. The fractions (150 µl) were eluted with a gradient of 0 to 80% n-propanol containing 0.12 % TFA at a flow rate of 0.15 ml/min. Aliquots (15 µl) of each were dried by vacuum centrifugation, mixed with 1 µl 8 M guanidine hydrochloride for 30 min and then diluted 50-fold (v/v) with buffer A and the protein allowed to renature overnight at 4 °C. Samples with activity in the mobility shift assays were stored at 4 °C.

Renaturation of the binding activity after SDS gel electrophoresis

Renaturation was conducted according to Hager and Burgess (1980). After SDS gel electrophoresis, the gel was rinsed with ddH_2O and incubated for 5 min in ice-cold 0.25 M KCl, 1 mM DTT. The protein band was cut and soaked with two changes of 1 mM DTT in ddH_2O for 15 min. The liquid was decanted, the gel mixed with 1.0 ml of elution buffer (0.1% SDS, 0.05 M Tris-HCl, pH 7.9, 0.1 mM EDTA, 5 mM DTT, 0.1 mg /ml BSA, and 0.20 M NaCl), and then crushed with three or four strokes of a Teflon pestle. After an hour incubation, the supernatant was transferred to an Eppendorf tube and was precipitated with 4 volumes of ice-cold acetone for 30 min at -20 °C. After centrifugation, the pellet was washed with 80% ice-cold acetone to remove traces of residual SDS, treated with 8 M guanidine–HCl and then diluted 1:50 in buffer A and renatured overnight at 4 °C.

Analysis of the molecular weight of LuxT by gel filtration

Partially purified LuxT was precipitated with saturated ammonium sulfate and dissolved in 1 ml of buffer A containing 0.5 M KCl before passing through a 1 x 90 cm Sephacryl S-300 (Pharmacia) column in the same buffer along with known standards (ovalbumin, 45 kDa; chymotrypsinogen, 25 kDa; ribonuclease A, 13.7 kDa). Activity of LuxT was monitored by the mobility shift assay.

Protein sequence analysis. The protein was cut from the SDS- PAGE gel and sent to Harvard Microchem (Harvard University, Cambridge, MA) for trypsin digestion and peptide sequencing.

DNase I footprint analysis

The protein-DNA binding conditions were the same as described in the mobility shift assay except 10 µg of poly (dI-dC) was added. Ten ng of a PCR amplified and ^{32}P-end-labeled (10000 cpm) DNA fragment (144 bp) encompassing the putative binding site

was mixed with varying amounts of the bacterial extracts and the DNA–protein mixture was incubated at room temperature for 30 min. The endonuclease reaction was started by the addition of 1 µl of 20 µg ml^{-1} of DNase I, stopped after 2.5 min with 40 µl stop buffer (10 mM Tris–HCl, pH 7.6, 20 mM NaCl, 20 mM EDTA, 0.2% SDS, 100ug/ml tRNA) and incubated with proteinase K (0.4 mg/ml) at 37 °C for 30 min. The DNA was then precipitated and resolved by electrophoresis in a 6% polyacrylamide sequencing gel.

RESULTS AND DISCUSSION

Binding of V. harveyi *extracts to the proximal upstream DNA of* luxO

A labeled 330 bp DNA fragment extending from -253 to +77 nucleotides from the first nucleotide of the *luxO* initiation codon (probe 1) was used in mobility shift assays to detect proteins binding to the *luxO* promoter region (Fig. 1). Cell extracts of *V. harveyi* but not *E. coli* caused a small distinct mobility shift of this fragment. The bound protein was not LuxR as extracts of a *V. harveyi luxR* null mutant MR1130 also caused this shift (lane 3, Fig. 1) and E.coli extracts containing the *luxR* expression vector had no effect on mobility of this probe (lane 5, Fig. 1). We therefore attributed this activity to a *V. harveyi luxO* binding protein which we have referred to as LuxT.

The specificity of binding of LuxT in *V. harveyi* extracts to the labeled probe was tested (Fig 2) by addition of increasing amounts of a specific cold probe (probe 1, Fig. 3) as well as a non-specific probe (probe 3, Fig. 3). Binding activity could be abolished by adding 25-fold molar excess of the cold probe while the mobility shift was unaffected by a 150-fold excess of a non-specific probe. All assays also contained a 3000-fold (w/w) excess of poly(dI-dC) .

The LuxT binding site was limited by cutting probe 1 (Fig. 3) at the unique TspEI or DraI site into two fragments and the mobility shifts measured with *V. harveyi* extracts (Fig. 3). In both cases, the mobility shift was observed only with the larger fragment (arrows) limiting the binding site to a 97 bp region located between the DraI and TspEI sites. Two additional DNA probes were then synthesized by PCR (probes 2 and 3, Fig. 3) to further define the binding site. Extracts caused a mobility shift with probe 2 but not

Fig. 1. Mobility shift of the *V. harveyi luxO* promoter region with extracts from different bacteria. Extracts (1 μg) were incubated with probe 1 and poly(dI-dC) as described in Materials and Methods. Lane 1, free DNA; lane 2, *V. harveyi* extract; lane3, *V. harveyi* MR1130 (*luxR*⁻) extract; lane 4, *E. coli* MM294 extract; lane 5, *E. coli* MM294 (+ pT7-*luxR*) extract.

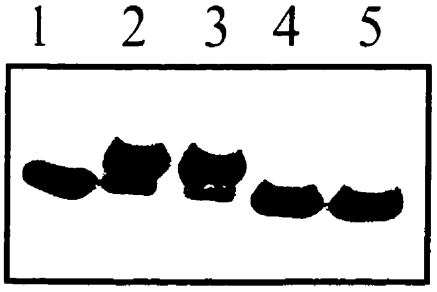

1 2 3 4 5

Fig. 2. Competition of labeled probe 1 with unlabeled probes (see Fig. 3) for binding to LuxT in *V. harveyi* extracts. The molar excess of cold probe added to the mobility shift assay mixture containing 1 μg of extract, 1 ng of labeled probe 1 and 3 μg of poly (dI-dC) is indicated.

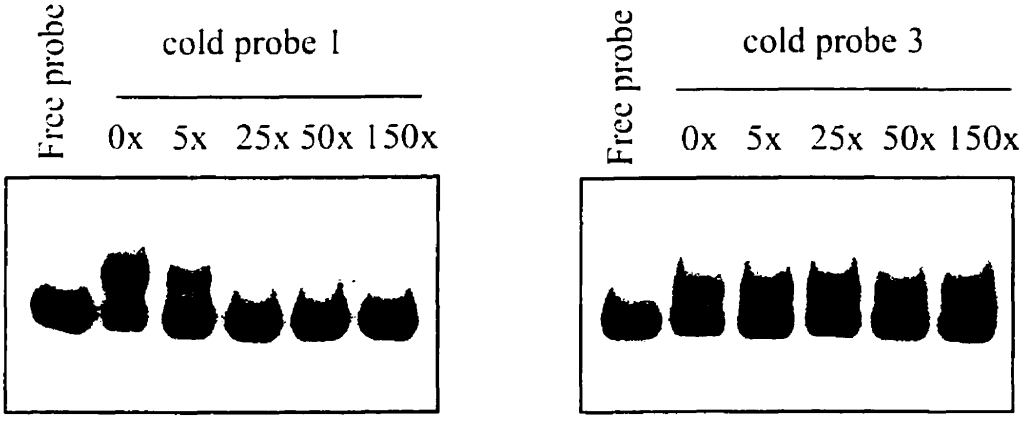
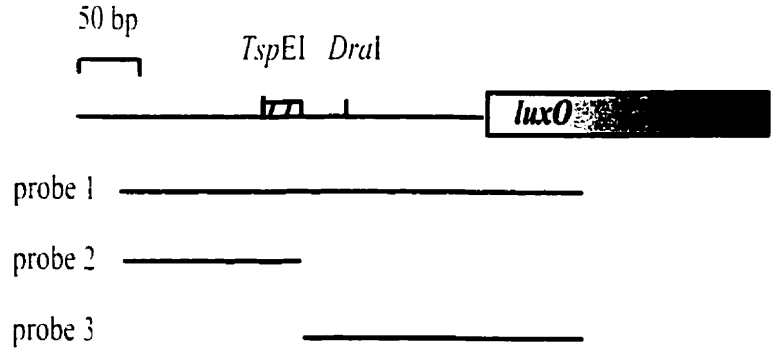
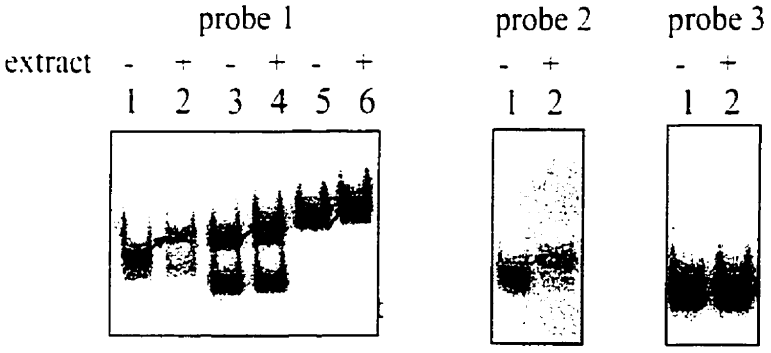

Free probe
cold probe 1
0x 5x 25x 50x 150x
Free probe
cold probe 3
0x 5x 25x 50x 150x

Fig. 3. Identification of the protein binding region of *luxO* by mobility shift assays with different DNA probes. A. Schematic diagram of protein binding region (slashed box) and the probes used for mobility shift assays. Two restriction sites, *Tsp*EI and *Dra*I, used to identify the binding region, are also indicated. Probe 1 was the fragment used for monitoring the DNA binding activity during protein purification. Probes 2 and 3 were generated by PCR to define the binding region. B. Electrophoresis of probes 1, 2 and 3 as well as probe 1 cut with DraI or TspEI in the presence and absence of 1 μg of *V. harveyi* extract . Arrows indicate mobility shifts.

A.


50 bp
TspEI
DraI
luxO
probe 1
probe 2
probe 3

B.


probe 1
probe 2
probe 3
extract - + - + - +
1 2 3 4 5 6
- +
1 2
- +
1 2

with probe 3. From these results, a 28 bp AT-rich region located between 166 bp and 139 bp upstream from the *luxO* initiation codon (slashed box, Fig. 3) was identified as being important for the LuxT binding activity.

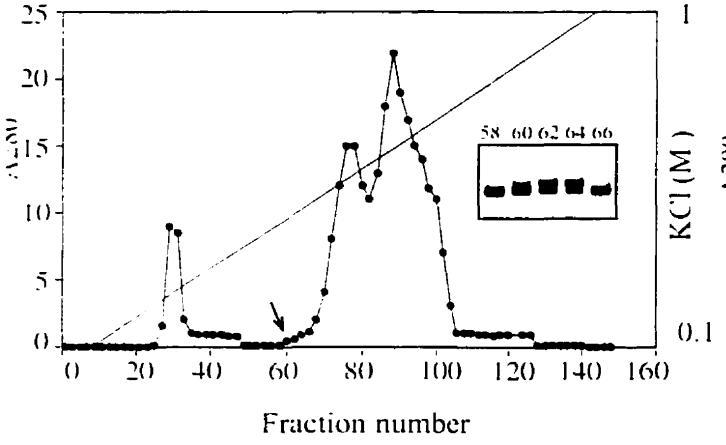
Purification of LuxT

Using the mobility shift with the *luxO* promoter as an assay, LuxT was purified through five different chromatography steps. Fig. 4 gives some of the key data concerning resolution of LuxT from other proteins. LuxT was eluted before the majority of the proteins on DEAE-Sepharose chromatography (Fig. 4A) and after most of the protein on CM-Sepharose (Fig. 4B) suggesting that it might be a positively charged protein. After chromatography on a non-specific DNA cellulose column, LuxT was resolved on a DNA affinity column containing the 28 bp oligodeoxynucleotide implicated in the binding activity (Fig. 2). Fig. 4C shows the mobility shifts and the corresponding silver-stained SDS-PAGE for the fractions containing the binding activity eluted from the affinity column. LuxT was then purified further by reverse phase HPLC in n-propanol and TFA (Fig. 4D). The propanol and TFA in the fractions were removed by vacuum centrifugation and the protein pellets were dissolved in 8 M guanidine hydrochloride. Samples were renatured by dilution in buffer A and incubated at 4 °C overnight before analyses by mobility shift assays and SDS-PAGE (Fig. 4D). Binding activity was detected in the second major peak obtained after HPLC (fractions 38 and 39); the same fractions contained a major band on the silver-stained SDS-PAGE with a molecular weight in the range of 14 to 17 kDa. The extra bands at 25 and 14 kDa are due to contamination by marker proteins which were overloaded in the next lane (not shown) in this particular purification while the weaker bands of 10 kDa at the front and 55kDa are found in the silver stained gel even in the absence of an applied sample.

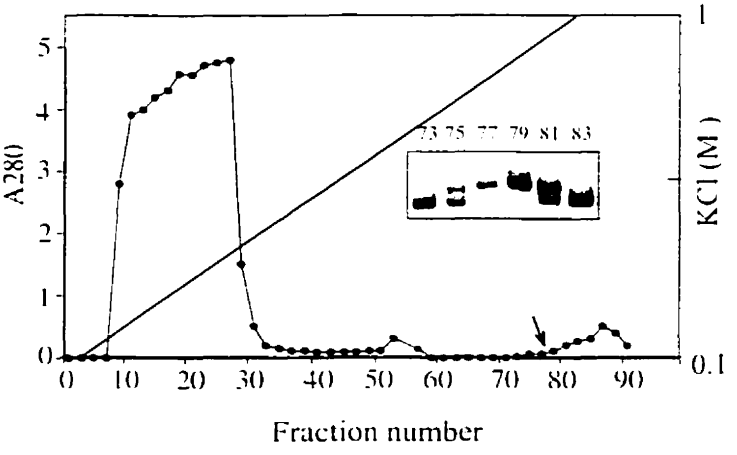
The purification of the binding activity from the crude extract to the DNA affinity chromatography is summarized in Table 1. Although the specific activity increased only

Fig. 4. Purification of LuxT. A. DEAE-Sepharose chromatography. Fractions (4 ml) were collected from the 500 ml KCl gradient and protein monitored by absorbance at A_{280}. Mobility shift assays were conducted with 0.2 µl of every second fraction and the relevant samples with activity shown (inset). Fractions 60 to 64 were pooled for further purification. B. CM -Sepharose chromatography. Fractions (3 ml) were collected from the 250 ml KCl gradient and protein monitored by absorbance at A_{280}. Mobility shift assays were conducted with 0.2 µl of every second fraction and the mobility shifts of the relevant fractions shown (inset). Fractions 75 to 81 were pooled and purified further. C. DNA affinity chromatography. A 10 ml DNA affinity column was eluted with 20 ml of a 0.1 to 1.0 M KCl gradient in buffer A and fractions of 0.4 ml collected. Shown are the mobility shift assays and the silver-stained SDS-PAGE gel for 1 µl and 10 µl respectively of the indicated fractions. The molecular weight markers are ribonuclease A (14 kDa) and chymotrypsinogen (25 kDa). Fractions 48 to 50 were pooled for further purification. D. Reverse phase HPLC. Fractions (150 µl) were collected with a gradient of 0 to 80% n-propanol in 0.12% TFA at a flow rate of 0.15 ml/min and monitored by absorbance at A_{280} shown in arbitrary HPLC analyzer units. The position of the activity is indicated by an arrow. Fractions 35 to 40 (15 µl each) were analyzed on a SDS-PAGE gel followed by silver staining (lower left). Mobility shift assays (lower right) were conducted with 15 µl of the same fractions renatured as described in Materials and Methods.

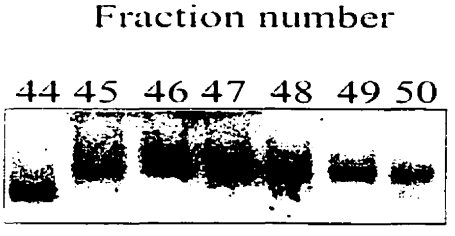
A. DEAE Sepharose chromatography

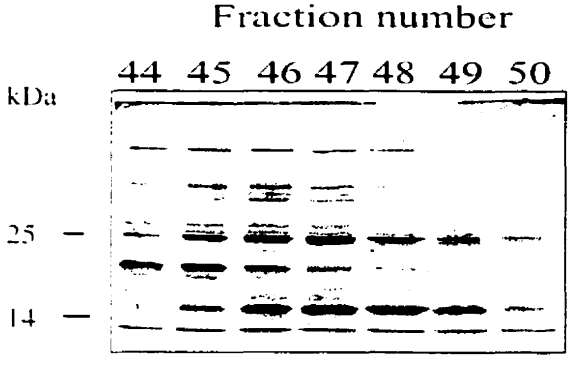


B. CM Sepharose chromatography

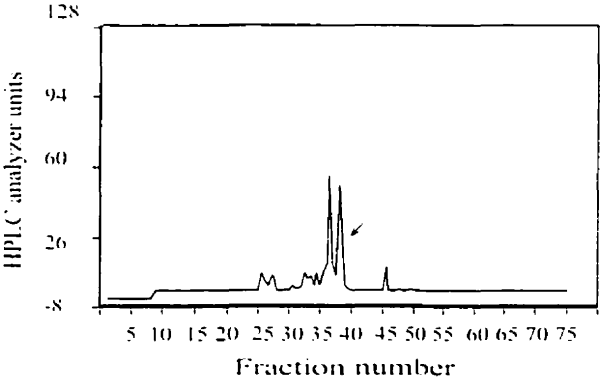


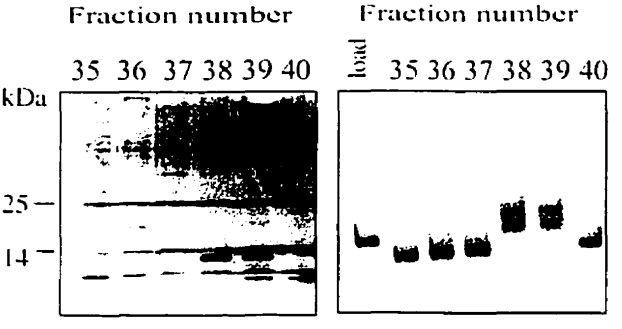
C. Affinity Chromatography





D. Reverse phase HPLC





250-fold during this process, less than 0.01% of the total protein in the crude extract remains associated with the LuxT binding activity. This result reflects a substantial loss of activity during purification as well as the difficulty in quantification of the level of activity. At this stage, most of the protein in the sample is composed of a single polypeptide (14-17 kDa) on SDS-PAGE (Fig. 5A). From the SDS gel , it is evident that chromatography on the non-specific DNA column had only a small effect in resolving the binding activity from the other proteins (lane 4, Fig. 5A) and indicated this step could be omitted without affecting the final purity of the protein. However, this step is convenient for rapidly concentrating the protein sample. In contrast, chromatography on the DNA affinity column (lane 5, Fig. 5A) had a dramatic effect in removal of most of the proteins even though the apparent specific binding activity increased only 3- fold in this step (Table 1). These results clearly indicate that LuxT is highly specific for the DNA sequence attached to this column.

As a minor contaminant and not the major polypeptide in the sample could be responsible for the binding activity, regions in the SDS gel (arrows) were excised and eluted, the protein precipitated with acetone and then dissolved in guanidine hydrochloride before renaturation and measurement of the binding activity. Fig. 5B shows that the strongest shift in the DNA mobility occurs on mixing with extracts from the gel containing the major polypeptide (lane 2, Fig. 5B). Traces of binding activity in the areas flanking the major polypeptide leading to a weaker mobility shift could be detected due to the high amount of extracts tested (lane 3 and 4, Fig 5B), while the extract from the area far from the major band did not cause a mobility shift (lane 1, Fig. 5B). A second band observed in the mobility shift assays both here and after HPLC (Fig. 4D) arose from the very high concentrations of LuxT used to monitor the activity after the renaturation process.

Tryptic peptides of LuxT

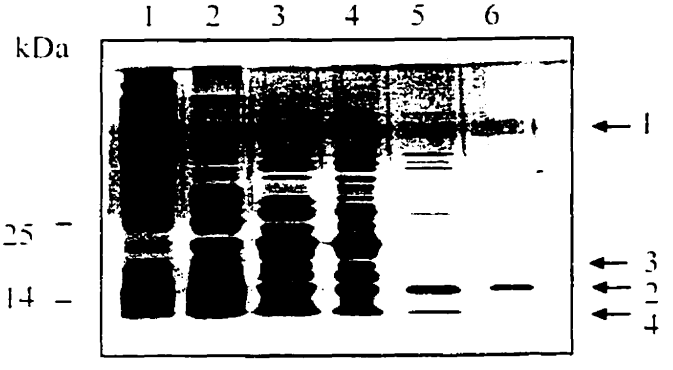
Table 1. Purification of DNA binding activity from *V. harveyi*[a]

Fraction	Protein concentration (mg/ml)	Volume (ml)	Total protein (mg)	Total Activity[b]	Specific activity	Yield (%)
Crude extract	9	170	1500	6400	4.2	100
DEAE Sepharose	1	54	54	2000	36	31
CM Sepharose	0.15	18	2.7	800	300	12
dsDNA cellulose	0.2	5	1	360	360	5
DNA affinity	0.05	2	0.1	110	1100	1.7

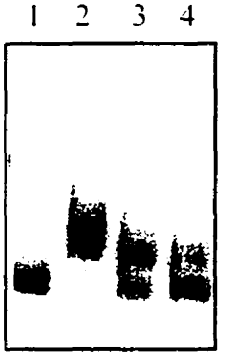
[a] From 28 g of wet cells.
[b] One unit of activity corresponds to 1 pmol of bound DNA/mg of protein in the standard mobility shift assay.

Fig. 5. Purification of LuxT monitored by SDS-PAGE. A. Silver stained SDS-PAGE gel of DNA binding activity at different stages of purification. Lane 1, crude extract (2 μg); lane2, dialyzed DEAE-Sepharose pool (2 μg); lane 3, CM-Sepharose pool (2 μg); lane 4, ds-DNA cellulose pool (2 μg); lane 5, DNA affinity pool (1 μg); lane 6, HPLC pool (0.5 μg). B. Mobility shift assays with proteins renatured from the SDS gel. Gel slices (1-4) from lane 6 in A were eluted and renatured as described in Materials and Methods before analyses of the mobility shifts with probe 1.

A.



B.



The purified binding protein was subjected to trypsin digestion, three peptides isolated and the sequences determined: peptide I, MSYTTLSQQTGVSR, peptide II, MAESQFGAGSDKELEWLIGR, and peptide III, SWITALEDSEFLAILR. Blast searches with the three peptides provided no protein match with any sequence in the data bank showing that LuxT is a new DNA binding protein from *V. harveyi*.

Characterization of LuxT

Movement of the partially purified LuxT protein through a gel filtration column compared with that of known standard revealed that the LuxT migrated as a protein of 34 kDa (Fig. 6). As the purified protein showing an apparent molecular weight of 14-17 kDa on SDS-PAGE, it was concluded that LuxT is a dimer in solution, which is a common characteristic of many DNA binding proteins.

Using the purified LuxT protein, it was possible to determine the dissociation constant for the interaction of LuxT with the *luxO* promoter (Fig. 7). The dissociation constant was estimated using the equation, $Kd = P_f \bullet D_f/D_s$, where D_f and D_s correspond to the amounts of the free and shifted DNA probe, respectively, and P_f is the unbound LuxT. As a large molar excess (~10 times) of LuxT over the DNA probe (0.2 nM) was needed to cause 50% of the DNA to shift in the assay, the free protein concentration (P_f) could be directly equated to the total protein concentration (P) without correcting for the low amount of protein bound (P_b) to the DNA probe (i.e. $P_f = P\text{-}P_b \approx P$). An apparent dissociation constant of only 2 nM was estimated explaining the high degree of

Fig. 6. Molecular size determination of LuxT. Elution position of purified protein (open circle) on gel filtration is compared to the positions of protein standards (solid circles). Ovalbumin (45 kDa); chymotrypsinogen (25 kDa); ribonuclease A (13.7 kDa).

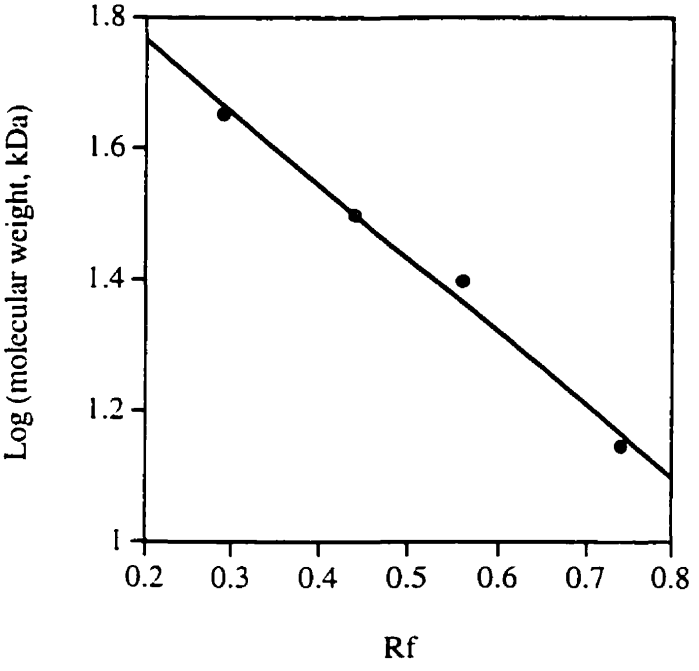
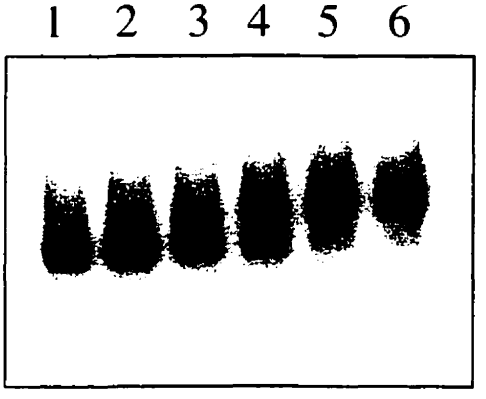

1.8
1.6
1.4
1.2
1
Log (molecular weight, kDa)
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Rf

Fig. 7. Estimation of the dissociation constant of LuxT and the *luxO* promoter by mobility shift assays with increasing amounts of purified LuxT protein. Lane 1, free probe 1 (1 ng); lanes 2 to 6 contain 0.1, 0.2, 2, 4, and 10 ng of protein, respectively. All assays contained 3 μg of poly(dI-dC).


1 2 3 4 5 6

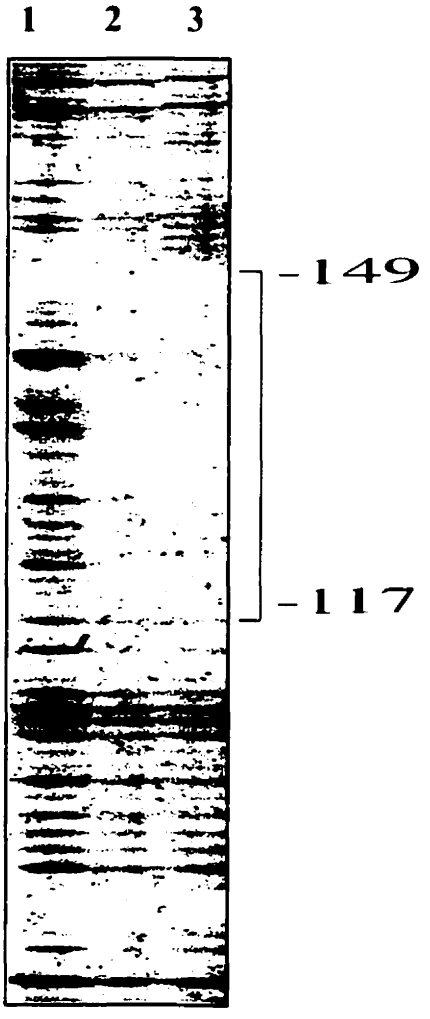
purification of LuxT on a DNA affinity column containing a 28 bp oligonucleotide from the promoter region.

Footprint analysis was conducted using partially purified LuxT to identify the binding sequence on the *luxO* promoter. It could be demonstrated that LuxT bound to a region located between -117 to -149 bp upstream of the initiation codon (Fig. 8). Although the 33 bp protected area overlapped the sequence of the oligonucleotide attached to the DNA affinity column, the footprint extended downstream another 22 bp. The results suggest that the overlapping 11 bp sequence TTCGGTTTACT is the critical region for the interaction with LuxT. As closely spaced short (5-6 bp) DNA repeats can be implicated in binding regulatory proteins in some eukaryotic (Lin *et al.*, 1997; Rhodes and Klug, 1986; Perisic *et al.*, 1989) and prokaryotic transcription systems (Escolar *et al.*, 1998), the possibility can be raised that the closely spaced GTTTA repeats in the footprint region might be a recognition sequence for LuxT. This might also explain why LuxT binds to the 28 bp affinity oligonucleotide which contains part of the footprint but also contains an imperfect GTTGA sequence separated by 6 bp from the upstream GTTTA from the footprint region.

The tight binding of LuxT to the *luxO* promoter provides evidence that this protein may be controlling *luxO* expression. Consequently, LuxT should affect luminescence and the cell density dependent induction of light. Cloning of the *luxT* gene will therefore be necessary to identify its biological role and characterize its genetic as well as biochemical properties.

Fig. 8. DNase footprinting analysis. A 144 bp DNA fragment (PCR amplified by the primers indicated in arrows in B) containing the *luxO* promoter region was digested with DNase I as described in Materials and Methods. Lane 1, 10 μg BSA; lanes 2 and 3, 2μg and 5μg of partially purified LuxT. The protected region is indicated by the bracket from –117 to –149 bp upstream from the initiation codon of *luxO*. B. Nucleotide sequence containing the promoter region of *V. harveyi luxO*. The first nucleotide of the *luxO* translational start site is designated as +1. The protein binding region defined by the footprint (-117 to –149) is boxed. The oligonucleotide identified as being important for binding (Fig. 3) and attached to the DNA affinity column is given in bold. A potential recognition sequence GTT(T/G)A, important for binding is highlighted.

A.



B.

-244 GAACATGTCGTTCACTAACAACGTCAGTTGGATAGGTGACCCGAGGGGTCCAAAAGTATA
-184 CAGCATGGTTTGTGCCAT**AATTTAACCTGTTGATA**TTCGGTTTACTTTGTTTAGAATACC
-124 CACAGTCTAACAAATGATTATTTGCAAAATGCAAAGCGTAATGCGATTATTATTTAAAAC

-64 ACAACGAAAAATCGGCTAGGCTATACAACAAATAACAGAAGGTCAAAAGTCTCGTTATCT
+1
-4 ACTTATGGTAGAAGACACCGCATCCGTTGCGGCACTTTACCGCTCTTACCTCACGCCACT

CHAPTER 3

Cloning and functional studies of a *luxO* regulator LuxT from *Vibrio harveyi*

PREFACE

This chapter presents the cloning and functional studies of LuxT purified as described in the previous chapter.

The following chapter has been submitted to *Biochim. Biophys. Acta* as presented : Lin, Y.H., Miyamoto, C., and Meighen, E.A. (2000)

CONTRIBUTION BY OTHER WORKERS

Carol Miyamoto constructed plasmid pMGMOP for chloramphenical acetyltransferase (*cat*) assays of *luxO* promoter

ABSTRACT

LuxO is the central regulator integrating the quorum sensing signals controlling autoinduction of luminescence. We have previously purified to homogeneity a new *lux* regulator, LuxT, that binds to the *luxO* promoter. Based on the sequence of the tryptic peptides of LuxT, degenerate oligonucleotides were designed for PCR of the genomic DNA. A 273 bp PCR DNA fragment containing sequences coding for the tryptic peptides was extended by inverse PCR to obtain the complete gene (*luxT*) coding for a protein of 153 amino acids which shares homology with the AcrR/TetR family of transcriptional regulators. The recombinant and native LuxT gave the same footprint binding between 117 bp and 149 bp upstream from the *luxO* initiation codon. Gene disruption of *luxT* in *V. harveyi* increased *luxO* expression and affected the cell density-dependent induction of luminescence showing that LuxT was a repressor of *luxO*. As LuxT also affected the survival of the *V. harveyi* cells at high salt concentration and homologous proteins are present in other bacterial species, including the pathogen, *V. cholerae*, the LuxT regulatory protein appears to be a general rather than a *lux*-specific regulator.

INTRODUCTION

Luminescence in *V. harveyi* is cell density dependent. The bacteria remain dim in the early stages of growth and small molecules (autoinducers) synthesized and excreted by the bacteria are required to accumulate in the media to a threshold concentration before causing the induction of light expression. The sensing of autoinducers is controlled by a pair of parallel signal-response systems which respond to two different autoinducers; N-(3-hydroxybutanoyl)-homoserine lactone (AI-1) (Cao *et al.*, 1989) synthesized by LuxLM (Bassler *et al.*, 1994a), and an unidentified autoinducer (AI-2) synthesized by LuxS (Surette *et al.*, 1999). These autoinducers are recognized by their respective transmembrane two-component sensor kinases, LuxN and LuxQ (Bassler *et al.*, 1993; 1994a). In the early stages of cellular growth, when the concentrations of autoinducers are low, LuxN and LuxQ autophosphorylate their sensor domains and subsequently transfer the phosphoryl group to the respective response domains. The two phosphorelay signals are then integrated by transfer of the phosphoryl group via LuxU (Freeman and Bassler, 1999a) to LuxO (Bassler *et al*, 1994b), the central regulator. LuxO in its phosphorylated form acts as a repressor of the induction of luminescence (Bassler *et al.*, 1994b; Freeman and Bassler, 1999b). As the cell density increases, the autoinducers accumulate in the media. Interaction of the autoinducers with their respective sensor proteins LuxN and LuxQ causes these molecules to preferentially act as phosphatases rather than kinases and dephosphorylate LuxO resulting in the loss of its repressor activity and leading to the induction of the expression of the *lux* operon (Freeman and Bassler, 1999b; Freeman *et al.*, 2000). In addition to the two-component regulators, a

transcriptional activator, LuxR, binds to the *lux* operon and is required for light emission (Showalter *et al.*, 1990; Swartzman and Meighen, 1993; Swartzman *et al.*, 1992).

Homologues of *V. harveyi* LuxO and LuxU with over 70% sequence identity are present in *V. cholerae* (gnl/TIGR/*V. cholerae*-666-1741, *V. cholerae* unfinished fragment of complete genome) and *V. fischeri* (Miyamoto *et al.*, 2000), and have recently been implicated in regulating luminescence in the latter species. In both *V. cholerae* and *V. fischeri* the *luxU* gene is immediately downstream of *luxO* with the initiation codon of *luxU* in *V. fischeri* overlapping the end of *luxO* in a manner similar to that observed in the *V. harveyi* genome. Based on closely related sequences, LuxO belongs to a large family referred to as the sigma 54-dependent family of transcriptional regulators (Klose *et al.*, 1998; Shingler, 1996). Many of the proteins that have been studied in this family have been shown to activate and/or repress the expression of the promoters of target genes dependent on sigma54 and/or other sigma factors. Whether *V. harveyi* LuxO affects the expression of the *lux* operon by direct or indirect action or through sigma 54 or another sigma factor is still unknown. Although a number of studies of members of the sigma54 family of regulatory proteins have been concerned with the role of phosphorylation in regulation, studies on the transcriptional control of the regulators themselves have not to our knowledge been previously conducted.

In previous studies, a *luxO* promoter binding protein, LuxT, has been purified to homogeneity from *V. harveyi* (Lin *et al.*, 2000). In this report, we have cloned this gene using degenerate oligonucleotides based on the sequences of tryptic peptides of LuxT and then disrupted the gene in the bacterial genome in order to characterize the phenotype.

Blast research shows that LuxT is related in sequence to the AcrR/TetR family of regulators implying it is a transcriptional factor. Our data also shows that LuxT negatively regulates *luxO* transcription and consequently modulates luminescence expression during cell growth. Evidence is also presented that LuxT affects cell survival at high salt concentrations and that a homologous regulator is present in *V. cholerae*, *V. fischeri*, and *Shewanella putrefaciens* suggesting that LuxT is a general transcriptional factor rather than a *lux*-specific regulator.

MATERIALS AND METHODS

Bacterial strains, plasmids and cell growth

Bacteria and plasmids used in this study are given in Table 1. Cells were generally grown in LB medium. For the analyses of bioluminescence levels, cells were grown in AB medium (0.3 M NaCl, 0.05 M $MgSO_4$, and 0.2% vitamin free casamino acids (Difco), adjusted to pH 7.5 with KOH, which after autoclaving was supplemented with 10 ml of sterile 1 M potassium phosphate (pH 7.0), 1 ml of 10 μg ml^{-1} riboflavin, and 1 ml of 1 mg ml^{-1} thiamine per liter of medium). *V. harveyi* was grown at 27 °C. *E. coli* MM294 was grown at 37 °C. Where indicated, the concentrations of the antibiotics in the medium were: ampicillin, 100 μg ml^{-1} ; kanamycin, 30 μg ml^{-1}; gentamicin, 25μg ml^{-1}.

Cloning of the luxT gene by PCR and inverse PCR

Forward and reverse degenerate oligodeoxynucleotides were designed according to the sequence of the tryptic peptides identified from the purified protein. PCR was performed at 95 °C for 5 min followed by 30 cycles at 95 °C of 1 min, 1 min at 54 °C and 40 sec at 72 °C, and completed by incubation at 72 °C for 10 min. The PCR product (273 bp) was purified by electrophoresis on a low melting agarose gel and sequenced at the Core DNA Sequencing Facility Center, Kingston, Ontario. Inverse PCR was performed by cutting 0.5 μg of *V. harveyi* genomic DNA with various four-base-cut restriction enzymes and the cut DNA ligated at different dilutions to preferentially cause selfligation into circular DNA. The circular DNA was then PCR-amplified with divergent primers to obtain the flanking sequences of the initial 273 bp PCR product. Based on this sequence, two fragments (987 bp and 2.1 kbp) encompassing the *luxT* gene were synthesized by direct PCR.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or feature	Reference or source
V. harveyi BB392	wild type	lab collection
BB392T⁻	*luxT*::kn	this study
E.coli MM294	F⁻ endA1 thi⁻1 hsdR17 supE44 lambda⁻	ATCC[a]
pT7	Apr, colE1, T7 promoter	Tabor and Richardson, 1985
pRK2013	broad host range; KnR	ATCC
pKT230	broad host range; SmR	ATCC
pJQ200	GenR, sacB, p15A	ATCC
pMGM100	SmR, cat reporter gene	Miyamoto *et al.*, 1990
pT7T	pT7 with 987 bp fragment containing *luxT*	This study
pT7TB	pT7 with *luxT*, 2.1 kb fragment containing *luxT*	This study
pT7TBKn	pT7 with *luxTB::kn*	This study
PKT230T	PKT230 with 987 bp fragment containing *luxT*	This study
PMGMOP	*luxO* promoter::*cat*	This study
pJQT::kn	pJQ200 with *luxTB*::*kn*	This study

a. American type culture collection

Expression of LuxT in E.coli

The 987 bp PCR fragment encompassing the *luxT* gene was transferred into pT7 or pKT 230 and the resulting plasmids (pT7T and pKT230T, respectively) were then transformed into *E.coli* MM294 for expression.

Gene replacement

The 2.1 kbp fragment containing the *luxT* gene was cloned into pT7-5 (pT7TB) using *Bam*HI and *Pst*I. The kanamycin *(kn)* gene (1.4 kbp) excised with *Eco*47III from pKT230 was then ligated into a blunt-ended *Bss*HII site in pT7TB resulting in pT7TB::*kn*. The *luxT* gene with the inserted *kn* gene was excised with *Bam*HI and *Pst*I and inserted into the same sites in pJQ200 to give pJQT::*kn*. This plasmid was then transferred into *V. harveyi* by conjugation and the colonies containing pJQT::*kn* were selected on ampicillin, kanamycin, and gentamicin (AKG) plates. The selected colonies were spread on ampicillin, kanamycin and 5% sucrose plates to kill the bacteria harboring the plasmids and to select the ones containing the *luxT* gene with *kn* inserted in the genome (knockout strain, BB392T^{-}). The knockout strains obtained were verified by Southern Blot and mobility shift analyses.

Mobility shift assay

The DNA binding assay was performed using a 330 bp DNA probe containing the *luxO* promoter region extending from 243 bp upstream to 87 bp downstream of the *luxO* initiation codon. The reaction mixtures (20 µl) containing approximately 1 ng of a 5'-^{32}P labelled DNA fragment and the indicated amount of protein extract was incubated in 10 mM Hepes, pH 7.9, 100 mM KCl, 2 mM DTT, 0.4 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA, 10% glycerol with 3 µg of poly(dI-dC) for 30 min at room temperature. The incubation mixtures were resolved by electrophoresis on a 5% polyacrylamide gel in 0.5 x TBE .

DNase I footprint analysis

The footprint assays were conducted using a 144 bp DNA fragment encompassing the putative LuxT binding site located between 87 to 222 bp upstream of the initiation codon of *luxO*. The protein-DNA binding conditions were the same as described in the mobility shift assay except 10 µg of poly (dI-dC) was added. Ten ng of the PCR amplified and ^{32}P-end-labeled (10000 cpm) DNA fragment was mixed with varying amounts of the bacterial extracts and the DNA–protein mixture was incubated at room temperature for 30 min. The endonuclease reaction was started by the addition of 1 µl of 20 µg ml^{-1} of DNase I, stopped after 2.5 min with 40 µl stop buffer (10 mM Tris–HCl, pH 7.6, 20 mM NaCl, 20 mM EDTA, 0.2% SDS, 100ug/ml tRNA) and incubated with proteinase K (0.4 mg/ml) at 37 °C for 30 min. The DNA was then precipitated and resolved by electrophoresis in a 6% polyacrylamide sequencing gel.

Bioluminescence assay

Wild type and mutant cells were grown overnight and cells of similar OD_{660} and light units were diluted to OD_{660} = 0.001 in AB medium. Luminescence of growing cells was measured with cell density using a photomultiplier tube where 1 light unit (LU) corresponds to 1.3×10^{10} quanta s^{-1} based on the light standard of Hastings and Weber (1963).

Chloramphenical acetyltransferase (cat) assays

The *luxO* promoter region amplified by PCR was cloned into pMGM100 and the resulting plasmid (pMGMOP) was transconjugated into both *V. harveyi* wild type and *luxT* mutant. Cat assays were performed as previously described (Miyamoto *et al.*, 1994).

Survival rate of BB392T⁻ and V. harveyi wild type cells on high salt medium

Overnight cultures of *V. harveyi* and BB392T⁻ cells were plated on LB agar plates containing 1% and 4% NaCl. Wild type and mutant cells were grown on 1% NaCl plates for 24 hours and on the 4 % NaCl plates for 36 hours. The same number of cells were plated on the 1% and 4% NaCl plates.

Sequence Data analysis

Protein database searches were performed using the Blast network service of the National Center of Biotechnology Information. Protein sequences *of V. cholerae* and S. *putrefaciens* were obtained from the Institute for Genomic Research website at http://www.tigr/org. Alignments of proteins were made by using the CLUSTAL W (version 1.8) program.

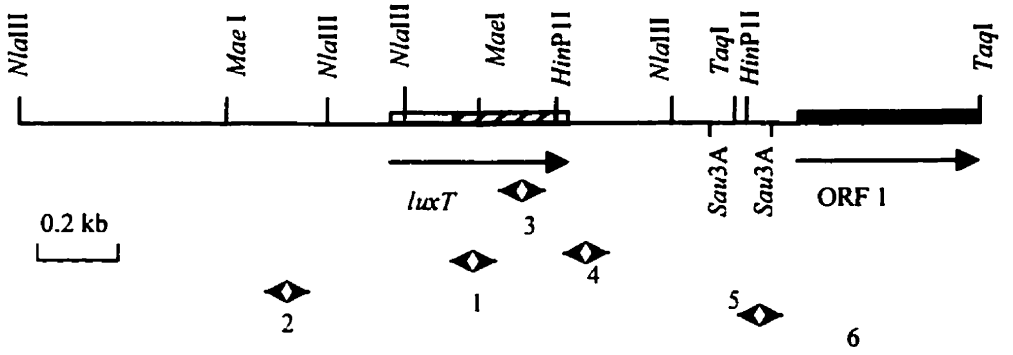
RESULTS

Cloning of the luxT gene

Part of the *luxT* gene of *V. harveyi* was initially cloned by PCR based on the sequences of three peptides (I to III) obtained from tryptic digestion of the native LuxT; I, MSYTTLSQQTGVSR, II, MAESQFGAGSDKELEWLIGR, and III, SWITALEDSEFLAILR. Degenerate oligonucleotides in the forward and the reverse directions for each of peptide I, II, and III, were used in different combinations for PCRs. The longest PCR product (273 bp) was obtained with a forward primer from peptide I and a reverse primer from peptide II. This DNA fragment could be translated into a peptide of about 90 amino acids with the sequence for peptide III found in the center and peptide II on the carboxyl terminal side indicating that it encoded part of LuxT. Inverse PCR was sequentially conducted to obtain the complete gene as well as the flanking sequences using pairs of divergent primers as outlined in Fig. 1A.. The slashed area shows the position of the 273 bp PCR product. The DNA was sequentially extended to the upstream *Mae*I site and then the *Nla*III site using two pairs of primers and then downstream to the *Taq*I site with four pairs of primers. An ORF coding for the *luxO* binding protein (LuxT) was found to extend 456 bp. Downstream, part of an ORF coding for a protein with homology to transcription factor MexT from *Pseudomonas aeruginosa* (Kohler *et al.*, 1999) and nodulation factors D from various bacteria (Davis and Johnston, 1990; Gillette and Elkan, 1996; Scott, 1986) was also detected.

Fig. 1. Cloning of *luxT* gene and its flanking region. A. Strategy for inverse PCR. A 273 bp fragment cloned by PCR using primers based on the sequence of tryptic peptides is given by the slashed area in the open box representing the *luxT* gene. The upstream sequence was cloned using inverse PCR with selfligated *Mae*I-restricted genomic DNA and two divergent primers (pair 1) to obtain the sequence to the upstream *Mae*I site. Pair 2 primers were then used with selfligated *Nla*III-restricted DNA to extend the sequence to the upstream *Nla*III site. Similarly, primer sets 3-6, respectively, were used sequentially with *Nla*III, *Hinc*PI, *Sau*3A and *Taq*I restricted and selfligated DNA to clone the downstream sequence. The open reading frame downstream (ORF1) shares homology with transcriptional factor MexT from *Pseudomonas aeruginosa* and nodulation factors from numerous bacteria. B. DNA sequence and protein translation of *luxT* A putative -10 AND -35 promoter sequence for *luxT* (open box) and a Shine-Dalgarno (SD) ribosome binding site are indicated. The sequence of the three tryptic peptides identified in the purified binding protein (I, II, and III) are underlined.

A.



B.

```
1    GAGAATAGTTTGGATGAATGATTTGGTGCAAGGTTTTGATGGAGTGGA 48

49   CTTTTCGCCATATTTTGTGTCACATGTATTGACTGAAATATCACTGTA 96

97   AATTGCACGTTACGCAGTGCAGTTTAGTTATGATTGGTCCTCTAACTT 144
       -35                  -10
145  TGATGTTTGAGGTATACAGTACATAATGCTGAGGATGATTTTCATTCT 192
                                                  SD
193  GGCCTCATTTAATTGTGAGTTAGGATGGATTTACAAGGAGGAGGAGCT 240

1          M  P  R  R  S  K  E  D  T  E  I  T  I  Q   14
241  TAAAATATGCCAAGGCGTAGTAAAGAGGATACGGAAATCACTATCCAG 288

15   K  I  M  D  A  V  V  D  Q  L  L  R  L  G  Y  D   30
289  AAGATCATGGATGCCGTTGTTGATCAGCTTCTGAGACTTGGATATGAC 336
                      I
31   K  M  S  Y  T  T  L  S  Q  Q  T  G  V  S  R  T   46
337  AAAATGTCTTACACCACGTTAAGCCAGCAGACAGGTGTATCGCGTACA 384

47   G  I  S  H  H  F  P  K  K  T  D  F  T  A  A  L   62
385  GGTATTAGCCACCATTTCCCTAAGAAAACGGACTTTACCGCCGCTTTA 432

63   D  G  R  I  F  K  M  F  V  E  H  L  E  F  D  K   78
433  GACGGTCGTATTTTCAAAATGTTCGTTGAACATCTAGAATTCGATAAA 480
                                  III
79   G  L  E  A  F  S  K  S  W  I  T  A  L  E  D  S   94
481  GGTCTAGAAGCGTTTTCAAAGAGCTGGATCACGGCTCTAGAAGACTCT 528

95   E  F  L  A  I  L  R  L  L  F  H  H  I  V  T  S  110
529  GAATTCTTAGCGATTTTGCGTCTATTATTCCACCATATTGTTACTTCT 576

111  E  S  A  H  E  F  A  A  N  G  I  D  R  L  Y  K  126
577  GAGAGCGCGCACGAGTTTGCGGCAAACGGCATTGATCGTTTGTACAAA 624
                      II
127  M  A  E  S  Q  F  G  A  G  S  D  K  E  L  E  W  142
625  ATGGCTGAGAGCCAGTTTGGCGCAGGCAGTGACAAAGAGCTAGAGTGG 672

143  L  I  G  R  S  L  I  Q  M  S  K  *
673  TTGATTGGTCGCTCATTGATCCAAATGAGCAAGTAACACCACAGTAAA 720
```

The nucleotide sequence of the *luxT* gene is shown in Fig. 1B coding for a protein of 153 amino acids. Upstream a classic Shine-Dalgarno sequence can be recognized along with a –10 and –35 promoter site. The sequence of the three tryptic peptides (I, II, III, underlined) are contained within LuxT confirming that the *luxT* gene encodes the purified DNA binding protein.

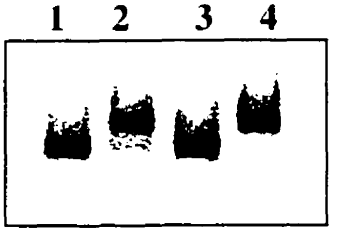
Expression of recombinant LuxT

The *luxT* gene was inserted into the pT7 plasmid and expressed in *E.coli.* Both the recombinant and native LuxT proteins gave the same mobility shift with the *luxO* promoter region DNA (Fig 2 A). Identical footprints were also observed for the recombinant and native proteins (Fig. 2B) further establishing that this gene encodes a protein that binds to the *luxO* promoter region.

The Blast sequence similarity search program was used to search for the amino acid sequence similarities of LuxT to other proteins. The results showed that LuxT shares a high degree of identity (Fig. 3) with open reading frames in *V. cholerae* and *Shewanella putrefaciens* over the entire protein sequence and with members of the AcrR/TetR family of transcriptional regulators in the N- terminal domain. Transcriptional regulators in this family usually share homology only in the N-terminal region and as many of them are repressors involved in the resistance of the bacteria to antibiotics or toxic substances in the environment, it is suggested that LuxT might have a similar function.

Fig. 2. Expression of recombinant LuxT in *E. coli*. A. Mobility shift assays using the probe containing the *luxO* promoter region. Lane 1 free probe; Lane 2. *V. harveyi* extract; Lane 3. *E.coli* MM294 extract; Lane 4. *E.coli* MM294(LuxT) extract. B. DNase I footprinting analysis. A 144 bp DNA fragment containing the *luxO* promoter region was digested with DNase I. Lane 1 and 4, 10 μg BSA; lanes 2 and 3, 2 μg and 5μg, respectively, of partially purified LuxT from *V. harveyi*; lanes 5 and 6, 5 μg and 10 μg, respectively, of an extract of *E.coli* MM294 (LuxT). The protected region is indicated by the bracket from –117 to –149 bp upstream from the initiation codon of *luxO*.

A.



B.

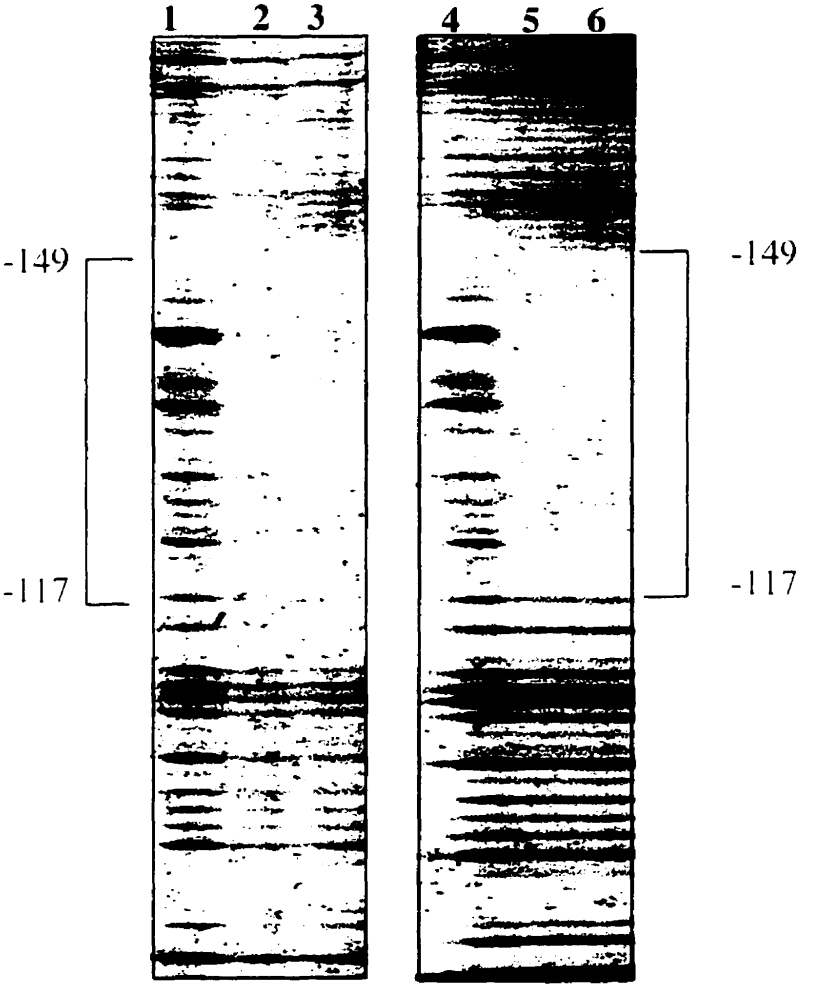
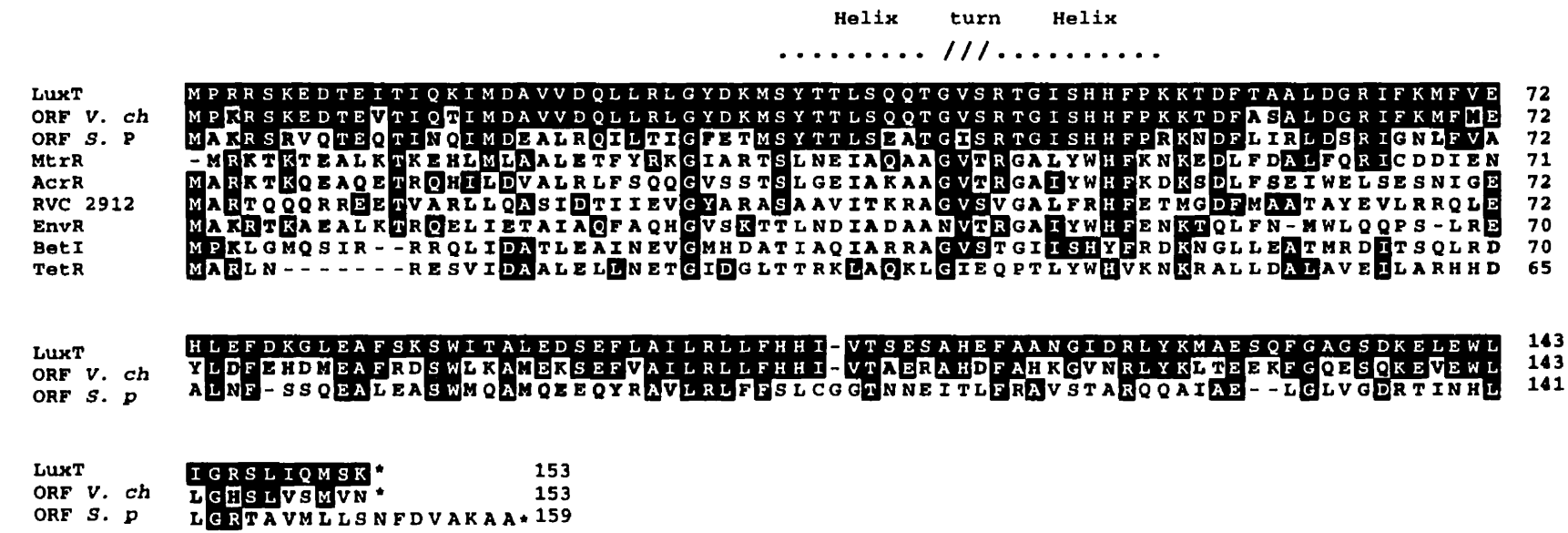


Fig. 3. Alignment of the amino acid sequence of LuxT, proteins coded by the *V. cholerae* and *S. putrefaciens* genomes and the family of AcrR/TetR transcriptional factors. Amino acid residues of LuxT are aligned with open reading frames from *V. cholerae* and *S. putrefaciens* genomes (ORF *V. ch* and ORF *S. p.*) as well as the N-terminal regions of MtrR of *Neisseria gonorrhooeae* (Pan and Spratt,, 1994) AcrR of *E.coli* (Ma *et al*, 1993) hypothetical transcriptional factor RV2912C of *Mycobacterium tuberculosis* (Cole *et al.*, 1998), EnvR of *E.coli* (Klein *et al.*, 1991), BetI of *E.coli* (Lamark *et al*, 1991), and TetR class D from *E.coli* (Waters *et al.*, 1983). Residues that are identical to LuxT are shaded and similar residues are highlighted. The predicted helix-turn-helix region involved in DNA binding is also indicated.

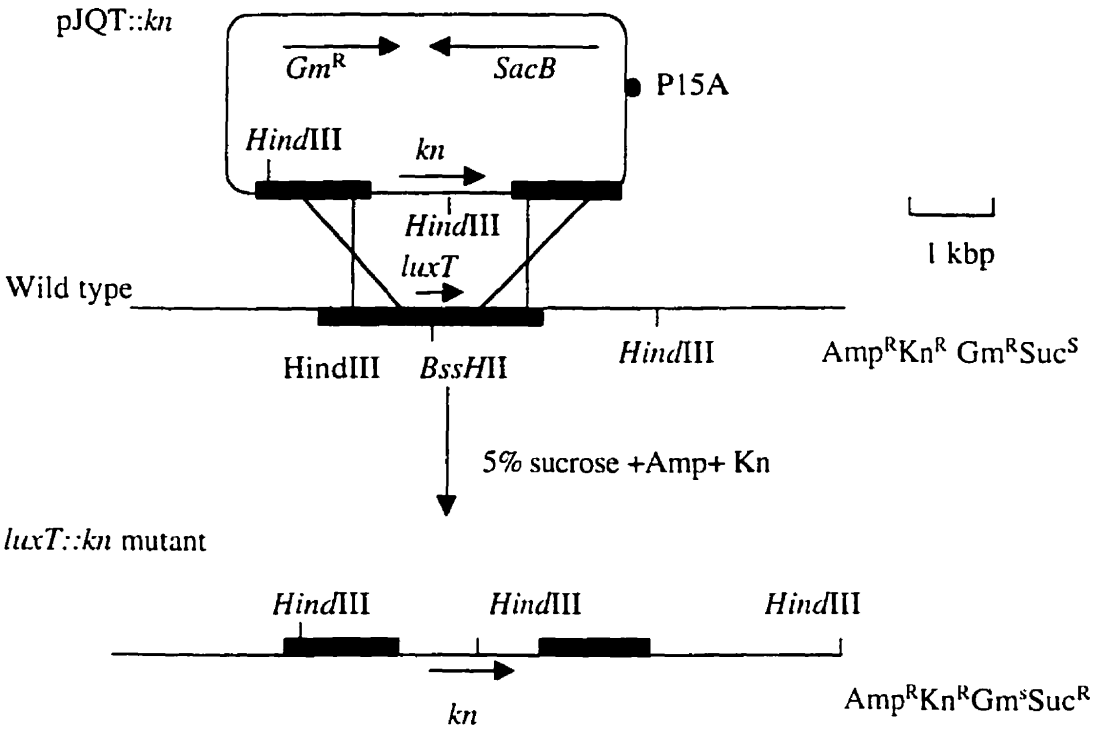

Helix turn Helix
LuxT MPRRSKEDTEITIQKIMDAVVDQLLRLGYDKMSYTTLSQQTGVSRTGISHHFPKKTDFTAALDGRIFKMFVE 72
ORF V. ch MPKRSKEDTEVTIQTIMDAVVDQLLRLGYDKMSYTTLSQQTGVSRTGISHHFPKKTDFASALDGRIFKMFME 72
ORF S. P MAKRSRVQTEQTINQIMDEALRQILTIGFETMSYTTLSEATGISRTGISHHFPRKNDFLIRLDSRIGNLFVA 72
MtrR -MRKTKTEALKTKEHLMLAALETFYRKGIARTSLNEIAQAAGVTRGALYWHFKNKEDLFDALFQRICDDIEN 71
AcrR MARKTKQEAQETRQHILDVALRLFSQQGVSSTSLGEIAKAAGVTRGAIYWHFKDKSDLFSEIWELSESNIGE 72
RVC 2912 MARTQQQRREETVARLLQASIDTIIEVGYARASAAVITKRAGVSVGALFRHFETMGDFMAATAYEVLRRQLE 72
EnvR MAKRTKAEALKTRQELIETAIAQFAQHGVSKTTLNDIADAANVTRGAIYWHFENKTQLFN-MWLQQPS-LRE 70
BetI MPKLGMQSIR--RRQLIDATLEAINEVGMHDATIAQIARRAGVSTGIISHYFRDKNGLLEATMRDITSQLRD 70
TetR MARLN-------RESVIDAALELLNETGIDGLTTRKLAQKLGIEQPTLYWHVKNKRALLDALAVEILARHHD 65
LuxT HLEFDKGLEAFSKSWITALEDSEFLAILRLLFHHI-VTSESAHEFAANGIDRLYKMAESQFGAGSDKELEWL 143
ORF V. ch YLDFEHDMEAFRDSWLKAMEKSEFVAILRLLFHHI-VTAERAHDFAHKGVNRLYKLTEEKFGQESQKEVEWL 143
ORF S. p ALNF-SSQEALEASWMQAMQEEQYRAVLRLFFSLCGGTNNEITLFRAVSTARQQAIAE--LGLVGDRTINHL 141
LuxT IGRSLIQMSK* 153
ORF V. ch LGHSLVSMVN* 153
ORF S. p LGRTAVMLLSNFDVAKAA* 159

Gene disruption of luxT *in* Vibrio harveyi

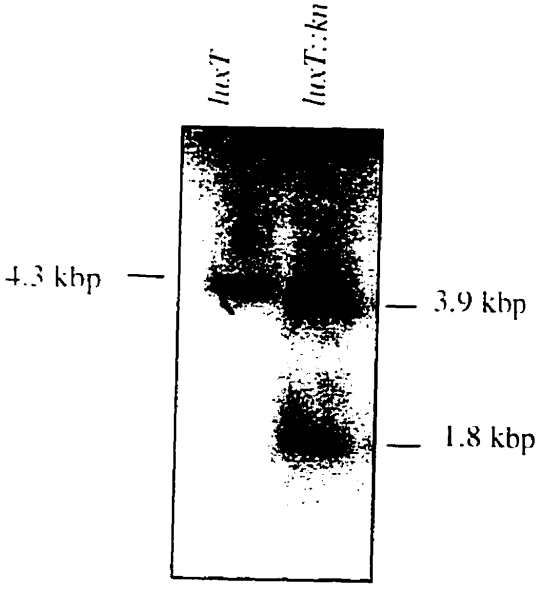
To further investigate the function of this protein, we constructed a *luxT* null allele in the chromosome of *V. harveyi* (Fig. 4A). A "suicide vector" pJQ200 was used for the gene replacement procedure (Quandt and Hynes, 1993). The plasmid, pJQT::*kn*, contained the *kn* inserted *luxT* gene, a P15A replicon origin which functions only in *Enterobacteria*, a gentamicin resistance marker, and the *sacB* gene encoding levansucrase, production of which is lethal in the presence of 5% sucrose in several Gram negative bacteria. The pJQT::*kn* plasmid transconjugated into *V. harveyi* was not expected to replicate in *V. harveyi* and thus only single recombinants with the whole plasmid inserted into the genome would be expected in selection with AKG (ampicillin for *V. harveyi*, kanamycin for the *luxT::kn* insert and gentamicin for the vector) in the LB plate. As this plasmid replicated in *V. harveyi*, it was not possible to detect single recombinants. Nevertheless, we could continue to screen by adding 5% sucrose to kill any colonies containing *sacB* in the plasmid and select the double recombinants. The double recombinants were then screened further to confirm their sensitivity to gentamicin thus demonstrating they were not single recombinants or did not harbor plasmids with a defective *sacB* gene. Southern blot analyses were conducted to confirm that the *luxT* gene was interrupted by the *kn* cassette in the mutant, BB392T⁻ (Fig, 4B). As shown in Fig. 4B, Southern hybridization using the 987bp *luxT* fragment as the probe to the *Hind*III digested chromosomal DNA of the wild type *V. harveyi* shows a single 4.3 kbp fragment while the mutant gives two fragments of 3.9 kbp and 1.8 kbp which is consistent with the

Fig. 4. Gene replacement of *luxT* in *V. harveyi*. A. Strategy for gene replacement (see text). Physical maps of the *luxT* (solid bars) region of the chromosome (wild type and *luxT::kn* mutant) and the pJQT::*kn* plasmid are illustrated. The *BssH*II site for insertion of the kanamycin gene (*kn*) and the *Hind*III sites for Southern analysis are indicated. B. Southern analysis of *V. harveyi* and mutant (BB392T$^-$) genomic DNA digested with *Hind*III. C. Mobility shift assays using a probe containing the *luxO* promoter region: Lane 1, *V. harveyi* wild type extract ; Lane 2 BB392T$^-$ extract; Lane 3. free probe.

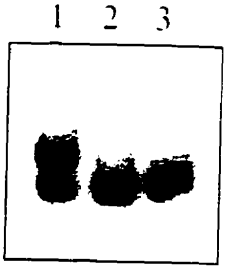
A.


pJQT::*kn*
*Gm*R
SacB
P15A
*Hind*III
kn
*Hind*III
luxT
1 kbp
Wild type
HindIII
*BssH*II
*Hind*III
AmpRKnR GmRSucS
5% sucrose +Amp+ Kn
luxT::kn mutant
*Hind*III
*Hind*III
*Hind*III
kn
AmpRKnRGmSSucR

B.


luxT
luxT::kn
4.3 kbp
3.9 kbp
1.8 kbp

C.


1 2 3

expected sizes of the kanamycin disrupted *luxT* region in the genomic DNA. Mobility shift assays using extracts of the confirmed clones further demonstrated that LuxT is not present (Fig. 4C).

Function of LuxT

The phenotype of the null mutant was investigated to understand the function of the *luxT* gene in *V. harveyi* by following the luminescence during growth of both the wild type and the mutant (Fig. 5). The dependence of light emission on cell growth was greater for the null mutant than the wild type cells with luminescence intensity becoming at least 20 fold lower in the early stages of cell growth. At higher cell density, luminescence in the mutant reached the same level as that of wild type cells.

To further clarify the role of LuxT, we cloned the *luxO* promoter in front of the *cat* reporter gene in pMGM100. The resulting plasmid, pMGMOP, was transconjugated into both the wild type and the mutant *V. harveyi* cells. Measurement of the cat activities at different points of the cell growth showed that the *luxT* null mutant had 2-fold higher *cat* activity than wild type cells (Fig. 6) indicating that LuxT negatively regulates the level of *luxO*. Throughout cell growth, it downregulates the level of *luxO* transcription and expression. Consequently in the null mutant, light levels are reduced at the early and middle stages of growth due to the higher expression of *luxO*. During luminescence induction, the functional (phosphorylated) form of the LuxO repressor would be inactivated due to the accumulation of autoinducers which would cause a decrease in the level of phosphorylation of LuxO in both the wild type and the mutant. As seen in Fig. 5, the maximum light intensity is not reached by the null mutant until a higher cell density presumably due to higher levels of LuxO.

Fig. 5. Effect of deletion of *luxT* in the *V. harveyi* genome on light induction. Cultures of wild type and *luxT:: kn* mutant BB392T$^-$ were grown overnight in AB medium. The overnight cultures were diluted 1000 fold in fresh AB medium and light intensity (LU) was measured as a function of cell density measured by OD_{660}. The solid circles represent the activity of wild type *V. harveyi* and the open circles represent that of the mutant BB392T$^-$.

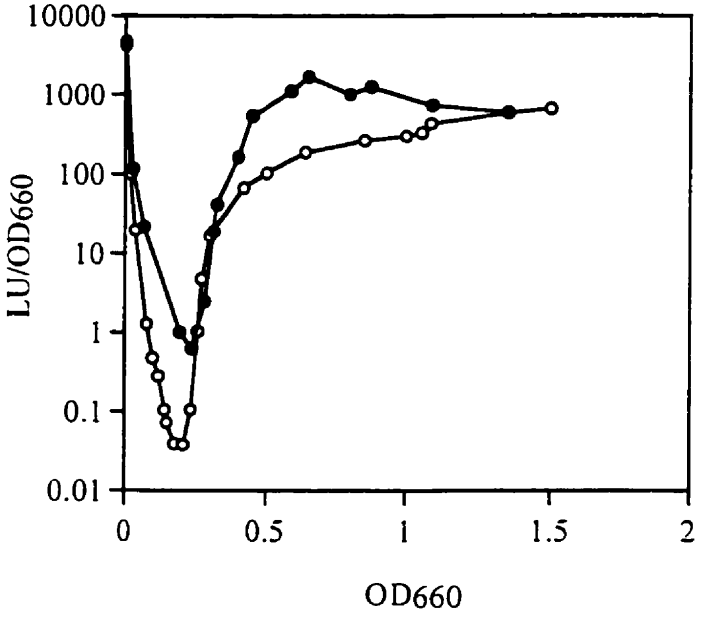
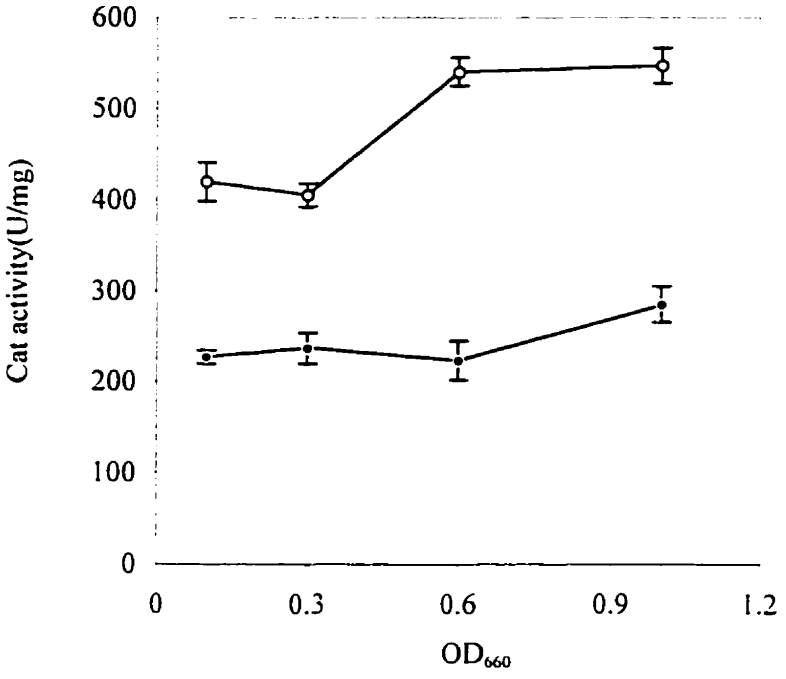

10000
1000
100
10
1
0.1
0.01
LU/OD660
0
0.5
1
1.5
2
OD660

Fig. 6. Expression of a *luxO* promoter::cat fusion (pMGMOP) in the wild type and mutant *V. harveyi*. The samples were collected at different growth stages (OD_{660}= 0.1, 0.3, 0.6, 1.0) and the specific activity of chloramphenical acetyltransferase (cat) was measured at least five times in independent assays and the cat units averaged. Standard deviations are shown as vertical bars. The solid circles represent the activity of wild type *V. harveyi* and the open circles represent that of the mutant BB392T$^-$.

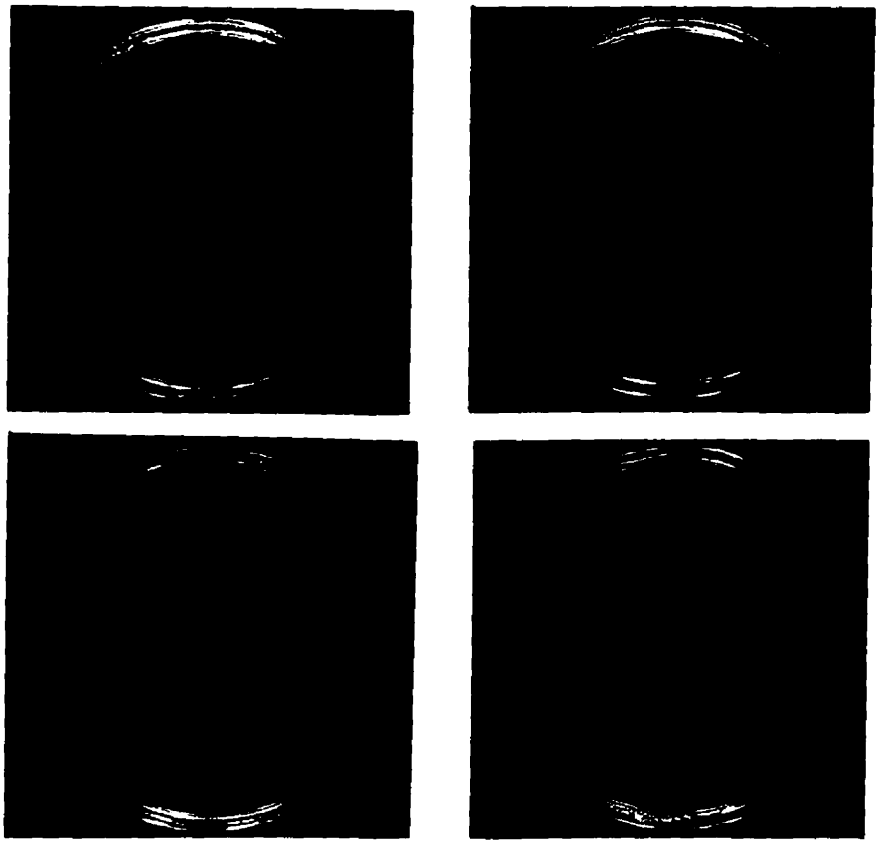

600
500
400
300
200
100
0
Cat activity(U/mg)
0
0.3
0.6
0.9
1.2
OD660

As LuxT appears to belong to the AcrR/TetR family of transcriptional regulators, many of which are involved in resistance to stress including high salt levels, we suspected LuxT might also control a similar physiological function. Survival of the wild type and the mutant cells on high concentrations of NaCl were tested. The null mutant BB392T$^-$ was much more resistant to high levels of salt (4% NaCl) than the wild type cells (Fig 7) indicating that LuxT might also play a role in the regulation of osmolarity.

Fig. 7. Survival rate of BB392T⁻ and *V. harveyi* wild type cells on high salt medium. A. Growth of *V. harveyi* cells on LB plates containing 1% (top) and 4% (bottom) NaCl. B. Growth of BB392T⁻ cells on LB plates containing 1% (top) and 4% (bottom) NaCl. Wild type and mutant cells were grown on 1% plates for 24 hours and on the 4 % plates for 36 hours. The same numbers of cells were plated on the 1% and 4% NaCl media.

A.

B.



DISCUSSION

Based on the amino acid sequence of tryptic peptides of LuxT, the gene coding this function and flanking regions were cloned and disruption of the *luxT* chromosomal gene in *V. harveyi* was accomplished. Although light intensity at high cell density was not affected, a 10 to 20-fold drop in light intensity for the *luxT* null mutant compared to that of the wild type cells was observed at low and intermediate cell densities. As LuxO is a negative regulator (Bassler et al., 1994b), this result combined with the higher activity of the *luxO* promoter in the *luxT* deletion mutant suggested that LuxT might function as a repressor. The effect of LuxT on light levels occurs mainly prior to the induction of luminescence consistent with LuxO being inactivated in response to the accumulation of autoinducers at the later stages of cellular growth.

Closely related homologues of LuxT were found in *V. cholerae* and *S. putrefaciens* with 74% and 42% identity, and 87% and 59% similarity, respectively, with *V. harveyi* LuxT. In contrast, only the N-terminal region of the other members of the AcrR/TetR family of transcriptional regulators could be easily related in sequence to LuxT (20~28% identity) as only very weak similarities in sequence can be detected in the carboxyl terminal regions. The very close relationship between LuxT and the translated ORF in *V. cholerae* is interesting as a number of strains of *V. cholerae* are known to emit light (Hada *et al.*, 1985). Moreover, even in nonluminescent strains of *V. cholerae*, homologues of other "*lux*" regulators have been found. HapR, which is an activator of the *hap* gene encoding the HA protease implicated in virulence in *V. cholerae* (Jobling and Holmes, 1997) is a homologue of the *V. harveyi lux* regulator, LuxR (Showalter *et al.*,

1990). Recently a closely related homologue of *V. harveyi luxO* has been reported in the *V. cholerae* genome (Klose *et al.*, 1998) along with other genes coding for *V. cholerae* proteins that appear to be part of the sigma 54 family of regulator proteins. The relationship of LuxT to an ORF in *S. putrefaciens*, which is widely distributed in fresh water and marine environments (Nealson and Saffarini, 1994) may also be of interest. Although this species is not luminescent, another member of this genus, *S. hanedai* is classified as a luminescent bacteria (Meighen and Dunlap, 1993). Using mobility shifts with the *V. harveyi luxO* promoter to screen for LuxT-like DNA binding proteins in extracts of other bacteria, we have recently demonstrated that proteins that give mobility shifts almost identical to that for *V. harveyi* LuxT can readily be recognized in extracts of *V. cholerae* and *V. fischeri* (Lin *et al.*, and unpublished experiments]. The presence of a LuxT-like protein in *V. fischeri* extracts is consistent with the discovery that *luxO* may be involved in regulation of luminescence in *V. fischeri* (Miyamoto *et al.*, 2000) even though another completely different regulatory system for luminescence is also present in this species. The possibility that LuxT is a conserved regulatory protein found in bacteria that are linked directly or indirectly to luminescence and/or found in freshwater or marine habitats seems likely particularly in view of the large number of members of the sigma 54 family of proteins related to LuxO and the demonstration that closely related LuxO homologues are present in *V. cholerae* and *V. fischeri*.

Only a few members of the AcrR/TetR family of regulators have been studied in any detail. The TetR protein is involved in regulating resistance to tetracycline in *E. coli* (Waters *et al.*, 1983). BetI represses the *bet* operon which encodes proteins responsible

for the accumulation of the osmoprotectant glycine betaine (Lamark *et al.*, 1991) MtrR is a repressor of the *mtrCDE* operon which encodes proteins involved as part of an efflux pump for hydrophobic antibiotics and detergents and is responsible for the resistance of *Neisseria gonorrhoeae* to these compounds [Pan and Spratt, 1994; Hagman and Shafer, 1995; Hagman *et al.*, 1995) Disruption of the *mtrR* gene results in a 1.6 -fold increase in the expression of the *mtrC* gene and an increase in resistance to hydrophobic agents. AcrR and EnvR are proposed to be involved in regulating the susceptibility of *E. coli* to acridines, dyes, antibiotics, detergents and other hydrophobic growth inhibitors [Ma *et al*, 1993; 1996; Klein *et al.*, 1991). The transcription of the *acrAB* gene is slightly enhanced in an *E.coli acrR* null mutant (1.5 fold in logarithmic phase and 3.5-fold at the onset of the stationary phase), and AcrR was proposed as a weak repressor to prevent unwanted overexpression of *acrAB* (Ma *et al*, 1996). Both the AcrR and MtrR regulators appear to function as modulators involved in fine tuning the expression of their respective systems in a role analogous to which might also be played by LuxT in controlling the expression of *luxO*.

The *mtrR* and *acrR* null mutants as well as the *luxT* null mutant grow at slightly slower rates than the respective wild type cells suggesting that they are pleiotropic regulators. As the *luxT* null mutant was much more resistant to high levels (4%) of NaCl than the wild type cells, it is of particular interest in view of its natural habitat in the ocean. As many of the proteins in this family of regulators control functions related to transport of compounds across the cellular membrane, the possibility exists that LuxT might also control similar functions. It seems that LuxT might be involved in the

regulation of multiple cellular functions and may play an even more significant role than controlling the expression of luminescence.

CHAPTER 4

Cloning, sequencing, and functional studies of the *rpoS* gene from *Vibrio harveyi*

PREFACE

The following Chapter focuses on the role of RpoS from *V. harveyi.* This project is independent of the previous two Chapters. RpoS has been reported to control the production of acyl-homoserine lactones or to be regulated by homoserine lactones in other bacteria. The relationship of RpoS to quorum-sensing and the effects of deletion of the *rpoS* gene on luminescence and survival were examined in *V. harveyi.*

The following chapter has been submitted to *gene* as presented : Lin, Y.H., Miyamoto, C., and Meighen, E.A. (2000)

CONTRIBUTION BY OTHER WORKERS

Carol Miyamoto PCR amplified and sequenced the initial 150 bp DNA fragment of *rpoS.*

ABSTRACT

The *Vibrio harveyi rpoS* gene which encodes an alternative sigma factor (σ^S or σ^{38}), has been cloned and characterized. The predicted protein sequence is highly homologous to RpoS proteins in other bacteria with up to 86% sequence identity. A *rpoS* null mutant of *V. harveyi* was constructed and the phenotype studied. Comparison of the properties of the *V. harveyi* wild type and *rpoS* deletion mutant showed that *rpoS* affected the survival of cells only under specific types of environmental stresses. The *rpoS* null mutant had a lower survival rate compared to the wild type parental strain at high concentrations of ethanol and in the stationary phase, In contrast to other bacteria, deletion of *rpoS* in *V. harveyi* did not affect the resistance of the cells to high osmolarity or hydrogen peroxide, suggesting the existence of alternative systems in *V. harveyi* responsible for resistance to these stresses. RpoS appears not to be involved in the control of luminescence in *V. harveyi* even though it is implicated in regulation of other acyl-homoserine dependent quorum sensing systems.

INTRODUCTION

The *rpoS* gene encodes the alternative sigma factor, RpoS (Hengge-Aronis, 1993; Loewen and Hengge-Aronis, 1994), also known as σ^{38} or σ^{S}, which has been identified as the central regulator of the stationary phase in *Escherichia coli.* On entering stationary phase, the nutrients become limited causing bacteria to differentiate and develop stress resistance before the nutrients are exhausted. The developmental changes requiring the induction of specific genes at the onset of starvation are partially regulated by RpoS. It has been reported that RpoS is involved in the survival under conditions of famine, oxidative stress, and osmotic shock and low pH in *E. coli* (Lange and Hengge-Aronis, 1991). A number of *rpoS* genes have been cloned and studied in other bacteria (Fang *et al.*, 1992; Badger and Miller, 1995; Yildiz and Schoolnik, 1998; Flavier *et al.*, 1998; Ramos-Gonzalez and Molin, 1998; Anderson *et al.*, 1998; Suh *at al.*, 1999).

Bioluminescence in marine bacteria is regulated by the process of quorum sensing, in which the bacteria monitor the amount of specific small signal molecules (autoinducers) secreted by the cells into the media and then respond to the signals by the expression of genes involved in light emission (Bassler, 1999; Hastings and Greenberg, 1999). This autoinduction process has been employed by *Vibrio fischeri* and *V. harveyi* to induce light emission at high cell density. In nonluminescent bacteria, quorum sensing has been used to control a variety of physiological functions, including virulence.

N-acyl-homoserine lactones have been identified as the autoinducers in most gram-negative bacteria, including N-(3-hydroxyl-butanoyl) homoserine lactone in *V. harveyi* and N-oxohexanoyl homoserine lactone in *V. fischeri* (Cao and Meighen, 1989; Eberhard *et al.*,

1981). In a number of cases in nonluminescent bacteria, the expression of the *rpoS* gene has been related to the level of homoserine lactone derivatives. An *E. coli* mutant defective in production of homoserine lactone has been shown to have a lower level of *rpoS* expression (Huisman and Kolter, 1994). In *Pseudomonas aeruginosa*, the promoter of *rpoS* has been shown to be directly controlled by the regulator RhlR and its cognate autoinducer, N-butanoyl-L-homoserine lactone, which are parts of the autoinduction system controlling virulence of this bacteria (Latifi *et al.*, 1996). In *Ralstonia solanacearum*, however, the acyl-homoserine lactone level, which also affects virulence, was decreased in an *rpoS* deletion mutant due to a decrease in transcription of *solR* and *solI*, the genes responsible for the quorum sensing system (Flavier *et al.*, 1998). In contrast, RpoS does not appear to be involved in the production of any of the three acyl-homoserine lactone inducers in *Pseudomonas putida* strain WCS358 (Kojic *et al.*, 1999).

As a two-component system involving phosphoryl group transfer is implicated as the signal-sensing mechanism controlling luminescence in *V. harveyi* quorum sensing (Bassler, 1999) and differs from that found in *V. fischeri* and other gram-negative bacteria (Fuqua *et al.*, 1994), the relationship of RpoS to light and N-(3-hydroxyl-butanoyl) homoserine lactone levels as well as response to stress was investigated in *V. harveyi.* In this report, we cloned the *rpoS* gene from *V. harveyi* and studied the role played by RpoS in these functions.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

Both *V. harveyi* B392 and *E. coli* MM294 were from laboratory collections, and were grown at 27 °C and 37 °C respectively in LB media. Plasmid, pJQ200, was purchased from ATCC. For the purpose of bioluminescence analysis, cells were grown in 1% complex medium (10 g of NaCl, 3.7 g of Na_2HPO_4, 1.0 g of KH_2PO_4, 0.5 g of $(NH4)_2HPO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 2 ml glycerol, 5 g of bactotryptone, and 0.5 g of yeast extract per liter). Where indicated, the concentrations of the antibiotics in the medium were: ampicillin (100 μg/ml), kanamycin (30 μg/ml) and gentamicin (25μg/ml).

Cloning of the rpoS gene from V. harveyi

Forward and reverse primers (+ strand 5' CGGGATCCCGGCGCGTCGCGCA CTGCGTGG; CGGGATCCCGCGTGCCAGCACTTCACGCTG) were designed according to the sequence of the most conserved region of the *E. coli rpoS* gene. PCR was performed at 95 °C for 5 min, 30 cycles at 95 °C of 1 min, 1 min at 54 °C and 40 sec at 72 °C, and the reaction was completed by incubation at 72 °C for 10 min. The PCR product (149 bp) was purified by electrophoresis on a low melting agarose gel and sequenced at the Core DNA Sequencing Facility Center, Kingston, Ontario. Inverse PCR was performed by cutting 0.5 μg of *V. harveyi* genomic DNA with various four-base-cut restriction enzymes and the cut DNA ligated at different dilutions to preferentially cause self-ligation into circular DNA. The circular DNA was then PCR-amplified with divergent primers to obtain the flanking sequences of the initial 149 bp PCR product. Based on the flanking sequence, a 1.79 kbp DNA fragment containing the *rpoS* gene was generated by PCR.

Insertional inactivation of rpoS

The procedure of gene replacement was conducted as described previously (Lin *et al.*, 2000). The 1.79 kbp PCR fragment containing the *rpoS* gene was transferred into the *Hind*III and *Eco*RI restriction sites of pT7-5 (pTRPOS). The kanamycin *(kn)* gene (1.7 kbp) was excised with *Bam*HI and *Acc*I from pKT230 followed by blunt ending. The DNA fragment was then ligated into an *Eco*RV site in pTRPOS. The *rpoS* gene interrupted with the *kn* gene was excised with *Pvu*II *and Fsp*I and inserted into the *Sma*I sites in pJQ200 to give pJQRPOS::*kn*. This plasmid was subsequently transferred into *V. harveyi* by conjugation, and the colonies containing pJQRPOS::*kn* were selected on plates containing ampicillin, kanamycin, and gentamicin. The selected colonies were spread on plates containing ampicillin, kanamycin and 5% sucrose to kill the bacteria harboring the plasmids, and select the cells containing the *kn*-interrupted *rpoS* gene inserted in the genome (knockout strain, BB392RPOS$^-$). The knockout strains were then verified by Southern blots.

Survival assays

Hydrogen peroxide (88 mM) and ethanol (18% vol/vol) was added to overnight *V. harveyi* wild type or RpoS$^-$ cultures (about $4x10^9$ cells/ml) in LB media at 25 °C.. At the selected time points, samples were taken and diluted before plating on LB agar for determination of viable cell numbers. For salt challenge experiments, the overnight cultures were centrifuged and suspended in LB medium containing 2.5 M NaCl. For

stationary phase survival studies, the overnight cultures in LB medium were left at 25 °C, and the viable cells were counted each day.

Bioluminescence

Wild type and mutant cells were grown overnight and diluted to OD_{660} = 0.001 in 1% complex medium. Luminescence of the growing cells was measured at the indicated cell densities with a photomultiplier tube where one light unit corresponds to 1.3×10^{10} quanta s^{-1} based on the light standard of Hastings and Weber (1963).

Chloramphenical transferase (cat) assays

A 530 bp fragment of PCR fragment containing the *rpoS* promoter region was subcloned into pMGM100 (Miyamoto *et al.*, 1990) upstream of the *cat* gene. The resulting plasmid pMGMRP was then transconjugated into *V. harveyi* wild type or into autoinducer negative cells (D1). Cells were grown from a starting O.D. of 0.01, and harvested at two different time points, O.D. = 1.0 and 3.0. The protein pellets were subjected to *cat* assays according to established procedures (Miyamoto *et al.*, 1994).

Sequence Data analysis

Protein database searches were performed using the Blast network service of the National Center of Biotechnology Information. Alignments of proteins were made using the CLUSTAL W (version 1.8) program.

RESULTS

Cloning and characterization of V. harveyi rpoS

Two primers designed according to the *E. coli rpoS* gene were used to amplify a 150 bp sequence from *V. harveyi* genomic DNA by PCR. Inverse PCR was sequentially conducted to obtain the complete gene as well as the flanking sequences using pairs of divergent primers outlined in Fig. 1. The slashed area shows the position of the 150 bp PCR product. The DNA was sequentially extended to the upstream *HinP*I site and then to the *Taq*I site and another *HinP*I using three pairs of primers, and downstream to the *HinP*I site using two pairs of primers. An open reading frame designated as *rpoS* was found to extend for 981 bp.

The nucleotide sequence of the *V. harveyi rpoS* gene is shown in Fig. 2 coding for 327 amino acids. The Shine-Dalgarno sequence can be recognized along with the -10 and -35 promoter sites. The blast sequence similarity search program showed very high homology between the RpoS of *V. harveyi* and the RpoS in other organisms (Fig. 3) with the highest identity (86%) to RpoS from *V. cholerae*.

The *rpoS* genes are reported to be flanked by the *nlpD* and *mutS* genes, which code for a novel lipoprotein (NlpD) and a DNA mismatch repair protein (MutS), respectively, in several bacteria (LeClerc *et al.*, 1996 Robbe-Saule *et al.*, 1995, Ichikawa *et al.*, 1994). The same arrangement is present in the *rpoS* region of *V. harveyi* (Figs. 1 and 2). An incomplete open reading frame transcribed in the same direction was found immediately upstream the *rpoS* gene which encoded the carboxyl terminal segment of a protein with high sequence identity to the NlpD protein. The stop codon of this

Fig. 1. Cloning of the *V. harveyi rpoS* gene and its flanking regions. A 150 bp fragment cloned by PCR using primers based on the sequence of the *E. coli rpoS* gene is given by the slashed area in the gray box representing the *rpoS* gene. The upstream sequence was cloned using inverse PCR with self-ligated *Bgl*II-restricted genomic DNA and two divergent primers (pair 1) to obtain the sequence to the *Bgl*II site. Pair 2 and pair 3 primers were then used sequentially with self-ligated *Taq*I and *Hin*PI restricted DNA, respectively, to further extend the sequence to the upstream *Hin*PI site. Similarly, primer sets 4 and 5 were used sequentially with *Bgl*II and *HinPI* restricted and then self-ligated DNA to clone the downstream sequence. The partial open reading frames predicted to code for NlpD and MutS proteins are also indicated.

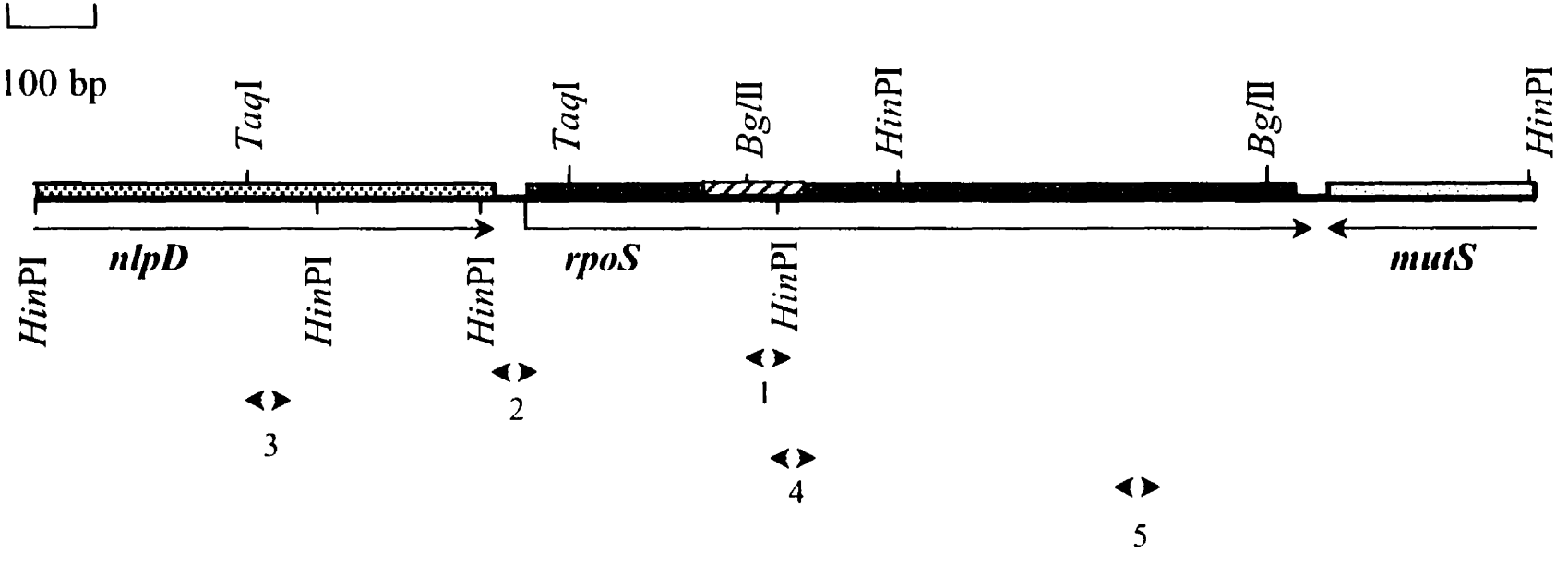

100 bp
TaqI
TaqI
BglII
HinPI
BglII
HinPI
HinPI
nlpD
HinPI
HinPI
rpoS
HinPI
mutS
1
2
3
4
5

Fig. 2. DNA sequence and protein translation of *rpoS*. The putative -10 and -35 promoter sequences and a Shine-Dalgarno (SD) ribosome binding site for *rpoS* are indicated. Sequences coding for part of the NlpD and MutS proteins flanking RpoS are also shown.

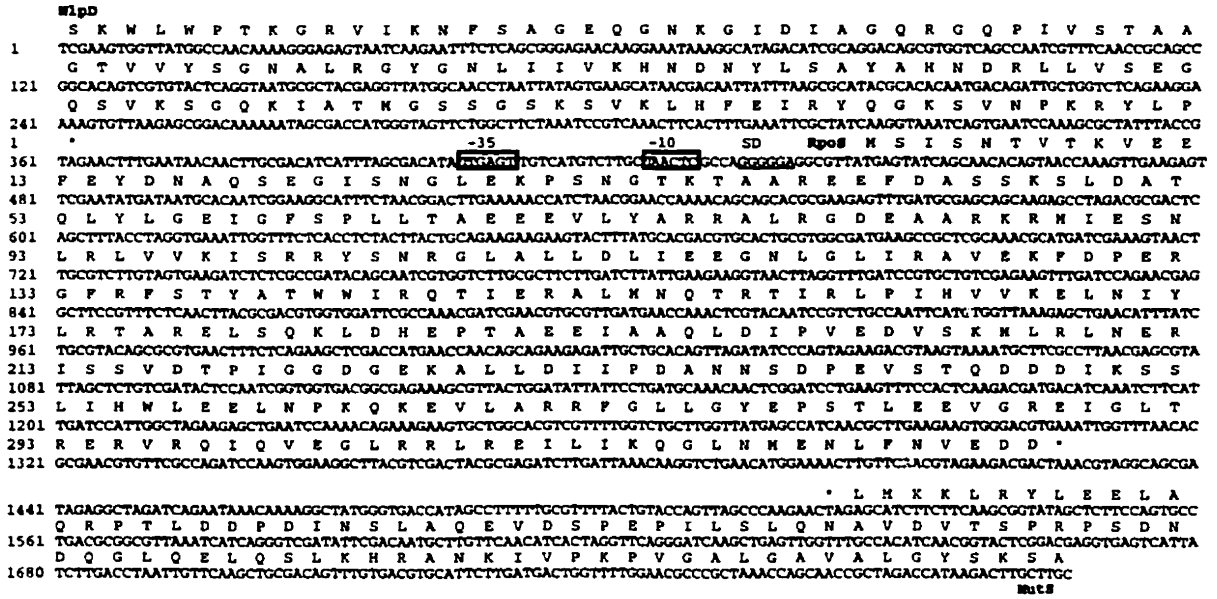

MlpD
-35
-10
SD
RpoS
MutS

Fig. 3. Alignment of the amino acid sequences of RpoS from *V. harveyi* (this study), *V. cholerae* (051804), *Salmonella dublin* (P39699), *E. coli* (P13445), *S. typhimurium* (P37400), *Shigella flexneri* (P35540), *Yersinia enterocolitica* (P47765), *Pseudomonas aeruginosa* (P45684), *Bacillus halodurans* (066381), *Listeria monocytogenes* (P52331), and *Clostridium acetobutylicum* (P33656) with the accession number of the databases indicated in parentheses. Identical amino acid residues present in five or more RpoS proteins are boxed in black. Similar residues are highlighted in gray.


V. harveyi
V. cholerae
S. dublin
E. coli
S. flexneri
Y. enterocolitica
P. aeruginosa
B. halodurans
L. monocytogenes
C. acetobutylicum

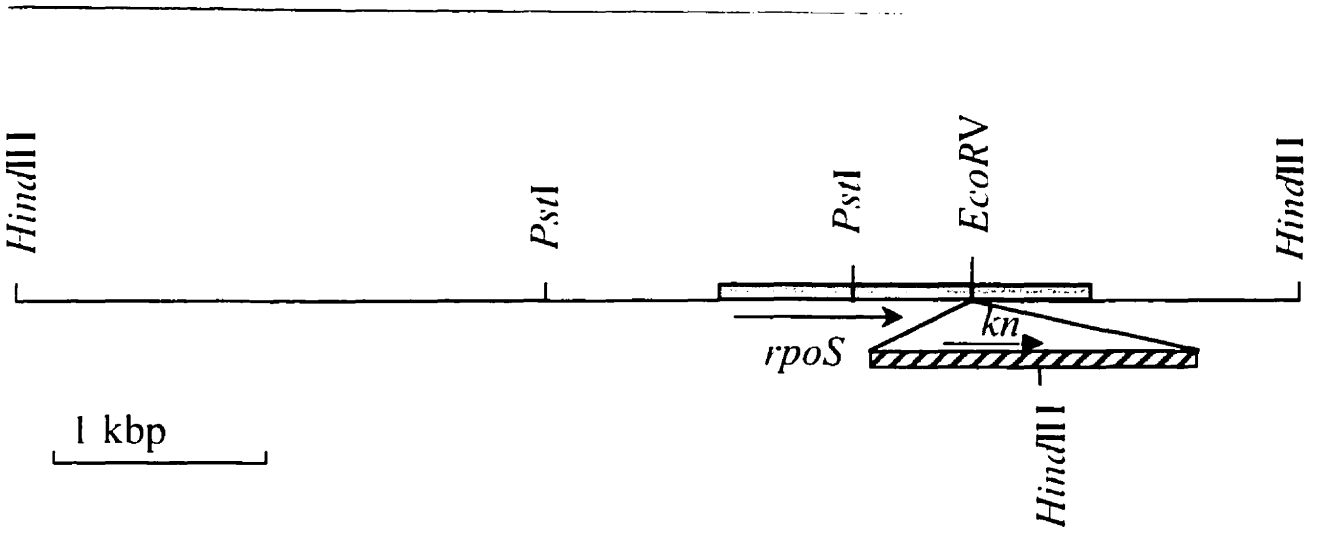
predicted NlpD protein is located just 80 bp upstream of the translational start site of the *rpoS* gene. Downstream of the *rpoS* gene, a convergent open reading frame codes for the carboxyl terminal region of MutS with the stop codons of the two genes separated by 90 bp.

Gene disruption of rpoS in V. harveyi

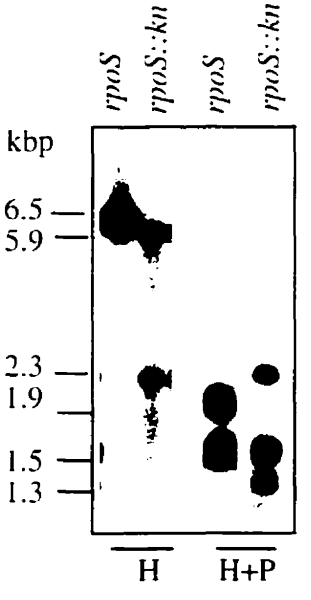
To identify the *rpoS*-associated phenotype, a *rpoS* null allele in the chromosome of *V. harveyi* was constructed (Fig. 4A). A kanamycin cassette was inserted at the *EcoR*V site of the PCR-generated, 1.79 kbp *rpoS*-containing fragment which had been subcloned in the pT7 plasmid. The DNA fragment containing the *rpoS::kn* from the resulting plasmid (PTRPOS::*kn*) was then subcloned to pJQ200, yielding pJRPOS::*kn* which was then introduced into *V. harveyi* by conjugation. The transconjugates harboring the pJRPOS::kn conferring ampicillin, gentamicin, and kanamycin resistance were selected. These transconjugates were then spread on LB plates containing ampicillin, kanamycin and 5% sucrose to select double recombinants which were verified by their gentamicin sensitivity followed by Southern analysis. As shown in Fig. 4B, Southern hybridization using the 1.7 kb PCR fragment as the probe to the *Hind*III digestion of the chromosomal DNA of the wild type *V. harveyi* shows a single 6.5 kbp fragment. The mutant, on the other hand, shows two fragments of 5.9 kbp and 2.3 kbp instead, resulting from the restriction of the *Hind*III site located in the kanamycin cassette. Testing with *Hind*III-*Pst*I double digestion also gave results consistent with the expected sizes and

Fig. 4. Generation and confirmation of the *rpoS* insertion mutation. A. A physical map of the *rpoS* (solid bar) region of the chromosome showing the *Eco*RV site where the kanamycin gene(*kn*) was inserted. The *Hind*III and *Pst*I sites for Southern analysis are also indicated. B. Southern analysis of *V. harveyi* wild type and *rpoS* mutant genomic DNA (*rpoS* and *rpos::kn* respectively) digested with *Hind*III (H), and the combination of *Hind*III and *Pst*I (H+P).

A.


HindIII
PstI
PstI
EcoRV
HindIII
kn
rpoS
1 kbp
HindIII

B.

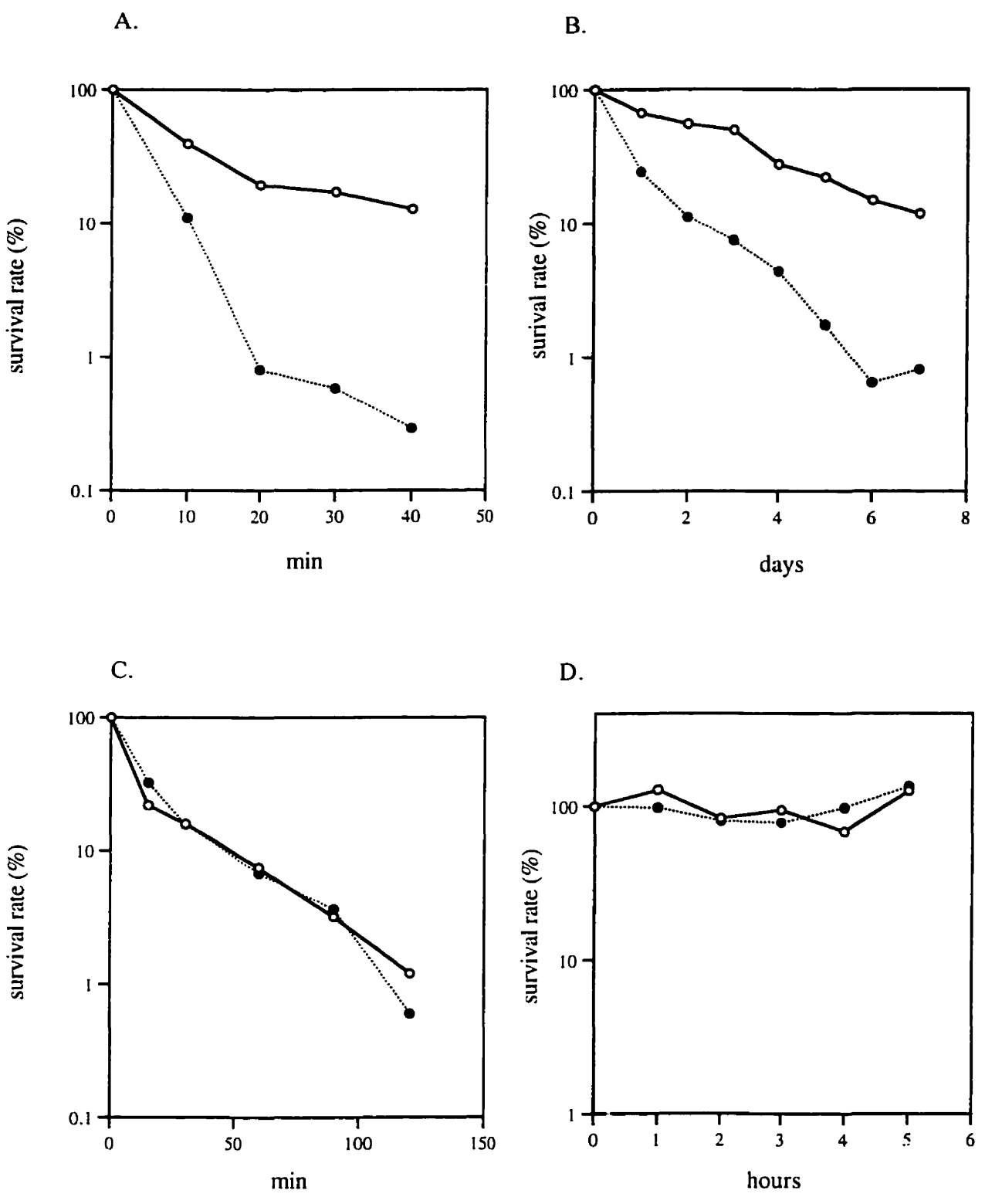

rpoS
rpoS::kn
rpoS
rpoS::kn
kbp
6.5
5.9
2.3
1.9
1.5
1.3
H
H+P

provided further confirmation that the *rpoS* gene was interrupted by the kanamycin cassette in the mutant.

Response of the rpoS-*deletion mutant to environmental stress*

The *rpoS* null mutants produced in other bacteria have been shown to be more susceptible to environmental stress than the corresponding parent strains (Lange and Hengge-Aronis, 1991; Fang *et al.*, 1992; Badger and Miller, 1995; Yildiz and Schoolnik, 1998; Kojic *et al.*, 1999; Ramos-Gonzalez and Molin, 1998; Anderson *et al.*, 1998). Examples of different stress conditions are hyperosmolarity, high concentrations of ethanol or H_2O_2 (oxidative stress), and the stationary phase. We therefore examined the effect of the *rpoS* deletion in *V. harveyi* on survival by determining the viable plate counts after exposure to various stress conditions. As shown in Figure 5, like the *rpoS* null mutants of most bacteria studied, the *rpoS* deletion mutant of *V. harveyi* is much less resistant to an environment of high concentration of ethanol (Fig. 5A) or being in the stationary phase (Fig. 5B). When challenged with hyperosmolarity (2.5 M NaCl), the viable plate counts of both the wild type and the mutant cells declined rapidly, but at the same rate (Fig. 5C). This result is surprising as the RpoS null mutants of most bacteria are much less resistant to salt than the wild type cells. For example, a 10-100 fold greater loss of the *rpoS* mutant cells was observed for *E. coli* and *Salmonella spp.* compared to the wild type cells at high salt concentrations (Lange and Hengge,1991; Robbe-Saule *et al*, 1995).

Fig. 5. Comparison of the stress response of *V. harveyi* wild type and the *rpoS* null mutant to different conditions of stress. *V. harveyi* wild type (open circles) and *rpoS* null mutant (closed circles) were exposed to various stress conditions and the survival rate determined as percentage of viable cell counts at different times. One hundred percent survival corresponds to the viable cell count determined prior to exposure to the indicated stress. Survival on exposure to (A) 18% ethanol, (B) stationary phase, (C) 2.5 M NaCl, (D) 88 mM H_2O_2.


A.
B.
C.
D.
survival rate (%)
surival rate (%)
100
10
1
0.1
0
10
20
30
40
50
min
2
4
6
8
days
150
hours

Survival under conditions of high oxidative stress was compared for the *rpoS* null mutant and wild type cells by adding H_2O_2 to the medium (Fig. 5D). The wild type cells showed complete resistance to 88 mM H_2O_2 over the six hour period examined. This is not surprising for the wild type *V. harveyi* cells since the marine habitat in shallow water requires that it to be resistant to H_2O_2 generated by the effects of UV radiation on water. However, the same resistance to H_2O_2 was observed in the *rpoS* deletion mutant, an unexpected result, since in most other bacteria studied, more than 99% of *rpoS* deletion mutants were killed after few minutes of exposure to a lower level of H_2O_2 (Lange and Hengge,1991; Anderson *et al.*, 1998; Yildiz and Schoolnik, 1998).

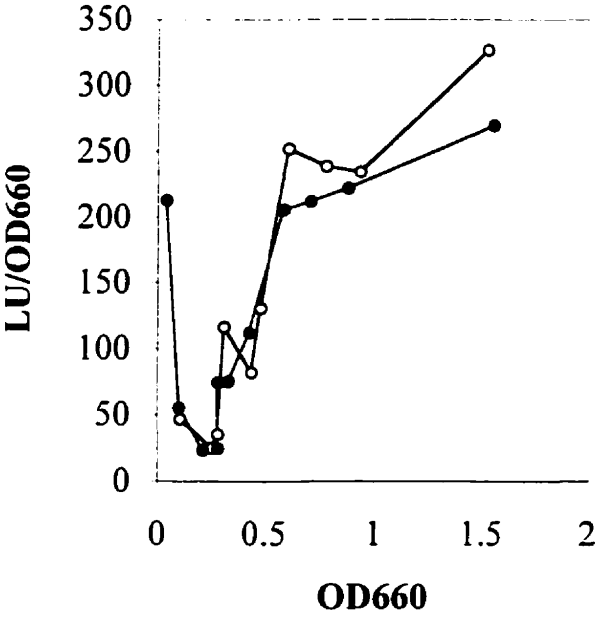
Luminescence and autoinduction is not affected by rpoS

Density-dependent luminescence was investigated for both the *V. harveyi* wild type and the *rpoS* mutant cells. Since the *rpoS* mutant cells showed the same luminescence-induction pattern as the wild type cells (Fig. 6), it appears that RpoS is not involved in regulating the production of the N-(3-hydroxyl-butanoyl) homoserine lactone autoinducers contrasting with results shown for *Ralstonia solanacearum*, which shows reduced acyl-HSL production in the *rpoS* deletion mutant compared to the parent strain (Flavier *et al.*, 1998).

The rpoS gene is not controlled by N-(3-hydroxybutanoyl)-homoserine lactone

The promoter region of *rpoS* gene was fused to a *cat* reporter gene. The resulting plasmid was then transconjugated into *V. harveyi* and D1 mutant cells and the *cat*

Fig. 6. Effect of deletion of *rpoS* in the *V. harveyi* genome on light induction. Cultures of *V. harveyi* wild type (open circles) and *rpoS::kn* mutant (solid circles) were grown overnight in 1% complex medium. The overnight cultures were diluted 500 fold in fresh 1% complex medium and light intensity (LU) was measured with time as a function of cell density (OD_{660}).


350
300
250
200
150
100
50
0
LU/OD660
0
0.5
1
1.5
2
OD660

activities were measured. The D1 mutant is defective in producing N-(3-hydroxybutanoyl)-homoserine lactone (HBHL). The *cat* activity in both strains is identical, suggesting that *rpoS* is not under the control of HBHL (Table 1). These results contrast sharply to those in *Pseudomonas aeruginosa*, in which *rpoS* expression was shown to be controlled by acyl-homoserine lactones (Latifi *et al.*, 1996).

Table 1. The *rpoS* promoter is not under the control of N(3-hydroxybutanoyl) homoserine lactone (HBHL)

V. harveyi strain[a]	Genotype	*rpoS* promoter-cat activity (units/mg)[b]
Wild type BB392	Wild type	800 ± 23
D1 mutant	HBHL$^-$	980 ± 110

[a] Each strain contains pMGMRP (*rpoS* promoter-cat fusion in pMGM100)
[b] Values shown are mean ± SD of three independent experiments. One unit corresponds to one nmole of chloramphinical acetylated per min at 37 °C under optimal assay conditions.

DISCUSSION

In this work, the *rpoS* gene of *V. harveyi* was cloned and shown to code for a protein with high sequence homology to RpoS in other bacterial species. The presence of the *nlpD* gene immediately upstream and a *mutS* gene immediately downstream confirm the identity of the *rpoS* gene, as this gene arrangement is conserved in several bacteria.

Although the *rpoS* gene was found to be important for survival of *V. harveyi* in response to 18% ethanol as well as in the stationary phase, the inactivation of the *rpoS* gene did not affect the survival of *V. harveyi* on exposure to high concentrations of NaCl or H_2O_2. These observations were surprising as the resistance to high osmolarity and oxidative stress has been a hallmark for recognition of the *rpoS* gene in other bacteria. The results indicate that there are additional and probably unique systems in *V. harveyi* involved in regulating the osmotic and oxidative stress resistance which could come to rescue the *rpoS* null mutant exposed to these particular conditions.

Cell-density-dependent luminescence does not appear to be affected by the deletion of *rpoS* gene suggesting that RpoS is not involved in regulating the quorum-sensing system in *V. harveyi*. The results also show that the *rpoS* gene is not regulated by HBHL as expression of the *rpoS* promoter::*cat* fusion gave the same activity in *V. harveyi* wild type and D1 mutant cells. Based on the data from this study, RpoS appears not to be involved in the two-component quorum-sensing system of *V. harveyi* even though it is implicated in regulation of other acyl-homoserine lactone dependent quorum-sensing systems. Moreover, RpoS for *V. harveyi* appears to be involved in responses to

only certain stress conditions and suggests that alternative systems must be available to protect against hyperosmolarity and oxidative stress in this marine bacteria.

CHAPTER 5

GENERAL DISCUSSION

In the past few years, a series of genetic studies on genes cloned by functional complementation of *V. harveyi* dim mutants have greatly increased the understanding of quorum sensing in *V. harveyi*. A model composed of a pair of parallel two-component phosphorelay systems with transmembrane kinases, LuxN and LuxQ (Bassler *et al.*, 1993; 1994a) was initially established. These transmembrane proteins respond to signals from autoinducers excreted into the media which were generated by LuxLM and LuxS, respectively. The signals are then relayed to the response regulator, LuxO, in the cytoplasm, via a phosphorelay protein LuxU. Although data showing LuxO bound directly to *lux* operon DNA was not available, phosphorylated LuxO was proposed as a *lux* operon repressor in the early stages of the cell growth based on experiments demonstrating the constitutive light emission of the *luxO* null mutant (Bassler *et al.*, 1994b; Freeman and Bassler, 1999b). Recent data, however, has provided evidence that LuxO, together with sigma 54, control the *lux* operon indirectly through control of a negative regulator of luminescence in *V. harveyi* (Lilley and Bassler, 2000).

In contrast to the well-characterized luminescence regulatory system of *V. fischeri*, *V. harveyi* appears to employ a different and much more complicated system for the regulation of bioluminescence despite the fact that both bacteria use acyl-homoserine lactones as autoinducers. In *V. fischeri*, the autoinducer(s), produced by LuxI, diffuses freely in and out from the cytoplasm and induces the expression of luminescence structural genes by activating LuxR, a *lux* operon activator (Engebrecht and Silverman, 1984). However, the recent discovery of the existence of a LuxO homologue in *V. fischeri*

(Miyamoto *et al.*, 2000) and the demonstration of the similar phenotype of the null mutant to *V. harveyi* LuxO$^-$ suggests that these two systems might have more in common than we have previously comprehended and that a two-component system regulating luminescence might also exist in *V. fischeri*. It is possible that the LuxO system is functioning as a second autoinduction system in *V. fischeri* in addition to the LuxR/LuxI system. This possibility is supported by the evidence that the proteins implicated in the synthesis of N-(3-hydroxylbutanoyl) homoserine lactone (HBHL) in *V. harveyi* (LuxM) and its sensor (LuxN) are related in sequence to the protein (AinS) (Gilson *et al.*, 1995) implicated in the synthesis of the secondary N-octanoyl-L-homoserine lactone autoinducer (OHL) in *V. fischeri* and its putative sensor (AinR), in addition to the high identity between the LuxO proteins from the two species. It is possible that this second autoinducer system in *V. fischeri* is similar to the LuxM-LuxN-LuxO system in *V. harveyi*. The elimination of luminescence autoinduction in the double mutant *ainS*$^-$*luxI*$^-$ (Kuo *et al.*, 1996) is also consistent with a possible interaction of the OHL autoinducer with AinR to repress LuxO in *V. fischeri* in the same manner as HBHL is proposed to interact with LuxN to cause the dephosphorylation of LuxO and prevent it from repressing expression of the *lux* operon in *V. harveyi*. It would be of interest to understand the functional and evolutionary implications of *V. fischeri*'s need to have two apparently divergent systems controlling luminescence induction while *V. harveyi* requires only one system to regulate light emission. It could be that for *V. fischeri*, found primarily in symbiotic relationships with marine organisms (Graf and Ruby, 1998), there is a need to control luminescence more closely in contrast to a species like *V. harveyi*

which is not found to have any specific relationship with other organisms and is isolated as a free-living species from the ocean and from the intestines of different marine organisms (Nealson and Hastings, 1979).

The existence of LuxO in both *V. harveyi* and *V. fischeri* also implies that LuxO might be a general regulator controlling various functions rather than a *lux*-specific system. This hypothesis is supported by the presence in various bacteria of a large number of proteins, referred to as σ^{54} -dependent proteins (Singler, 1997), which have a close sequence relationship with LuxO. The striking homology of LuxO to those proteins suggests that LuxO is a regulator dependent on the alternative factor σ^{54}. As the *luxO* gene has been cloned along with other σ^{54}-dependent regulators from a nonluminescent *V. cholerae* strain and has been suggested to modulate the expression of the virulence factors (Klose *et al.*, 1998), the evidence suggests that LuxO is a general regulator which could be found in different bacteria controlling diverse physiological functions. A recent report demonstrates that LuxO functions together with sigma 54 to control luminescence, as well as other physiological functions, including the production of siderophore and cell morphology, providing evidence that LuxO controls multiple functions in *V. harveyi* (Lilley and Bassler, 2000). The presence of proteins in *V. cholerae, V. fischeri*, and S. *putrefaciens* with similar properties or sequences to LuxO or to its regulator, LuxT, also suggests that the LuxT-LuxO system is a general regulator system (Miyamoto *et al.*, 2000; Lin *et al.*, 1998; Chapter 3, this thesis).

LuxS, responsible for synthesizing the second yet unidentified autoinducer can be found in a wide range of bacteria including both Gram-positive and Gram-negative bacteria

(Surette *et al.*, 1999). This discovery, along with the presence of LuxO and LuxT in multiple bacteria, and their pleiotropic functions in *V. harveyi*, seems to raise further the possibility that this two-component "Lux" quorum-sensing system in *V. harveyi* is a general signal transduction system that exists in many bacteria and controls multiple physiological processes. An explanation has been proposed for the presence of LuxS in many bacteria: the LuxS pathway might be responsible for inter-species cell-cell communications in contrast to the LuxLM proteins which are *V. harveyi* specific (Bassler, 1999). More evidence is needed to prove this proposed role for LuxS as LuxS could also be involved in intra-species cell-cell communication.

Despite the fact that LuxR has been shown to be a *lux* promoter binding protein and the only transcriptional factor required to turn on the *lux* operon transformed in the *E. coli* system (Swartzman *et al.*, 1992; Swartzman and Meighen, 1993), LuxR has not yet been directly implicated in signal-sensing and response in most regulatory models for quorum sensing in *V. harveyi*. The question arises: what is the mechanism of luminescence repression in the early stages of growth and how does this interface with the activation of the *lux* operon by LuxR? As the stimulation of *luxR* expression in the presence of autoinducers is increased two to three fold (Miyamoto *et al.*, 1996), it is possible that the LuxO pathway partially regulates the *lux* operon through LuxR. One possibility is that a yet unidentified negative regulator is activated by LuxO which prevents LuxR binding or functions as a LuxR inhibitor in the early stages of cell growth. Elimination (e.g. degradation) of these unknown regulators in later stages of cell growth would abolish the competition for the promoter binding site or the inhibition of LuxR

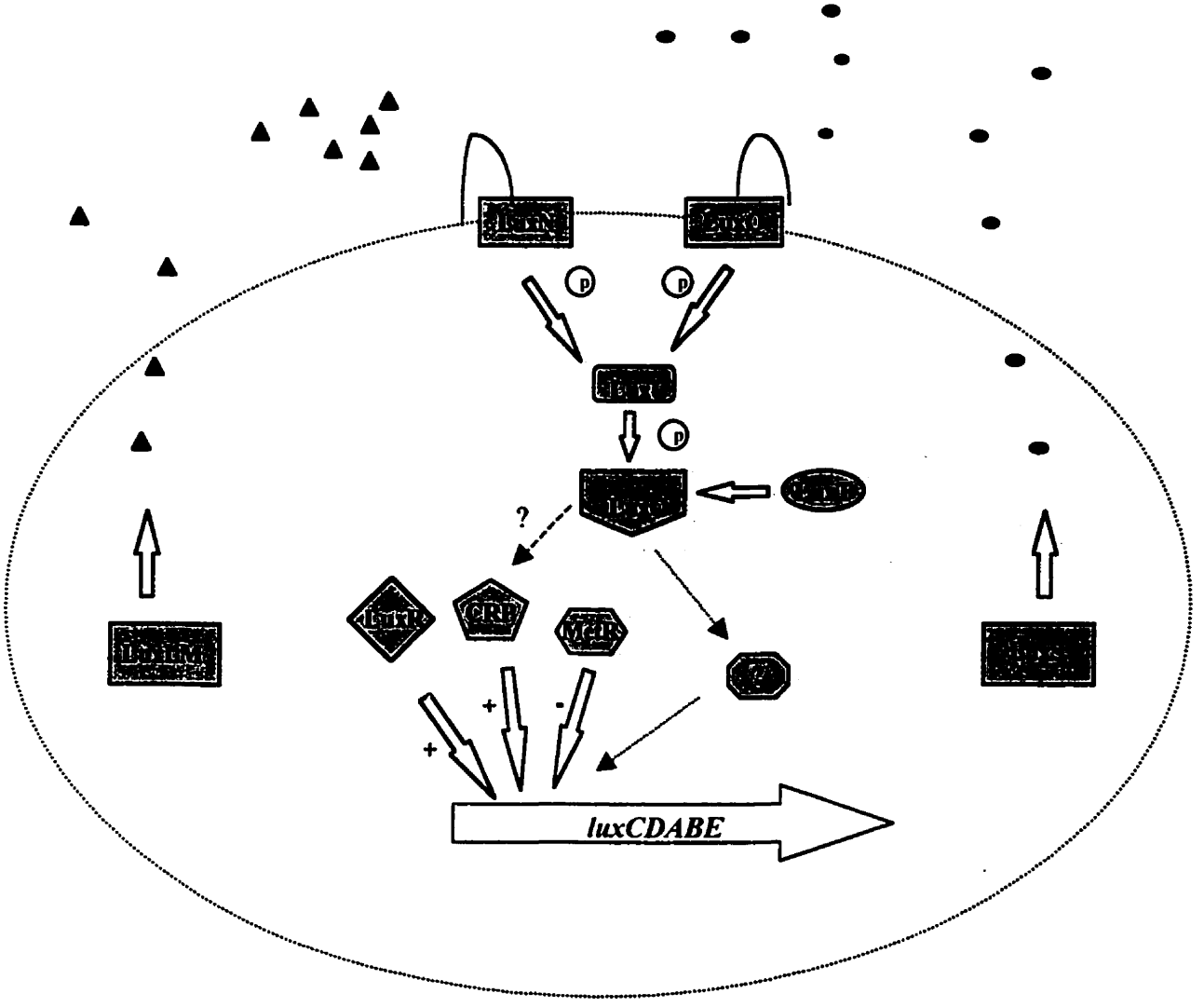
(Lilley and Bassler, 2000). Studies identifying and characterizing this negative *lux* regulator need to be conducted to prove this theory.

Recently, a couple of transcriptional factors (CRP, MetR) in addition to LuxR have been found to bind to the *lux* operon and to be implicated in regulating luminescence (Jaidip Chatterjee, personal communication). It is intriguing that multiple transcriptional regulators are involved in regulating the *lux* operon. Studies still need to be done to comprehend the interplay between those factors and their relationships with the LuxO pathway. A summary of the luminescence regulatory system in *V. harveyi* modified from the initial model (Fig. 3, Chapter 1) by including the additional proteins discovered or proposed to be involved as well as possible pathways, is illustrated in Fig. 1.

In this work, a new transcriptional factor, designated as LuxT, that binds and regulates the *luxO* operon was purified to homogeneity. Based on the tryptic sequences of the purified protein, a *luxT* gene was cloned, a *V. harveyi* LuxT null mutant constructed, and the phenotype studied. The deletion of the *luxT* gene was shown to decrease the LuxO expression by two to three fold and to affect the light induction in the early stages of cell growth. It was also demonstrated that the *luxT* null mutant is more resistant than wild type cells to a high concentration of NaCl.

The modulatory role played by LuxT which causes a two to three fold decrease in *luxO* expression is similar to AcrR/TetR regulators like MtrR and AcrR (Hagman *et al.*, 1995; Ma *et al.*, 1996). The level of the target genes seems to be under careful control by these modulators. It appears to be somewhat inefficient for bacteria to use one transcriptional factor to adjust the level of only one gene by two to three fold.

Fig.1. The modified luminescent regulatory system in *V. harveyi.* The effect of LuxO is indirect and the possible pathways are indicated by dashed arrows. The putative regulators are indicated by a question mark.


?
+
+
-
luxCDABE

Consequently, it is likely that a transcriptional factor such as LuxT has multiple promoter targets and therefore controls multiple functions. MtrR, a LuxT homologous protein, has been discovered to control the *farAB* efflux pump in addition to modulating the *mtrCDE*-operon which controls the membrane-associated efflux pump (Lee and Shafer, 1999). LuxT might have a similar role of acting on multiple promoters in *V. harveyi*. Mobility shift and footprint analyses of LuxT on the *luxT* promoter region DNA also suggests that *luxT* might be under autoregulation by itself and is one of its own target promoters (Lin *et al.*, preliminary results). The slower growth rate of the *luxT* deletion mutants compared to the wild type parent strain could provide additional evidence that LuxT has multiple target promoters. As the deletion of *luxO* in *V. harveyi* does not affect the growth of the cells, it is concluded that LuxT regulates cell growth not through LuxO, but another factor(s).

Does LuxT downregulate the *luxO* expression by responding to the signals from the environment? As LuxT belongs to the AcrR/TetR family of regulators whose members are known to sense environmental stresses and respond by subsequently regulating their target promoters, it is possible that LuxT has a similar mechanism to regulate *luxO* and/or other promoters under its regulation. The identification of the signals that LuxT responds to would be an area worthy of exploration. As the LuxT$^-$ mutant is more resistant to a high concentration of NaCl than the wild type, it is possible that osmotic stress could be one of the signals although in this case LuxT could be predicted to be a repressor of osmotic stress resistance genes.

RpoS has been linked to the quorum-sensing system in some bacteria. We have cloned the *rpoS* gene from *V. harveyi* and examined the phenotype of the *rpoS* null mutant. Our results show that deletion of the *rpoS* gene in *V. harveyi* has no effect on the induction of light. Therefore we concluded that it does not influence the production of autoinducers. The *rpoS* gene was also shown not to be under the control of HBHL. Whether or not the *rpoS* promoter is regulated by the second autoinducer in *V. harveyi* still needs to be tested. It is interesting that the relationship between *rpoS* and the quorum sensing system is different in diverse bacteria. For example, RpoS regulates the production of acyl-homoserine lactones in *Ralstonia solanacearum* (Flavier *et al.*, 1998) and is regulated by acyl-homoserine lactones in *Pseudomonas aeruginosa* (Latifi *et al.*, 1996). In enterohemorrhagic *E. coli*, it was shown that RpoS regulates the type III secretion gene transcription and protein secretion along with the LuxS quorum-sensing system (Sperandio *et al.*, 1999). In the case of *P. putida* (Kojic *et al.*, 1999) and *V. harveyi*, the RpoS null mutant apparently does not affect on the production of acyl-homoserine lactones and the *rpoS* promoter of the latter bacteria was shown in this study not to be regulated by the quorum-sensing system.

The survival of the *rpoS* null mutant on exposure to various stress conditions compared to wild type parent strain was examined. Unexpectedly, the deletion of the *rpoS* gene did not affect the resistance of *V. harveyi* to high oxidative stress and high NaCl concentration. It is possible that *V. harveyi* might have alternative pathways in addition to the RpoS pathway to cope with these stress conditions found in its marine habitat.

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