ABSTRACT

Title of Dissertation: ECOLOGICAL SIGNIFICANCE OF LUMINESCENCE IN *VIBRIO CHOLERAE*: OCCURRENCE, STRUCTURE, EXPRESSION, AND FUNCTION

Christopher J. Grim, Doctor of Philosophy, 2006

Dissertation directed by: Professor Rita R. Colwell
Marine Estuarine Environmental Sciences
University of Maryland, College Park

Several *Vibrio* species are bioluminescent, including *Vibrio cholerae*. Analysis of 224 non-pathogenic *V. cholerae* isolates collected from the Chesapeake Bay, MD, revealed that 52% were luminescent, and 58% of the isolates harbor the *luxA* gene. A significant association of *luxA* to the gene *stn* ($r = 0.40$) was observed and luminescent strains were found to have a significant association with sample fraction and time of sampling, especially from the observed interaction of these two traits. In contrast, 334 non-pathogenic *V. cholerae* strains isolated from two rural provinces in Bangladesh, yielded 21 luminescent (6.3%) and 35 *luxA*$^+$ (10.5%) isolates. None (0%) of 48 laboratory reference pathogenic strains from various geographic locations or 222 environmental and clinical isolated strains of *V. cholerae* O1 or O139 from Bangladesh were luminescent or harbored the *lux* operon. To improve success of isolation of *V. cholerae* from environmental samples, two colony blot hybridization methods were developed. Specificity of two probes was confirmed, using

laboratory reference strains, in addition to environmental and clinical isolates, and sensitivity of the probes was confirmed using water samples into which *V. cholerae* had been inoculated. The *lux* operon of *V. cholerae* was sequenced and its chromosomal location determined. The operon organization is most similar to that of *Shewanella hanedai* and the non-*luxF Photobacterium leiognathi*. Sequence analysis revealed that the *V. cholerae lux* operon is most similar in its genomic sequence to *V. harveyi* and *Photorhadbus luminescens* and it most likely originated from a common *Vibrionaceae* ancestor. Using a new bioassay method that measures expression level, several classes of defective luminescent *V. cholerae* were identified and characterized, including one class previously termed dark, or K variants, in *V. harveyi*. Multiple causes of the defects were identified, indicating several levels of luminescence control in *V. cholerae,* in addition to autoinduction and *lux* repression. Using *luxA* mutants, luminescence was implicated in conveying competitive advantage in growth under microaerophilic conditions, DNA repair by photoreactivation, and neutralization of reactive oxidative species. These results demonstrate that bioluminescence is a frequently occurring trait in non-pathogenic *V. cholerae,* the expression of which gives a selective advantage in specific habitats.

ECOLOGICAL SIGNIFICANCE OF LUMINESCENCE IN *VIBRIO CHOLERAE*: OCCURRENCE, STRUCTURE, EXPRESSION, AND FUNCTION

By

Christopher J. Grim

Dissertation submitted to the Faculty of the Graduate School of the
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Advisory Committee:

Professor Rita R. Colwell, Chair
Professor Sam W. Joseph
Associate Professor Anwar Huq
Dr. Estelle Russek-Cohen
Dr. John F. Heidelberg

Dedication

In loving memory of my mother, Virginia Elaine (1945-2003), and my father, Richard Leo Grim (1940-2005), who inspired me to persevere through faith.

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helped significantly in the direction and analysis of the *lux* operon sequencing (Chapter 4) and in investigations into *lux* expression mutants (Chapter 5); and Dr. David Karaolis, who provided materials and direction for the generation of *luxA* chromosomal *V. cholerae* mutants via allelic exchange.

Lastly, but most importantly, I would like to thank my family, especially my wife, Cheryl, for their support and encouragement during my graduate student career.

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Chapter 1 INTRODUCTION

1.1 Background and Introduction

1.1.1 *Vibrio cholerae* and cholera

Vibrio cholerae is the type species of the genus *Vibrio*. All species of the genus are halophilic and are found in the marine environment, with *V. cholerae* being somewhat unique in its ability to also flourish in freshwater environments. There are more than 200 serogroups of *V. cholerae*, each differentiated by antigenic properties of their cell surface structure. Two serogroups, O1 and O139 (Bengal), are capable of causing epidemic and pandemic cholera outbreaks, although serogroup O37 has caused local epidemics in Czechoslovakia and the Sudan (Datta-Roy et al., 1986). All other serogroups are collectively referred to as non-O1/non-O139 *V. cholerae* and are easily cultured from environmental samples. The O1 serogroup can be further classified into three distinct biotypes, Ogawa, Inaba, and Hikojima, and each biotype can display two phenotypes, referred to as Classical and El Tor.

Cholera is a severe diarrheal disease that affects many poor and/or developing countries that lack proper sanitation. Its transmission is predominantly via ingestion of water or seafood containing the bacterium. Endemic areas include Southeast Asia, Africa, and Latin and South America. Throughout recorded history, there have been seven pandemics, with the first six caused by O1 Classical *V. cholerae*. The current and seventh pandemic began in 1961 and is caused by *V. cholerae* O1 El Tor, which appears to be more persistent than Classical *V. cholerae* O1. In 1992, *V. cholerae* O139 emerged as an agent of epidemics and has persisted in the current pandemic, but *V. cholerae* O1 El Tor remains the predominant cause of cholera to date.

1.1.2 Bioluminescence

Bioluminescence, or biological chemiluminescence mediated by an enzyme catalyst, has evolved independently several times in numerous taxonomically different groups that include bacteria, unicellular algae, coelenterates, ctenophores, beetles, and fish. This is evidenced by a high diversity of the luciferase enzymes, luciferin substrates and chemistries, cellular localization, and phylogenetic unrelatedness among luminescent groups. Currently, 30 different and unique systems are found in nature (Wilson and Hastings, 1998). Bioluminescent bacteria emit light continuously, whereas higher organisms usually emit light in pulses, accomplished by localizing the systems into organelles that are regulated by pH change, calcium flux, and oxygen (Wilson and Hastings, 1998). Despite differences among the various bioluminescence systems, they are defined, as a group, as orchestrating the exergonic reaction of oxygen with a substrate, luciferin, catalyzed by an enzyme, luciferase, which results in light emission.

1.1.3 Luminescent bacteria

Traditionally, luminescent bacteria comprised nine species within four genera. The majority of these species are closely related, belonging to the same family, *Vibrionaceae*. The genus *Photobacterium* includes two species, *Photobacterium phosphoreum* and *Photobacterium leiognathi*, which are found as saprophytes on the surface of fish or crustaceans, as non-specific commensalists in the digestive tracts of fish, and as light organ symbionts in teleost fish (Nealson and Hastings, 1979). The genus *Vibrio* includes five recognized luminescent species. *Vibrio fischeri* inhabits niches similar to *Photobacterium*, but can also be found in the light organs of certain squids. *Vibrio harveyi* is a parasite of crustaceans, especially shrimp, and can be found in the digestive tracts and outer surfaces of

fish and invertebrates. *Vibrio splendidus* and *Vibrio logei* are also found in the digestive tracts and on the outer surfaces of fish and crustaceans. *V. cholerae* can be found in association with copepods and crustaceans, and on the surface of fishes. In addition to the five *Vibrio* species cited above, a single strain of *Vibrio vulnificus*, VVL1, was reported to be luminescent (Oliver et al., 1986). Recently, Fidopiastis et al. (1999) have shown that *Vibrio salmonicida*, which is closely related to *V. fischeri* and *V. logei*, contains a cryptic bioluminescence system. A related species, *Shewanella* (*Alteromonas) hanedai,* is also luminescent (Jensen et al., 1980). In addition to the niches listed above, all can also be found as free-living organisms in seawater or estuarine water.

Photorhabdus (*Xenorhabdus) luminescens* is a terrestrial luminescent member of the *Enterobacteriaceae* found in the digestive tract of nematodes that are parasites of insects (Thomas and Poinar, 1979; Colepicolo et al., 1989). Interestingly, all luminescent bacteria produce a chitinase (Nealson and Hastings, 1979). Furthermore, the chemical reaction, which produces light in bioluminescent bacteria, is essentially the same for all species (Nealson and Hastings, 1979), as follows:

$$FMNH_2 + O_2 + RCHO \text{ (e.g. tetradecanal)} \rightarrow FMN + H_2O + RCOOH + \text{light}.$$

This light emitting reaction is catalyzed by the enzyme luciferase, a mixed function oxidase, which concomitantly oxidizes reduced flavin mononucleotide ($FMNH_2$) and a long-chain (10-14 carbon, usually) aliphatic aldehyde. Bacterial luciferase is a heterodimer consisting of an α and β subunit (Baldwin et al., 1989b). The α subunit contains the active site (Baldwin et al., 1989b) and the β subunit is responsible for cytoplasmic stability (Sugihara and Baldwin, 1988).

1.1.4 The *lux* operon.

Using *V. fischeri* as a model, the genes responsible for light production are situated in two oppositely transcribed operons (Figure 1.1) (Engebrecht and Silverman, 1984; Baldwin *et al.*, 1989a; Swartzman *et al.*, 1990b). Seven genes comprise the right-sided operon (*luxICDABEG*), followed by a transcriptional terminator (Engebrecht and Silverman, 1984; Baldwin *et al.*, 1989a; Swartzman *et al.*, 1990b), while the left side is composed of a single gene, *luxR*. The two sides of the operon are separated by a regulatory region comprised of a cAMP receptor protein-binding site, (Shadel *et al.*, 1990), a 20bp inverted repeat segment ("*lux* box"), and a putative FNR-binding site (Müller-Breitkreutz and Winkler, 1993). The *lux* box is located -40 bp upstream from the *luxI* transcriptional start and is the proposed LuxR binding site (Devine *et al.*, 1989). The *luxR-luxI* regulatory pair is unique to *V. fischeri* among the bioluminescent bacteria. However, other bacteria use homologous quorum sensing regulatory genes to control expression of pathogenic and host interaction factors (Discussed below).

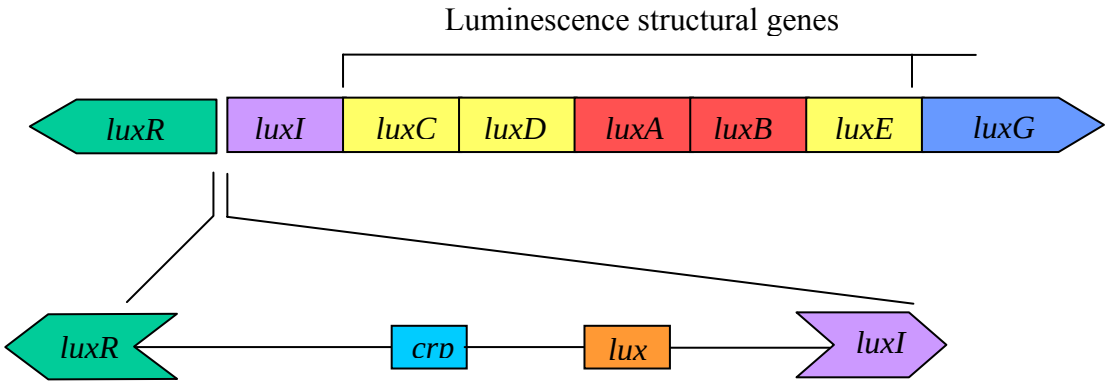


Figure 1.1. Organization of *lux* operon in *V. fischeri*. The *luxR* gene encodes an autoinducer-dependent transcriptional activator. *luxI* is the autoinducer synthase; *luxC*, *D* and *E* are responsible for making the substrate for luciferase; *luxA* and *B* encode structural components of luciferase and the function of *luxG* is unknown. *crp* box is a cAMP-receptor protein binding site and *lux* is the *lux* box.

The *luxA* and *luxB* genes code for the α and β subunits of luciferase, respectively. Sequence homology (30% amino acid sequence identity) suggests that *luxB* probably arose from gene duplication of *luxA*; however, their roles in the whole enzyme are different (Baldwin et al., 1989b). Three genes are responsible for the cycling of the aldehyde substrate through the luminescence pathway. *luxD* encodes an acyl-transferase, which redirects the target fatty acid from the lipid biosynthesis pathway into the luminescence pathway. *luxE* encodes an acyl-synthetase, which activates the fatty acid using ATP. *luxC* codes for a fatty acid reductase, which catalyzes the reduction of the activated fatty acid to the corresponding aldehyde. The function of the *luxG* protein is proposed as a probable flavin reductase (Lin et al., 1998), but its synthesis is not required for bioluminescence (Andrews, *et al.*, 1992).

Additional *lux* genes have been found in *lux* operons of other bioluminescent bacteria. *luxH* has been described in *Vibrio harveyi* (Swartzman et al., 1990a) and is thought to be involved in riboflavin synthesis, as it has 60% amino acid identity with the enzyme which catalyzes synthesis of a riboflavin precursor in *E. coli*. *luxF* has been described in *P. phosphoreum* and certain strains of *P. leiognathi* (Illarionov et al., 1990; Mancini et al., 1988; Mancini et al., 1989). It encodes a flavoprotein related to *luxB*, with almost 40% sequence identity in the carboxyl half of the *luxB* subunit, which appears to be important for those species found in deeper waters (Lee, et al., 1991).

Among all of the luminescent bacteria investigated, the *lux* operon contains a conserved *luxCDABE* motif, with each species having a slightly different arrangement. Figure 1.2 shows similarities and differences in the arrangement of the *lux* genes for luminescent bacteria described to date.

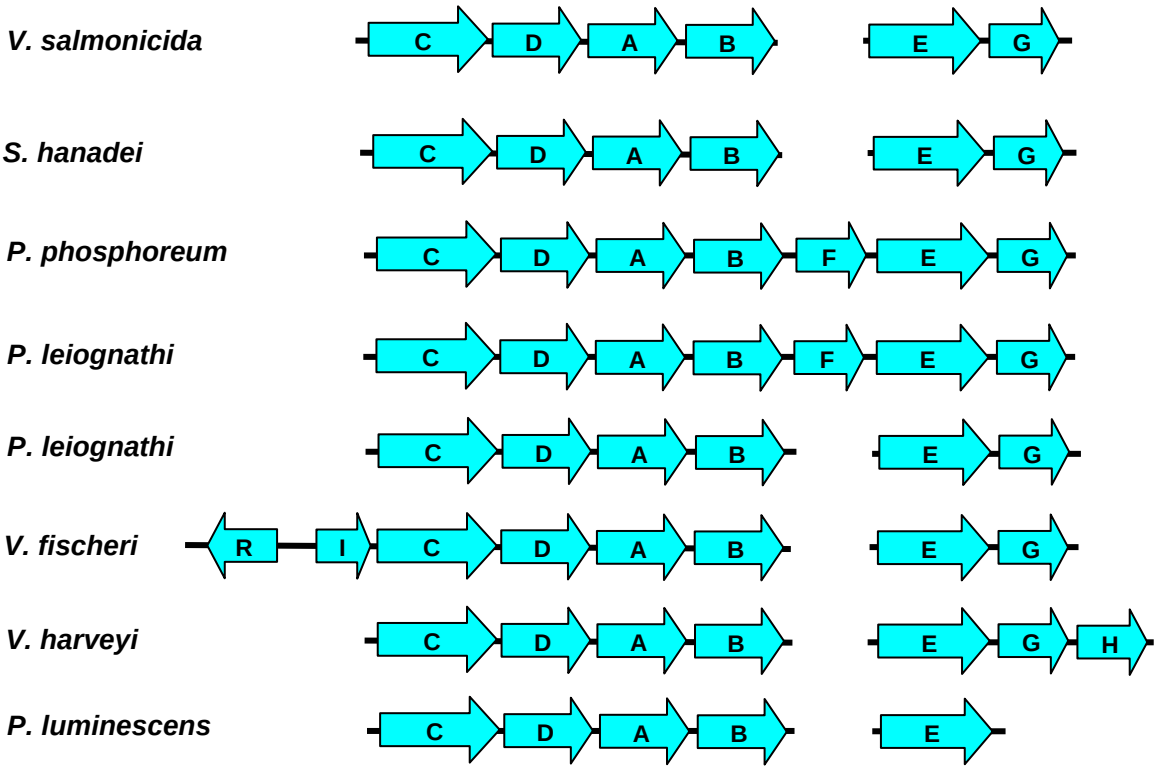


Figure 1.2. *lux* operon arrangement comparison among luminescent bacterial species. *lux* genes are indicated by corresponding letters. Two different operon arrangements are found within the *P. leiognathi* species, and both are shown.

1.1.5 *Lux* regulation and quorum sensing.

Regulation of bioluminescence is accomplished by autoinduction or quorum sensing, two terms that are interchangeable. In *Vibrio fischeri*, expression of bioluminescent genes, namely the *lux* operon, depends on the production of, accumulation of, and response to a small signal molecule or pheromone, *N*-3-(oxohexanoyl) homoserine lactone (HSL), called an autoinducer (Eberhard *et al.* 1981). Autoinducer (AI) is freely diffusible across the cell membrane. At low cell densities, AI passively diffuses out of cells and into the surrounding medium. At high cell densities (such as in the light organ of squids and fish), the

concentration of AI in the surrounding medium and cells increases, reaching a critical concentration, resulting in expression of the luminescent genes (Eberhard, 1972; Nealson, 1977). Autoinducer concentrations of 10 nM are adequate to initiate bioluminescence in *V. fischeri* (Kaplan and Greenberg, 1985). For *V. fischeri*, *luxI* encodes the autoinducer synthase, responsible for producing the autoinducer signal. A second autoinducer, N-octanoyl-HSL, produced by AinS, an acyl-HSL synthase also interacts with LuxR to control luminescence in *V. fischeri* (Gilson et al., 1995). Recently, it has become clear that regulation is controlled by multiple input signals, autoinducers, and corresponding sensors for the majority of the bioluminescent bacteria.

LuxR senses autoinducer levels and is the transcriptional activator of the *lux* system. The 250 amino acid residue polypeptide is divided into an N-terminal regulatory domain (c. residues 1-156), which is required for autoregulation, membrane association, and autoinducer binding, and a C-terminal domain (c. residues 162-250), which contains a helix-turn-helix motif responsible for DNA-binding and a transcriptional activation region (Devine *et al.*, 1988**;** 1989; Fuqua *et al*., 1996).

Currently, more than 25 quorum sensing regulatory systems have been described in Gram-negative bacteria, including both marine and terrestrial species and several pathogens (Swift et al., 1996; Greenberg, 1997; Zhu et al., 2002; Steidle et al., 2002; Schaefer et al., 2002). Many of these bacteria possess more than one autoinduction regulatory network controlling the same target or different target genes (Fuqua et al., 1996). For example, elastase, exotoxin A, exoenzyme S, alkaline protease, phospholipase, neuramidinase, hemolysin (rhamnolipid), and chitinase expression in *Pseudomonas aeruginosa* are

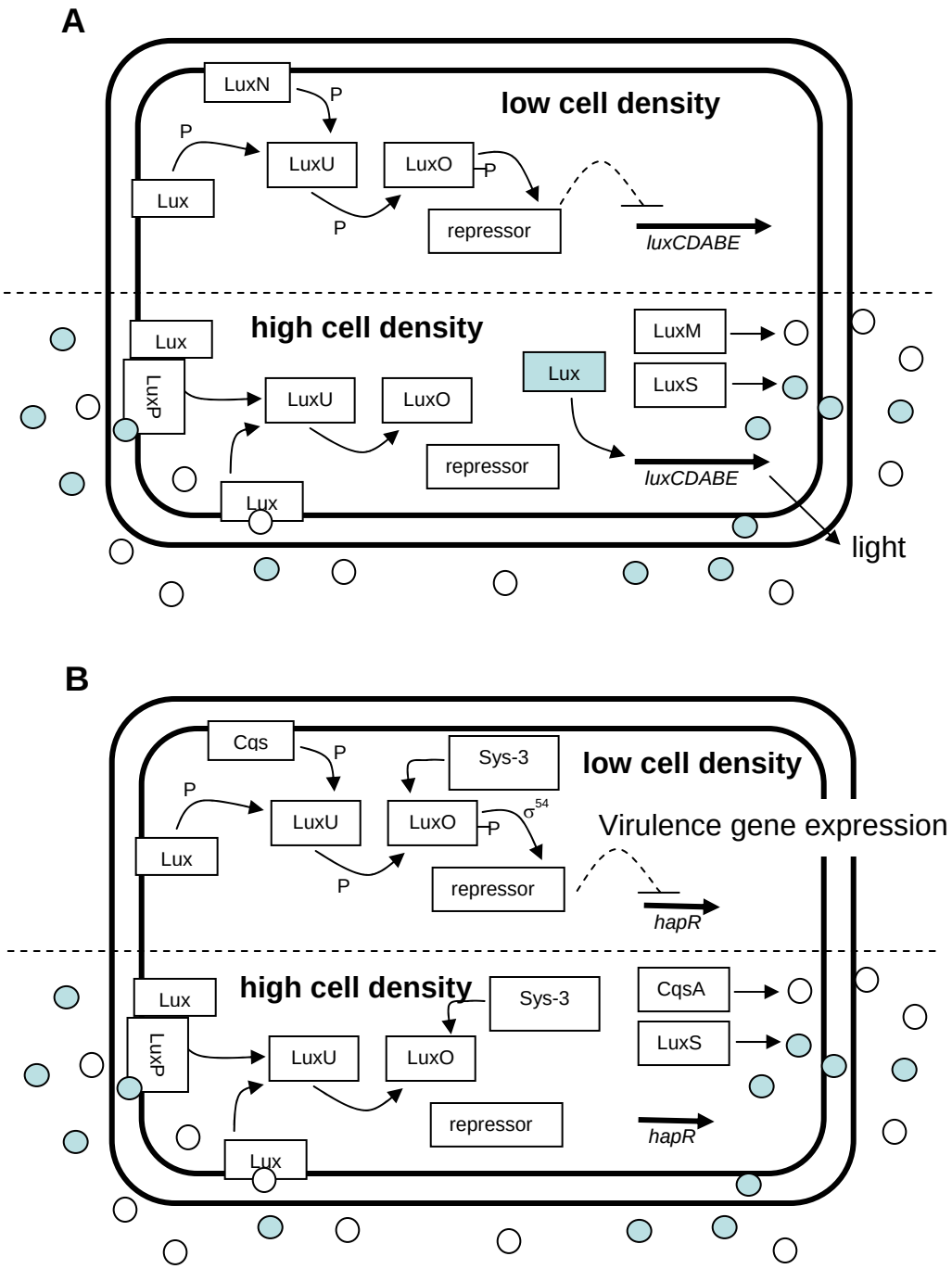


Figure 1.3. Model of genetic control of (a) luminescence in *V. harveyi* and (b) virulence in *V. cholerae* O1. Quorum sensing controls both phenotypes. At low cell density, expression of the *lux* operon or *hapR* is repressed, via a phosphorelay signal transduction cascade. Phosphorylated LuxO is active and allows production of an unknown repressor. At high cell concentrations, autoinducers accumulate and diffuse into the cell. In this condition, central components are de-phosphorylated and LuxO is inactive allowing transcription of the *lux* operon and HapR.

controlled by at least two quorum sensing regulatory networks that are similar to *luxI/R* (Swift et al. 1996).

In *V. harveyi* (and the other well characterized luminescent species of the *Vibrionaceae* species), there is no homologous *luxR-luxI* regulatory pair located immediately upstream of the *luxCDABE* operon, also referred to as the *lux* structural genes. In this case, lux regulation is accomplished by a multiple component signal transduction cascade involving groups of linked genes. Figure 1.3a is a schematic representation of the *lux* regulatory network of *V. harveyi*. LuxO, a two-component response regulatory protein, is the central, shared integrator protein that, when active, represses light production through a repressor protein. It is phosphorylated/dephosphorylated at residue Asp-47. As with many of the bioluminescent bacteria, *V. harveyi* possesses two quorum sensing systems which control light production, termed system 1 and system 2. Lux N and LuxPQ are the sensors for system 1 and 2, respectively. LuxM is responsible for production of N-(3-hydroxybutanoyl)-L-HSL, the autoinducer of system 1, similar in structure to the autoinducers of *V. fischeri*. LuxS catalyzes production of the system 2 autoinducer, a furanosyl borate diester (Chen et al., 2002). LuxU is a histidine phosphotransferase protein that is important in transducing the phosphorelay message from LuxN and LuxP/Q to LuxO. Additionally, there is a transcriptional activator, LuxR (not homologous with *V. fischeri* LuxR) that is repressed by LuxO (Miyamoto et. al., 2003).

Besides autoinducer, regulation of the luminescence system is controlled by other factors. *lux* gene expression is subject to catabolite repression, providing a link between metabolism and light production. Glucose repression is atypical, however, in that it may or may not be reversed by exogenous cAMP (transient or permanent). The oxygen level also

has a significant impact on luminescence expression, since it is a primary reactant. In addition, there have been several other reports of inducers and inhibitors of luminescence expression, but usually under specific growth conditions (in minimal media), so it is unclear whether the effect is direct on *lux* or through an indirect route. Proposed inducers, or positive regulators, include *lexA* (Ulitzer, 1989), *htpr* (Ulitzer and Kuhn, 1988), arginine (Coffey, 1967), and low salt concentration (Nealson and Hastings, 1979), while modified forms of the amino acids, lysine, cysteine, and histidine, inhibit the reaction by binding luciferase (Cousineau and Meighen, 1976; Meighen et al., 1971; and Nicoli and Hastings, 1974).

1.1.6 Dark or K variants

Luminescent strains of *V. fischeri* and *V. harveyi* can give rise to non-luminescent, or dark (K) variants, when cultured under normal laboratory conditions (Keynan and Hastings, 1961). The mechanism for this switch is not understood, but certain factors have been identified which select for the dark variant, namely cultivation at elevated temperature, static growth over several days (Keynan and Hastings, 1961), and treatment with an acridine dye (Nealson and Markovitz, 1970). Most dark variants are not completely non-luminescent, emitting low-level light. They produce normal levels of autoinducer, but are not induced by cAMP. The defect in *V. fischeri* and *V. harveyi* is pleiotropic, with the effect of dark variants also involving colony morphology, flagellation, and phage sensitivity (Hastings and Nealson, 1977). Dark variants are genetically stable, but can revert to the luminescent phenotype, as do the other affected characteristics (Keynan and Hastings, 1961).

1.1.7 *lux* regulatory genes present in *V. cholerae* O1

Several other members of the genus *Vibrio* contain genes homologous to the *lux* regulatory genes of *V. harveyi*, indicating that these species possess autoinduction controlled

phenotypes. Bassler et al. (1997) reported that *V. anguillarum, V. parahaemolyticus, V. alginolyticus, V. natriegens*, and *V. cholerae* O1 were able to induce bioluminescence in *V. harveyi* autoinduction reporter strains. The authors used two different reporters, as there are two independent autoinducer/sensor pairs that act on a shared central regulator in *V. harveyi*. Interestingly, *V. logei*, which itself is luminescent, did not induce luminescence in *V. harveyi*. But the same is true of autoinducer from *V. fischeri*, which does not react with and induce the *lux* operon of *V. harveyi*.

Recently, the genome of *V. cholerae* N16961 O1 El Tor was sequenced (Heidelberg et al., 2000). Several homologues of the *V. harveyi lux* regulatory network are present in this strain (Figure 1.3b). *V. cholerae* possesses homologues to *luxO, P, Q, R (hapR,* Jobling and Holmes, 1997*), S, U,* and *N* (*cqsS*, Miller et al., 2002). In addition, Miller et al. (2002) identified the synthase for the system 1 signal, *cqsA*, and a yet undescribed system 3 input circuit. The quorum sensing circuit in *V. cholerae* O1 controls virulence through HapR (Zhu et al., 2002). A *luxCDABEG* locus is not present in this strain of *V. cholerae*.

1.1.8 *Lux* operon of non-O1 *V. cholerae*

As mentioned above, *V. cholerae* includes luminescent strains, most notably, *V. cholerae* biovar *albensis*. Hada et al. (1985) reclassified *Vibrio albensis* (Shewan and Veron, 1974) as a luminescent non-O1 serovar of *Vibrio cholerae*. Results of a phenotypic characterization of 115 *V. cholerae* isolates showed that 10% of the strains were bioluminescent, when colonies were screened by visual examination of culture growth on an agar plate in the dark (West et al., 1986). Ramaiah et al. (2000) also screened colonies of 7801 environmental isolates from the Chesapeake Bay for luminescence by visual examination in the dark. In the latter case, all were non-luminescent; however, 136 of the

7801 (1.7%) isolates hybridized with a *luxA* probe designed from *V. harveyi* sequence. These data suggest that dark-adjusted visualization of colonies on agar plates is not a good method for detection of luminescent bacteria. A re-examination of 62 of these isolates, employing other methods, yielded an additional 5% that were dimly luminescent, and 16% that emitted light below levels detectable by the human eye (Palmer and Colwell, 1991). Furthermore, the authors found that 56% of the strains hybridized to a *V. fischeri luxA* probe; however, four of the 13 "weakly" luminescent strains did not hybridize to the probe.

Only one *lux* structural gene, *luxA*, has been identified in luminescent non-O1 *V. cholerae*, although it is assumed that at least *luxC, D, A, B* and *E* are present, since these have been shown to be essential in *V. fischeri* for production of light. Palmer and Colwell (1991) sequenced a 350-bp fragment and Ramaiah et al. (2000) sequenced a 745-bp fragment of *luxA* from luminescent *V. cholerae* isolates.

1.1.9 Horizontal gene transfer.

Prokaryotic evolution historically has been viewed as a straight-line vertical transmission of genetic variation from parent to daughter cells undergoing asexual cellular division. In this scheme, the main mechanism for genetic variation is mutation of the base sequence of their DNA molecule. Missense and nonsense mutations and changes in a single base pair occur approximately once in every 10^8 replications of DNA. Deletions or insertions of genetic material can occur through copy error or genetic recombination causing frame-shift mutations, which usually result in premature stops in translation. Nonsense and frame-shift mutations that are not corrected by the cellular proofreading or suppressor mutation system will prevent the translation of genes downstream (polarity), which are normally transcribed together. These mutations are then passed on to daughter cells, with

beneficial mutations being retained and maintained and deleterious mutations being selected against. Assuming that random genetic mutation should occur at a constant rate based on replication, we would expect species that diverged from a common ancestor to show approximately even departure from each other, with small differences due to the strength of the environmental selective pressure that each species has endured.

Many phylogenetic studies, made available through whole genome sequencing, present glaring inconsistencies (Doolittle, 1999). Horizontal or lateral gene transfer has recently emerged as a major component in the evolution of prokaryotic species, and is believed to explain these discrepancies. Lawrence and Ochman (1998) found that 755 out of 4288 ORF's (18%) from the genome of *E. coli* MG1655 were of foreign or alien origin, i.e. laterally transferred, in no fewer than 234 events. Some of the genes that have been introduced include lactose and phosphonate utilization, O antigen biosynthesis, citrate utilization, indole production, and propanediol utilization. Horizontal gene transfer has also gained recent interest as an important mechanism in the shuffling of entire operons between closely and distantly related species (Lawrence and Roth, 1996).

Lateral gene transfer involves two separate processes, namely introduction of new genetic material into a cell and stable integration of this new genetic material, either through genetic recombination between the new material and the bacterial chromosome, or as a stable extra-chromosomal element. DNA can be transferred by bacteriophages, conjugative plasmids, and transposons. Transducing phages pick up host gene(s) and incorporate them into their genome. The newly incorporated host genes usually displace essential viral genes, rendering the phages defective in host lysis. More and more strains of bacteria have been found to harbor transducing phages or remnants of these phages. Conjugative plasmids

permit donor cells to express a "sex" pilus that is responsible for attaching to the recipient cell and bridging the distance between the two cells, thereby allowing genetic exchange to take place. Dahlberg et al. (1998) showed that the conjugative plasmid pBF1 from *Pseudomonas putida* could be transferred to *Deleya marina, Vibrio fischeri* and another *Vibrio* sp. strain isolated from seawater under laboratory conditions (ASW). Antoine and Locht (1992) isolated a cryptic plasmid in *Bordetella bronchiseptica* that can be transformed or conjugated into *Escherichia, Vibrio, Rhizobium, Pseudomonas* and *Bordetella* and stably maintained. Many transposons are also transferred through conjugation. Insertion sequences have been identified as important mediators in the insertion of foreign DNA, including plasmids, into the host chromosome.

Theoretically, deleterious, neutral, and beneficial genetic material could be transferred laterally. Beneficial genes should persist through positive selection. Neutral genes should be weakly selected for, but may persist in the genome for some time before they are lost. Deleterious genes should be lost quickly.

The genome of *V. cholerae* (Heidelberg et al., 2000) contains several genes that establish a close relationship of the *Vibrionaceae* with the *Enterobacteriaceae*, as well as genes that establish close relationships among several members of the genus such as *V. parahaemolyticus*, *V. harveyi*, and *V. alginolyticus*. But, surprisingly, there are genes present in the genome of *V. cholerae* that establish a close relationship with species previously assumed to be distantly related. For example, there is an abundance of *nif* regulatory and functional genes, with close identity to genes from *Synechocystis* sp., *Rhizobium meliloti* and *Bradyrhizobium japonicum*.

Lateral gene transfer can also be responsible for the evolution of pathogenic strains from non-pathogenic parental strains of the same species. Waldor and Mekalanos (1996) showed that the CTX genetic element of *V. cholerae*, which contains genes that encode cholera toxin, as well as *zot, cep*, and *ace*, is encoded by a filamentous phage, CTXΦ. Filamentous phages are ideal vehicles for transmission of large pathogenic determinants, since their genomes can accommodate large pieces of foreign DNA. Karaolis et al. (1998) recently described a pathogenicity island in *V. cholerae*, termed VPI, containing genes encoding the TCP pili, the receptor for CTXΦ, as well as ACF and ToxT, associated with epidemic and pandemic strains. The VPI is absent in nonpathogenic strains of this species. Pathogenicity islands belong to a group of mobile elements, termed specialization islands, which encode specialized functions that are not essential for normal growth but allow alternate growth strategies or occupation of alternate environmental niches. Pathogenicity islands have been found in other bacterial pathogens, such as *E coli* O157:H7 and enteropathogenic *E. coli*, *Y. pestis, S. typhimurium*, and *D. nodosus* (Karaolis et al., 1998).

1.1.10 Luminescent *V. cholerae* and rationale of the study

As stated above, several members of the luminescent bacterial species have been studied extensively and are well characterized. However, the majority of research into these species has focused on characterization of the *lux* operon, quorum sensing regulation of the operon and the symbiotic relationship of these species with their hosts. Only recently has there been any investigations into the ecological role of luminescence expression outside of a mutualistic relationship and these investigations have only focused on one luminescent species, *V. harveyi* (Czyz et al., 2000; Szpilewska et al., 2003). Although the relationship is not clearly understood, this organism is classified as a marine endo-symbiont of certain fish.

The study of luminescence in *V. cholerae* is important for several reasons. First, it will greatly add to the overall knowledge of luminescent bacterial species, since little is known about luminescence in this species even though it was first described over a century ago. Second, the study of the ecological function of luminescence of *V. cholerae* will provide conclusions that can be used in conjunction with results obtained using *V. harveyi* to conclude whether these functions are group-specific or unique to each species. The use of *V. cholerae* is ideal in that this species represents a non-symbiotic luminescent bacterial species. Lastly, the study will conclude whether this phenotype is important for the survival of this organism in the environment and whether or not it is important in pathogenic strains of *V. cholerae*.

1.2 Objectives of this study

The major objective of the study reported here was to determine the significance, namely ecological function, combined with incidence and expression, of the *lux* operon in the non-symbiotic estuarine and marine bacterium, *Vibrio cholerae.* In order to achieve this overall objective, multiple experimental approaches were developed as objectives, as follows.

- Determine benefit, if any, of luminescence in a marine, non-symbiotic species, that is whether alternate roles for luminescence allow the genes to be maintained in the genome.
- Determine incidence of bioluminescence expression in non-O1/non-O139, O1, and O139 *V. cholerae* under optimized conditions of growth favoring expression of luminescence, employing both environmental and clinical isolates.

- Identify *lux* genes present in *V. cholerae* that allow expression of luminescence and determine organization of the *lux* operon.
- Determine the origin of *lux* genes that are present, that is, whether they arose from a common ancestor or were laterally transferred.
- Determine the expression profile patterns of luminescence of the bioluminescent *V. cholerae* to determine if previously identified traits in other bioluminescent species are universal.

In addition to the objectives listed above, new methods for isolation and detection of *V. cholerae* were developed, in order to obtain an accurate measure of the incidence of this phenotype in the environment.

Chapter 2 INCIDENCE AND DISTRIBUTION OF LUMINESCENT *VIBRIO CHOLERAE*

2.1 Introduction

Vibrio cholerae, an autochthonous aquatic bacterium, is the causative agent of epidemic cholera. More than 200 serogroups of *V. cholerae* have been identified, but of these, only O1 and O139 are associated with clinical disease, although non-O1/non-O139 serogroups have been linked to less severe outbreaks of diarrhoreal disease.

Bioluminescence is a characteristic of several bacterial species, notably those of the family *Vibrionaceae*, including environmental isolates of *V. cholerae* (Palmer and Colwell, 1991; West et al., 1986). Bioluminescence has been well characterized in several genera and species, including *Photobacterium phosphoreum*, *P. leiognathi*, *Shewanella* (*Alteromonas) hanedai* (Jensen et al., 1980), *Vibrio fischeri*, and *Vibrio harveyi* (Nealson and Hastings, 1979), *Vibrio splendidus*, *Vibrio logei,* and *Vibrio cholerae* (biovar *albensis*) (Nealson and Hastings, 1979). Oliver et al. (1986) reported the isolation of a single strain of *V. vulnificus*, VVL1 to be luminescent and Fidopiastis et al. (1999) described *Vibrio salmonicida*, closely related to *V. fischeri* and *V. logei*, as possessing a cryptic bioluminescence system.

Luminescence is controlled by the *lux* operon and is regulated in a cell-density dependent manner, termed quorum sensing or autoinduction, a response in target gene expression when extra-cellular signal molecules, autoinducers, reach a critical concentration. Bassler et al. (1997) observed that *V. cholerae* O1, as well as *Vibrio anguillarum, Vibrio parahaemolyticus, Vibrio alginolyticus,* and *Vibrio natriegens* were able to induce bioluminescence in *V. harveyi* reporter strains, indicating that *lux* regulatory genes were present. The genomic sequence of *V. cholerae* N16961 (O1 El Tor) includes several

homologues of the well characterized *V. harveyi lux* regulatory system (Heidelberg et al., 2000). *Vibrio cholerae* N16961 possesses homologues to *luxO, P, Q, R (hapR,* Jobling and Holmes, 1997*), S, U,* and *N* (*cqsS*, Miller et al., 2002). The *lux* quorum-sensing system controls multiple cell-dependent responses in *V. cholerae*, including virulence and biofilm formation (Hammer and Bassler, 2003; Zhu and Mekalanos, 2003; Zhu et al., 2002). Notably missing from the genome sequence of *V. cholerae* N16961 is the lux "structural" operon. The six gene operon, *luxCDABEG*, encodes a acyl-CoA reductase, an acyl-transferase, two luciferase subunits, an acyl synthetase, and a flavin reductase.

West et al. (1986) conducted an extensive numerical taxonomy study of *Vibrio* spp. and found that 11/115 (10%) strains of *V. cholerae* were bioluminescent. The authors used a simple assay that involves visualizing luminescent colonies on an agar plate in the dark after a period of adjustment, approximately 10 minutes. Palmer and Colwell (1991) re-examined 62 of the 115 non-luminescent strains from the above study, using a liquid scintillation counter in the single photon-monitoring mode to examine 6 hr LB broth cultures. They found that 5% (3/62) were dimly luminescent (visible by plate assay) and 16% (10/62) emitted "low-level" light; i.e., below the level detectable by the human eye. Interestingly, they found that 56% (35/62) hybridized to a *V. fischeri luxA* probe; however, 4 of the 13 "weakly" luminescent strains did not hybridize to the probe. Additionally, one of two *V. cholerae* O1 strains (ATCC 14033) used in the study hybridized with the *V. fischeri luxA* probe. These results indicate that the methods, then available, lacked sensitivity and specificity.

In this study, a more sensitive assay for expression of luminescence in *Vibrio cholerae* was developed. In addition, an improved genetic screen for *luxA* was constructed

and used to confirm luminescence in strains identified by the expression assay. A total of 47 strains from the West et al. (1986) study were evaluated and an additional 224 environmental isolates from Chesapeake Bay, 400 environmental and 156 clinical isolates from Bangladesh, and 48 *V. cholerae* clinical isolates from various sources were tested for luminescence. The results were confirmed by either *luxA* PCR or dot-blot hybridization. Phenotypic and genotypic characterizations of the 224 environmental *V. cholerae* isolates from Chesapeake Bay, MD were analyzed, including data on environmental parameters measured at the time of sampling, to estimate the distribution of luminescent *V. cholerae* in this estuarine ecosystem.

2.2 Materials and Methods

2.2.1 Bacterial strains

Five groups of strains from collections of *Vibrio cholerae* were included in this study. Group I comprised 47 strains of *V. cholerae* that had been characterized by West et al. (1986) and Palmer and Colwell (1991). West et al. (1986) originally characterized 115 isolates of *V. cholerae*, based on 133 phenotypic tests, including luminescence. Of the 115 strains, 62 characterized as non-luminescent were re-tested for luminescence and *lux* gene content by Palmer and Colwell (1991). Of these 62 strains, 47 were able to be recovered from the laboratory collection and included in this study.

Group II comprised 224 strains of *V. cholerae* isolated from the Chesapeake Bay in an earlier study by Louis et al. (2003). Four near-shore sites were sampled monthly during 1998-1999 and bi-weekly during the summer months (June-August). Additionally, samples were collected twice during each summer season from mid-bay on research cruises carried out on a North-South transect yielding a set of 278 isolates. The isolates were collected by direct plating (and subsequent colony blot hybridization, Chapter 3) and by traditional

enrichment-selective plating protocols (Huq et al., 2006). ERIC-PCR analysis was performed on all strains and the results were used to identify 54 replicates from the enrichment-selective plating method and the clones were subsequently removed from the collection. The remaining 224 individual environmental *V. cholerae* isolates were included in this study.

Group III comprised 48 clinical strains of *V. cholerae* O1 or O139 serogroups, from the laboratory collection or received from various sources.

Group IV comprised 156 clinical isolates of *V. cholerae* collected from Bangladesh during 1997-2000. Group V comprised 334 *V. cholerae* environmental non-O1/non-O139 isolates, 26 *V. cholerae* environmental O139, and 40 *V. cholerae* environmental O1 isolated from eight ponds located in the Bakerganj district and eight ponds located in the Mathbaria district, Bangladesh, during March, 2004 to April, 2005 (Alam et al., 2006).

2.2.2 Luminescence bioassay

Test strains were transferred from –80°C frozen stock onto agar plates after thawing and incubated overnight at 30°C. Single colonies were inoculated into fresh broth media and incubated overnight at 30°C, with shaking (200rpm). The overnight cultures were diluted 1:500 or 1:1000, into 125 ml flasks or 16 x 125 mm culture tubes containing fresh media and incubated at 30°C with shaking (200rpm). Luminescence was measured at successive time points using a model LB96P luminometer (EG&G Berthold, Oak Ridge, TN). Light units are defined as the number of events observed per second per 100 µl culture. Simultaneously, culture density measurements were made, by measuring absorbance at 600nm (OD_{600}), using a model DU640 UV/VIS spectrophotometer (Beckman Coulter, Inc., Fullerton, CA).

Five media were tested to achieve optimal luminescence: Luria Bertani medium + 1% NaCl (LBN); luminous medium (LUM, ATCC medium 731); autoinducer bioassay medium (AB, Bassler et al., 1993); *Photobacterium* medium (PB); and marine broth 2216 (MB, Difco, Detroit, MI).

Optimum oxygen concentration was determined, by varying the volume of medium in 16 x 125 mm tubes and 150 ml culture flasks, while maintaining constant shaking speed (200 rpm). Sixteen x 125 mm culture tubes were filled with 2.5, 5 or 10 ml and 150 ml Erlenmeyer flasks with 75, 50, 25 or 10 ml sample. Three strains from Group I: *V. cholerae* UM4057; UM4086; and UM4102 were assayed in triplicate.

V. harveyi ATCC14126, *V. fischeri* ATCC7744, and *V. cholerae* biotype *albensis* ATCC14547 were used as positive controls and *E. coli* DH5α and uninoculated medium were used as negative controls in these experiments. All experiments were performed in triplicate.

2.2.3 *V. cholerae luxA* PCR

To confirm luminescent in those strains identified as luminescent by the luminescence bioassay, *luxA* PCR amplification or *luxA* dot-blot hybridization was performed on genomic DNA extracted from each *V. cholerae* isolate included in this study. For groups I and IV, *luxA* PCR was used. First, degenerate *luxA* primers were designed, based on partial *V. cholerae luxA* sequence and complete *luxA* gene sequences from *V. harveyi* and *V. fischeri*. The primer pair 5'-wGAAGrkTGTGGyTTCGA-3' and 5'-AyTCTTCwGGyGTyCCTA-3' was used to amplify an 827-bp amplicon from *V. cholerae* biotype *albensis*, using *Pfu* polymerase (Stratagene, La Jolla, CA). PCR was carried out according to manufacturer's protocol. The blunt-ended PCR products were cloned using

Zero Blunt™ TOPO™ PCR Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). Five transformants were selected and their plasmid DNA extracted using the Qiaprep Spin Miniprep kit (Qiagen, Valencia, CA). Proper insertion of PCR amplicons was confirmed by digestion of 1.0 μg of plasmid DNA with *Eco*RI and gel electrophoresis. The PCR insert was sequenced from the plasmid DNA using M13 sequencing primer sites on the plasmid. Sequencing was performed on an ABI 373 sequencer. A new, highly specific *V. cholerae luxA* PCR primer pair, 5'-CGAAGCGGTTTGGTTGCTA-3' and 5'-CGGGTAGCATTGACGTAGGA-3', which amplify a 650-bp fragment, were designed from the 827-bp sequence. Confirmatory *luxA* PCR, using *Taq* polymerase (Promega, Madison, WI), was performed according to standard protocols.

2.2.4 *V. cholerae luxA* dot blot hybridization

To confirm luminescence in strains from Groups II, III, and V, *luxA*$^+$ strains were identified by dot blot hybridization. DNA was extracted using the DNeasy Tissue Kit (Qiagen) and dot-blotted onto MagnaCharge membranes (Osmonics, Westborough, MA), according to manufacturer's instructions. A 650-bp *luxA* probe labeled with DIG-dUTP was produced, using the PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals, Mannheim, Germany). The PCR product was analyzed by agarose gel electrophoresis. Hybridization was performed using the DIG Detection Starter Kit II (Roche Molecular Biochemicals). The dot blots were hybridized at 45°C. Autoradiography was performed for 20 min at 25°C.

2.2.5 Phenotypic characterization of *V. cholerae* from Chesapeake Bay

Twenty-five biochemical tests were used to characterize the *V. cholerae* isolates from the Chesapeake Bay (Group II) and included the following: O1/O139 serotyping;

luminescence; arginine dihydrolase; esculin hydrolysis; growth in nutrient broth containing different concentrations of NaCl (0, 6, and 8%); acid production from sucrose, arabinose, mannose, mannitol; lysine and ornithine decarboxylase; methyl red; Voges-Proskauer; oxidase; gelatinase; amylase; lipase (corn oil); chitinase; sensitivity to vibriostatic agent O/129; sensitivity to polymyxin B; and growth at 42°C (Choopun, 2004).

2.2.6 Genotypic characterization of *V. cholerae* from Chesapeake Bay

Genotypic characterization of Group II involved screening for presence/absence of nine selected genetic markers by dot blot hybridization. The genetic targets were six toxin-related genes (*ctxA, zot, tcpA, toxR, hlyA, stn*), two membrane protein-coding genes (*ompU* and *ompW*), and one luminescence-coding gene (*luxA*).

2.2.7 Statistical analysis

Phenotypic traits were coded as binary data (positive/negative response). The signal intensities from the dot-blot hybridizations were recoded into five classes, 0-4 (0 for negative and 1-4 for low to high positive, respectively) as nominal data. When a probe generated only two responses; i.e., strong intensity or no signal, the data were coded as binary (absence/presence). A series of nonparametric analyses were performed on phenotypic and genotypic traits of isolates by appropriate procedures implemented in SPSS version 11.5 (SPSS Inc., Chicago, Illinois). For correlation among traits, Spearman rank correlation was used. Exact test was used to test even distribution of responses by isolates. To test association between a pair of binary traits or multiple-nominal traits, the McNemar test or marginal heterogeneity test was used, respectively (Sokal and Rohlf, 1994).

For isolates from shore sampling sites (B, F, K, H, and S) (Figure 2.1), the habitat of the isolates was considered a potential factor associated with phenotypic and/or genotypic

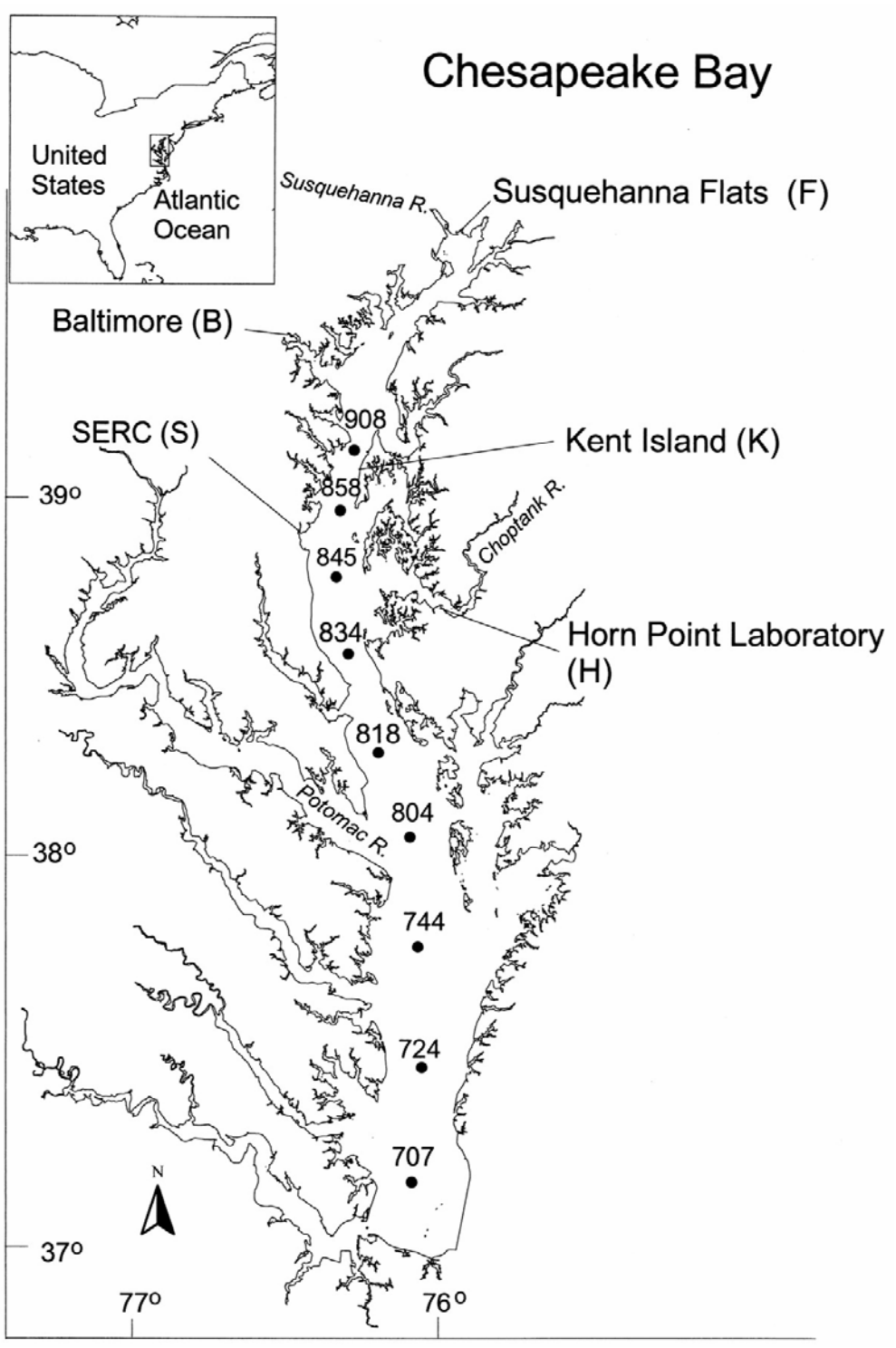


Figure 2.1. Map of sampling stations in Chesapeake Bay.

traits of the isolates. The habitat of an isolate was coded by three types of dimensional variables (fraction, site, and temporal variables), because samples used for *V. cholerae* isolation were generated by size-fractionation of surface water samples at each sampling location on a specific day. To differentiate annual and seasonal variation of traits of isolates, time of sampling was represented by a combination of year and round. When each site was sampled twice in a month (from June to August), each round corresponded to approximately biweekly intervals. For the rest of a year, round was coded as monthly interval. Log-linear analysis was performed to determine association of traits with specific habitat (Sokal and Rohlf, 1994). The log-linear model best fitting the data was searched by backward elimination from saturated models. For all the tests, unless specified, significant interactions were declared at type I error level of <0.05.

2.3 Results

2.3.1 Luminescence bioassay

In the past, standard procedures for assaying bioluminescence of *V. cholerae* and related species involved visual determination of cultures streaked onto an agar plate and incubated overnight, after a period of dark adjustment by the investigator (approximately 10-15 min) (West et al., 1986) or use of a liquid scintillation counter (chemiluminescent mode) to measure light production (single photon events) from broth cultures (Palmer and Colwell, 1991). Results from two studies employing these methods showed that examining broth cultures using a liquid scintillation counter was more sensitive and identified low-level luminescence. Bassler et al (1997) demonstrated that the latter was sufficiently sensitive to detect exogenous AI levels of luminescence above those of wild type *V. harveyi*.

Table 2.1. Phenotypic expression and genotypic confirmation of luminescence in 47 strains of *V. cholerae.*

V. cholerae strain UM-	Hyb. with *V. fischeri luxA* probe[a]	Low-level light emission[a,c]	Luminescence in LB[b,d]	Luminescence in MB[b,d]	*V. cholerae luxA* PCR[b]
4052	+	-	-	-	-
4054	+	-	-	-	-
4055	+	-	-	-	-
4056	+	6.9	-	15	+
4057	+++	10.1	5	2101	+
4058	+	-	-	-	-
4060	++	-	-	-	-
4065	+	-	-	-	-
4068	-	-	-	-	-
4069	+	-	-	-	-
4070	++	-	-	-	-
4071	-	-	-	-	+
4072	+++	-	-	-	+
4073	-	-	-	-	-
4075	+++	-	-	-	+
4078	++	-	-	-	-
4079	-	-	-	-	-
4080	-	-	-	-	-
4082	+++	-	-	-	+
4083	-	-	-	-	-
4086	+++	9.5	6	1167	+
4089	+++	11.8	9264	209630	+
4091	-	7.0	-	18	+
4092	-	-	-	-	-
4093	+	-	-	-	-
4094	-	-	-	-	-
4096	-	-	-	-	-
4097	-	6.0	-	-	-
4098	++	-	-	-	-
4100	-	8.8	-	-	-
4102	+++	11.1	164	18471	+
4103	+++	7.1	-	19	+
4104	+	6.8	-	-	-
4105	++	5.6	-	-	-
4157	+++	10.0	-	412	+
4178	-	6.3	-	-	-
4180	-	-	-	-	-
4181	-	-	-	-	-
4182	+	-	-	-	-
4183	-	-	-	-	-
4184	-	-	-	-	-
4187	-	-	-	-	-
4188	-	-	-	-	-
4189	-	-	-	-	-
4195	-	-	-	-	-
4196	++	-	-	-	-
4217	++	-	-	-	-

[a]Palmer and Colwell, 1991.
[b]This study
[c]Arbitrary light units = (mean cpm – cpm of blank)/10-ml (OD_{660})
[d]Relative light units = # of events per second per 100-µl culture

In this study, the luminescence bioassay (see Methods and Materials) was optimized for *V. cholerae*, using five previously characterized strains, *V. cholerae* UM4057, UM4086, UM4089, UM4102, and UM4157 from West et al. (1986) and Palmer and Colwell (1991), mainly to evaluate assay conditions, i.e. growth medium, sampling times, and air-to-culture volume ratios in assay vessels. West et al. (1986) found these strains to be non-luminescent by plate assay. However, Palmer and Colwell (1991) determined that the strains were either dimly luminescent by plate assay or emitted low levels of light when tested using liquid scintillation counting, and that they hybridized to a *V. fischeri luxA* probe (Table 2.1). The five strains of *V. cholerae* were grown in five different media and assayed for expression of luminescence over a 10 hour time period using the new bioassay. Results are presented in Figure 2.2A-E. Expression of luminescence was significantly higher when strains were grown in marine broth and expression of luminescence increased rapidly at approximately 4 hr post dilution, stabilizing at 5-6 hr post dilution, except in the case of *V. cholerae* UM4157 (Figure 2.2E), which stabilized 8 hr post dilution. None of the strains were luminescent when grown in luminous medium or *Photobacterium* medium, except *V. cholerae* UM4089, which was luminescent in all media (Figure 2.2C). *V. cholerae* UM4157 was luminescent only in the MB and AB media (Figure 2.2E).

To determine if differences in cell density could account for the above observations, culture density (OD_{600}) was also measured. Results for *V. cholerae* UM4089 are presented in Figure 2.3 as representative of the five strains tested. In all cases, strains grew to higher cell densities in LBN compared to the other media tested. Cultures grown in marine broth, luminous media, and *Photobacterium* broth grew to comparable cell densities at comparable growth rates, while the AB cultures exhibited lowest cell density and slower growth rates.

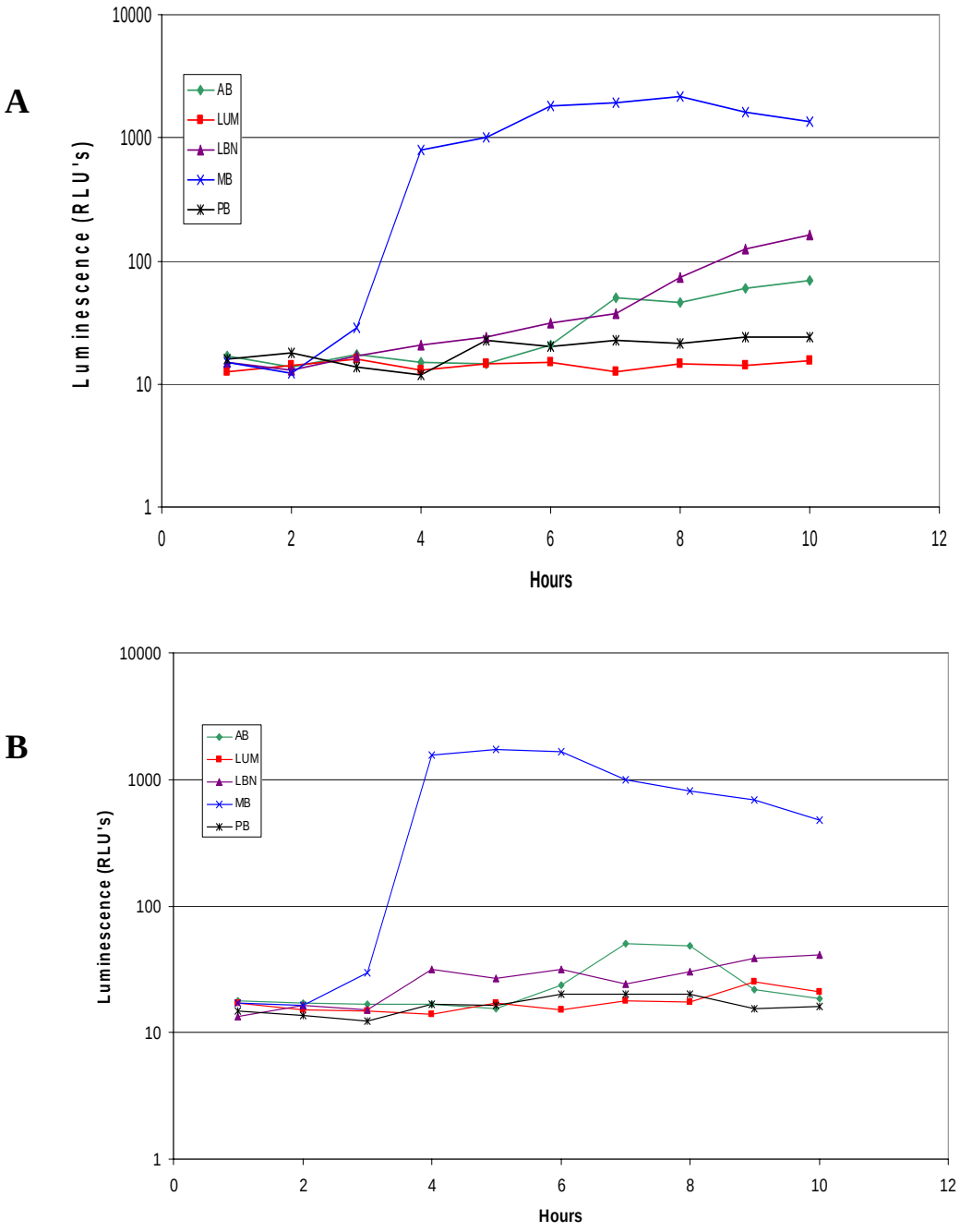
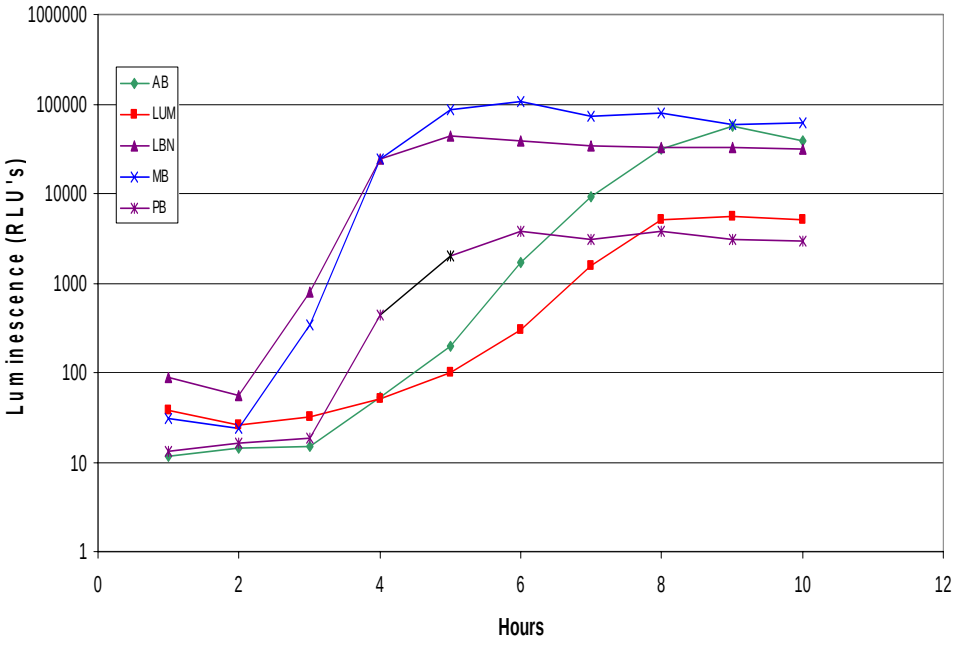


Figure 2.2. Expression of luminescence by five strains (n =9) of *V. cholerae* in various media over a ten-hour time period; A. UM4057, B. UM4086, C. UM4089, D. UM4102, and E. UM4157. Instrumentation background, 12-20 RLU, not removed

C



D

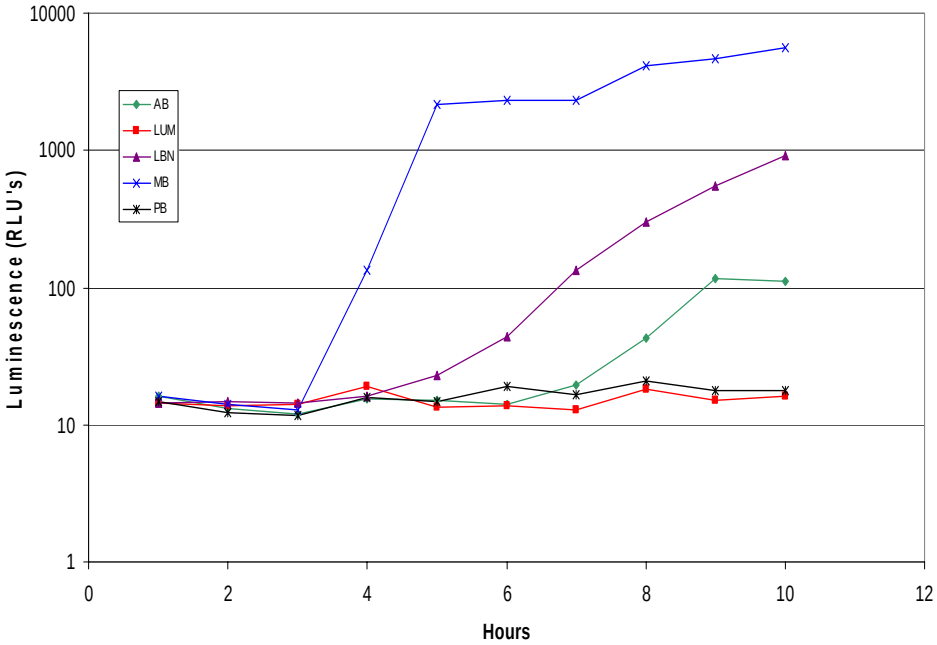


Figure 2.2. Expression of luminescence by *V. cholerae* (continued)

E

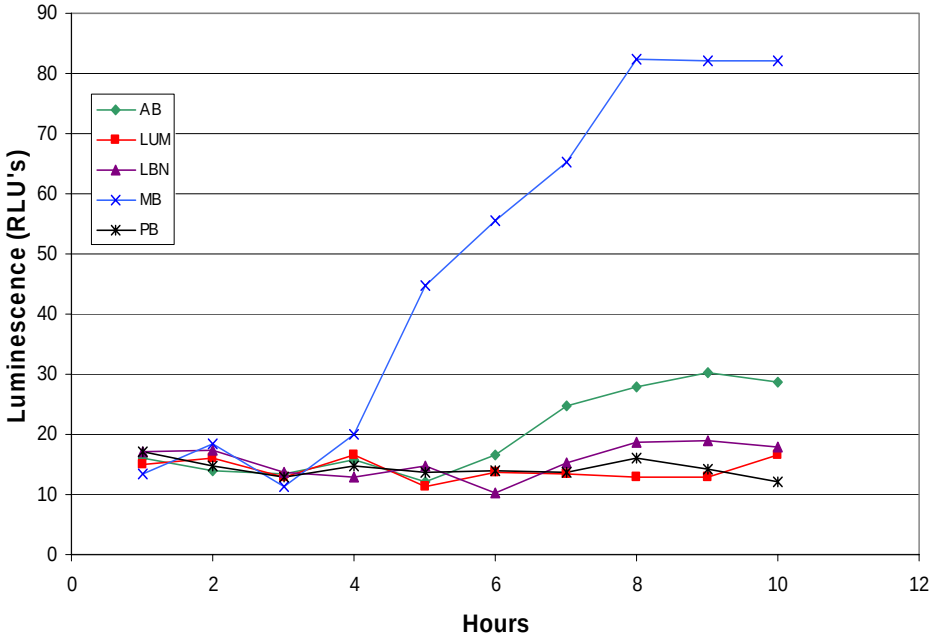


Figure 2.2. Expression of luminescence by *V. cholerae* (continued)

Hada et al. (1985) showed that luminescence in *V. cholerae* biotype *albensis* is oxygen dependent. In the bioassay employed in this study, oxygen concentration was influenced by a combination of factors, shaking speed (or aeration) and air-to-culture volume in the sample vessel. We tested 150-ml flasks (used for small-scale experiments) and 16 x 125-mm borosilicate culture tubes (used in large-scale experiments) with different air-to-culture medium volumes (2, 3, 6, and 15:1, and 2.5, 5, and 10:1, respectively), at a constant shaking speed of 200 rpm. The 150 ml flasks containing 25 ml (6:1 air to culture volume) of culture medium gave the highest expression of luminescence, while all samples yielded sufficient expression for detection (data not shown).

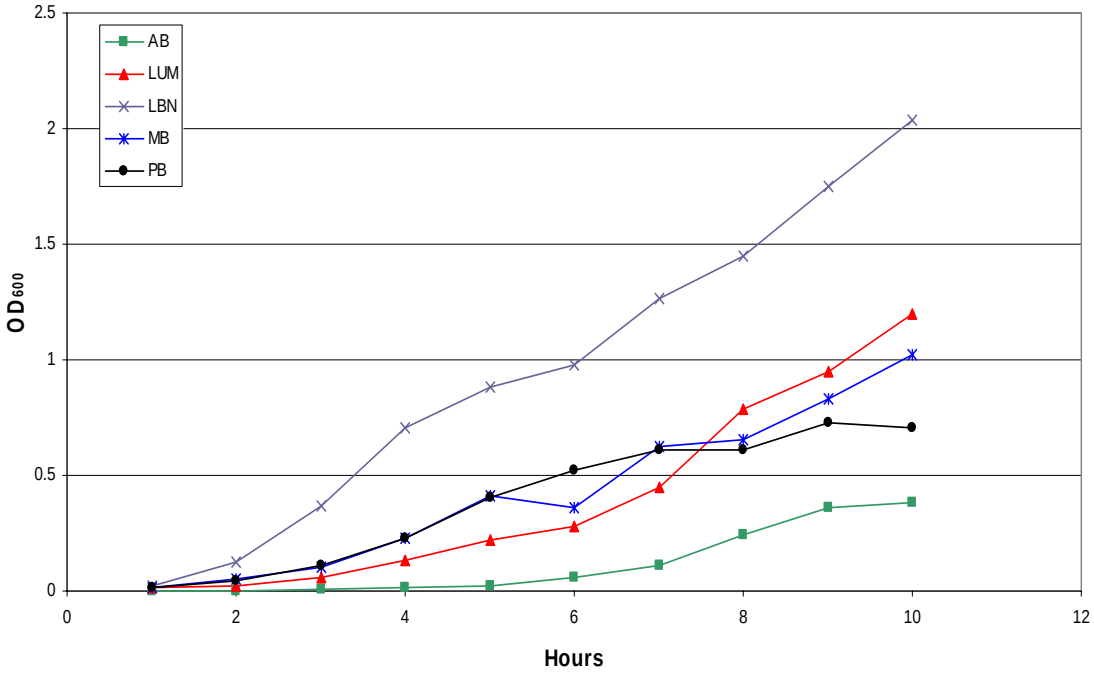


Figure 2.3. Growth of *V. cholerae* UM4089 (n=9) in five media over a ten-hour time period. LBN, Luria Bertani medium + 1% NaCl; LUM, luminous medium (ATCC medium 731); AB, autoinducer bioassay medium (Bassler et al., 1997), PB, *Photobacterium* medium; and MB, marine broth 2216 (Difco, Detroit, MI).

2.3.2 Bioassay evaluation

Using the optimized protocol, with marine broth as the growth medium and 7 hours post dilution as the time of measurement (7 hr presented the best choice for measuring luminescence when cultures were grown in MB, since the majority of strains showed peak or near-peak, stabilized luminescence at this time) (Figures 2.2A-E), the 47 previously characterized *V. cholerae* strains (Palmer and Colwell, 1991) of Group I were assayed for expression of luminescence. Table 2.1 shows that 8 out of the 47 strains (17%) expressed luminescence above background. These luminescent strains were confirmed by PCR, using

primers for *V. cholerae luxA*. Thus 12 of the 47 strains were found to possess *luxA*. In addition to the eight luminescent strains identified by the bioassay, *V. cholerae* UM4071, UM4072, UM4075, and UM4082 were also *luxA*$^{+}$.

2.3.3 Luminescence in *V. cholerae*

The 224 environmental *V. cholerae* isolates of Group II from the Chesapeake Bay were assayed for luminescence and 116 of the isolates were positive (52%) by the new assay.

Dot blots of extracted DNA from the 224 isolates were tested using a DIG-labeled 650-bp *V. cholerae luxA* probe. Fifty-eight percent (130/224) were positive for *luxA* by hybridization. In addition to the 116 strains that were luminescent by the bioassay, 14 strains were positive for *luxA,* but were not luminescent. The 14 strains were retested and gave the same results; i.e., lack of expression of luminescence.

Similarly, isolates of Groups III, IV, and V were assayed for luminescence and screened for *luxA* by PCR or dot-blot hybridization. For Group III and IV, none (0/48 and 0/156, respectively) were luminescent or harbored the *luxA* gene. For Group IV, none of the *V. cholerae* O1 or O139 serogroup isolates (0/40 and 0/26, respectively) were luminescent. Among the non-O1/non-O139 strains, 6% (21/334) were luminescent and 11% (35/334) harbored the *luxA* gene. For all groups, there were no cases in which an isolate was positive by the luminescence phenotypic assay and negative by genotypic screening.

2.3.4 Phenotypic characterization

Among the phenotypic traits assayed from Group II, the assay for luminescence (Lum) produced one of the two most diverse results (the other being acid production from mannitol), in which isolates were evenly distributed across positive and negative responses

(P>0.5, n=224, exact test). The rest of the biochemical tests showed one response (P<0.05, exact test).

2.3.5 Genotypic characterization

Similar to luminescence among the phenotypic tests, distribution of the luciferase α subunit gene (*luxA*) was the most variable of those included in this study: it was present in 58% (130/224) of the isolates. Unlike luminescence (Lum), the hypothesis of even distribution of isolates with regard to presence and absence of this gene was rejected with a marginal significance (P=0.02). Instead, the hypothesis of predominance of *luxA* by a 6:4 ratio was accepted (P=0.59, exact test). Variability within the 650-bp *luxA* DNA sequence was very limited. All of the 130 isolates that were positive produced a strong dot-blot hybridization signal, indicating high level of homology in the DNA sequence.

2.3.6 Correlation among traits

Table 2.2 shows correlation between the phenotypic (Lum) and genotypic (lux) luminescence traits and three environmental variables, five genotypic and 14 phenotypic traits. As expected, comparison of luminescence (Lum) and *luxA*, namely the phenotype and its genetic determinant showed the highest correlation (r=0.89, P<0.01). Of 224, 94 were negative for both tests, and 116 were positive for both tests. As stated above, there were no cases in which luminescence was observed in a strain that did not harbor the *luxA* gene. However, there were 14 isolates (5.8%), which were *luxA*$^+$ but did produce luminescence under the conditions established for this study. Since 100% correlation of *luxA* with luminescence expression was not found, the two luminescence traits (Lum and *luxA*) were analyzed individually. The two porin genes, *ompU* and *ompW*, were significantly correlated with luminescence and *luxA,* notably *ompU* (r=0.41 and 0.43, respectively), as was the heat-

stable enterotoxin gene, *stn* (r=0.40 and 0.34). These correlations were further analyzed using the McNemar test or the marginal homogeneity test to confirm these associations for each level of each trait. In the case of *stn*, association between *luxA* and *stn* and Lum and *stn* were rejected (exact method, P<0.01). In the case of *ompU*, association with luminescence was also rejected (exact method, P<0.01). These results indicated an "internal structure" within the luminescent Chesapeake Bay population with respect to these two traits.

Table 2.2. Spearman rank correlation coefficients among traits and environmental variables. Only significant correlations are shown. High correlation (>0.5) values are boxed, and correlations >0.35 are shown in bold. Negative correlations are underlined and in red.

	pH	Tem	Sal	*luxA*	*toxR*	*hlyA*	*ompU*	*ompW*	*stn*	Lum	Na6*	Mns*
pH	1.00											
Tem	0.31	1.00										
Sal		0.28	1.00									
luxA		0.18		1.00								
toxR				0.31	1.00							
hlyA			0.33		0.29	1.00						
ompU				**0.43**	0.30	0.16	1.00					
ompW	0.18			0.33	**0.68**	0.26	**0.37**	1.00				
stn		**0.36**	0.17	0.34	0.14	0.18	0.16	0.20	1.00			
Lum		0.19		**0.89**	0.26		**0.41**	0.31	**0.40**	1.00		
Na6	0.16			0.29	**0.38**	0.22	0.23	**0.38**		0.28	1.00	
Mns		0.20		0.17	0.31		0.16	0.19	0.17	0.28		1.00
Mnt*	0.18	0.20	0.31	**0.47**	0.33		0.21	0.26	**0.43**	0.34	0.16	0.21
Suc*			0.19		0.32	0.31	0.15	0.23				
Orn*											0.27	
MR*			0.21	0.16	0.17	0.22		0.19		0.23	0.13	0.21
VP*				**0.38**	**0.83**	0.29	0.34	**0.70**	0.18	0.32	**0.39**	0.27
Amy*				0.20	**0.38**	0.27		**0.36**		0.16	0.15	
Lip*					0.23			0.20			0.27	
Chi*					0.17	0.15		0.13			0.19	
V150*					0.17	0.15		0.13			0.19	
V10*					0.17	0.15		0.13			0.19	
PB*				0.36	0.22		0.30	0.29		0.30	**0.37**	
42C*					0.31	0.29		0.22				

*Na6 = growth in 6% salt (NaCl); Mns = acid production from mannose, Mnt = acid production from mannitol; Suc = acid production from sucrose; Orn = ornithine decarboxylase; MR = Methyl Red; VP = Voges Proskauer; Amy = amylase production; Lip = lipase production; Chi = chitinase production; V150 = resistant to vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine) at 150-µg; V10 = resistant to vibriostatic agent O/129 at 10-µg; PB = resistance to polymixin B; 42C = growth at 42°C.

Table 2.2. Spearman rank correlation coefficients among traits and environmental variables (continued).

	Mnt*	Suc*	Orn*	MR*	VP*	Amy*	Lip*	Chi*	V150*	V10*	PB*	42C*
pH												
Tem												
Sal												
luxA												
toxR												
hlyA												
ompU												
ompW												
stn												
Lum												
Na6*												
Mns*												
Mnt*	1.00											
Suc*		1.00										
Orn*			1.00									
MR*	0.23			1.00								
VP*	**0.35**	0.29		0.24	1.00							
Amy*	0.17	**0.38**		0.14	**0.47**	1.00						
Lip*					0.23	0.31	1.00					
Chi*					0.16	0.22		1.00				
V150*					0.16				1.00			
V10*					0.16					1.00		
PB*	0.27				0.25			0.16			1.00	
42C*		**0.77**			**0.37**	**0.39**		**0.44**				1.00

*Na6 = growth in 6% salt (NaCl); Mns = acid production from mannose, Mnt = acid production from mannitol; Suc = acid prodcution from sucrose; Orn = ornithine decarboxylase; MR = Methyl Red; VP = Voges Proskauer; Amy = amylase production; Lip = lipase production; Chi = chitinase production; V150 = resistant to vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine) at 150-µg; V10 = resistant to vibriostatic agent O/129 at 10-µg; PB = resistance to polymixin B; 42C = growth at 42°C.

2.3.7 Environmental characteristics

The occurrence of luminescent strains in Chesapeake Bay was analyzed with respect to environmental parameters at the sites where the samples were collected. Figure 2.4 shows plots of luminescent and non-luminescent isolates and pH, salinity, temperature, and time of

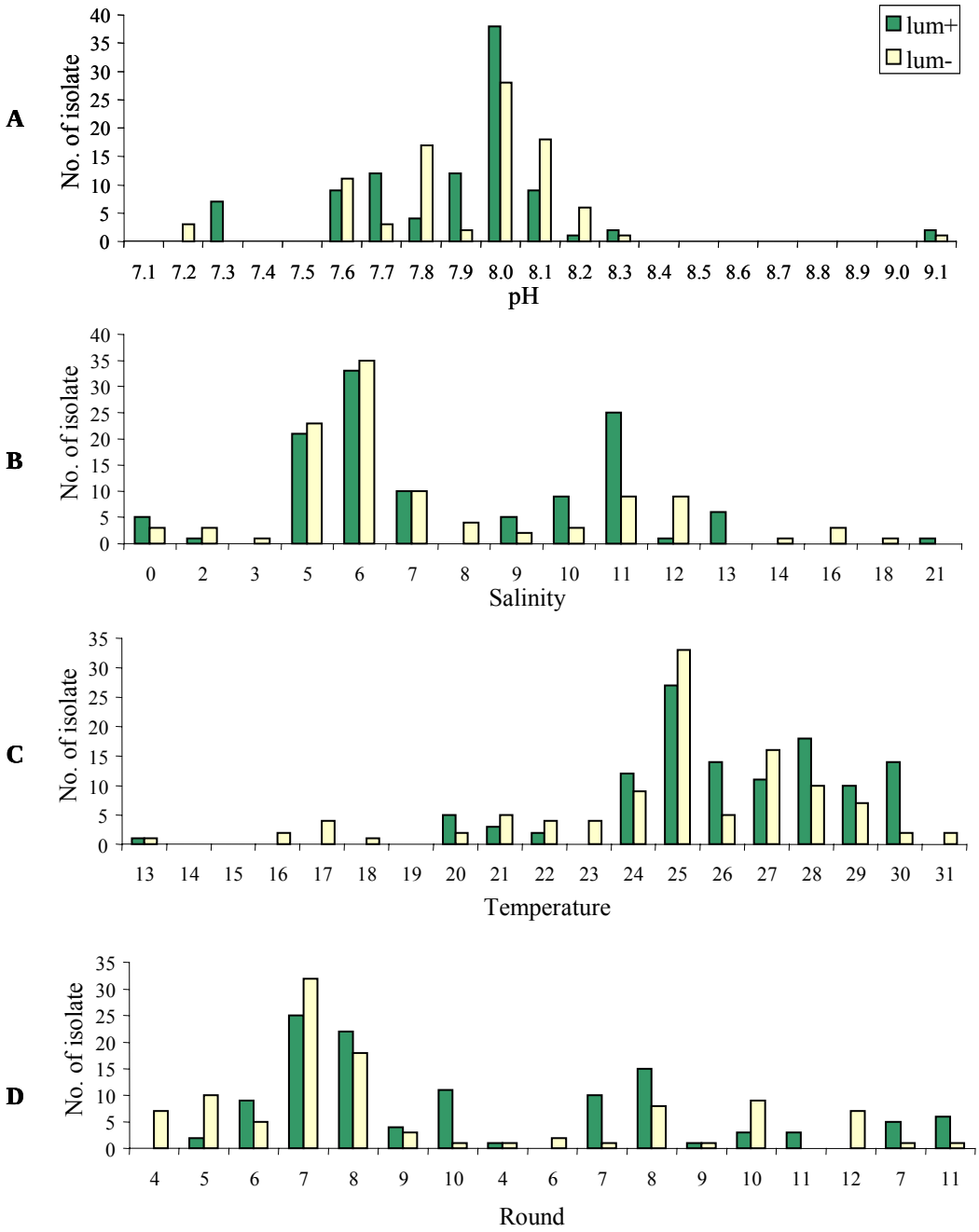


Figure 2.4. Comparison of luminescent (Lum+) and non-luminescent (Lum-) isolates with pH (A), salinity (B), temperature (C), and sampling time of year (D). For D, numbers on the X-axis indicate round of sampling (15 rounds/year) in each year, from 1998 to 1999, with mid-Bay transect samples at the far right.

isolation (round of sampling). A higher percentage of luminescent strains was achieved when the salinity was 9-11 ppt (Figure 2.4B) and a temperature of 28-30°C (Figure 2.4C), while significant correlation with pH or range was not observed (Figure 2.4A). In round 10, collected in 1998, and round 7, collected in 1999, corresponding to the first half of August and the second half of June, respectively, a significantly higher ratio of luminescent isolates was obtained (Figure 2.4D).

With regard to sample size fraction (water, or fraction < 20 µm; phytoplankton, or fraction > 20 µm and less than 64 µm; and zooplankton, or fraction > 64 µm), luminescent isolates were obtained from all three size fractions (Figure 2.5). Table 2.3 shows percent of

Table 2.3. Percent positive luminescent *V. cholerae* in three different sample size fractions collected from Chesapeake Bay (n=224).

	Percent positive (number of isolates) for each sample fraction		
Traits/Fraction	W[a] (n=104)	P[b] (n=65)	Z[c] (n=55)
Lum	55.8 (58)	47.7 (31)	49.1 (27)
luxA	58.7 (61)	53.8 (35)	61.8 (34)

[a]W = water, or size fraction <20 µm
[b]P = phytoplankton, or size fraction >20 µm and <64 µm
[c]Z = zooplankton, or size fraction >64 µm

V. cholerae isolates that were luminescent for each size fraction over the entire study. When the overall ratio, as well as, number of luminescent strains in each sample was considered, the smallest size fraction or water fraction showed greater likelihood of containing luminescent strains than of the larger size fractions.

2.3.8 Temporal and spatial distribution

The hypothesis that a certain habitat preferentially yields isolates with specific traits (in this case, luminescence) was addressed by log-linear analysis. In our study, four temporal-spatial factors determined the "habitat" for a particular water sample: SITE and FRACTION, for spatial and size separation, respectively, and YEAR and ROUND for annual and seasonal variation, respectively.

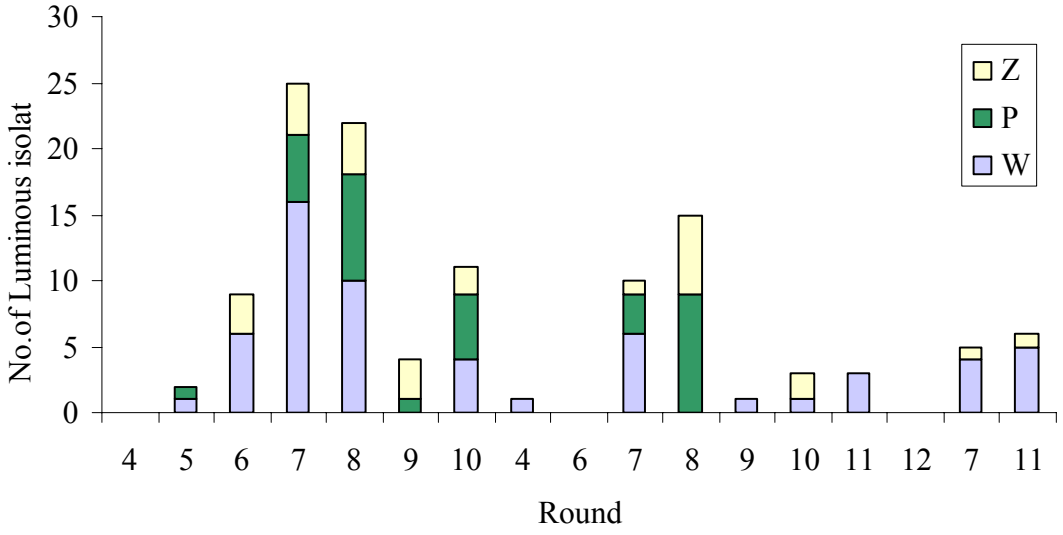


Figure 2.5. Composition of luminescent population in each sample size fraction (<20 m, (W), > 20 m and < 64 m, (P); and > 64 m, (Z)). X-axis indicates round of sampling (15 rounds/year) from 1998 to 1999, with mid-Bay transect samples at far right.

To conserve the power of analysis for association of a trait with habitat, confounding among these four factors had to be removed. By selecting a subset of 145 isolates, obtained in 1998 from three shore sites (H, S, K), the effect of isolation method, annual variation in the habitat, and sites yielding fewer than 10 isolates, could be removed. In addition, isolates from sample collections yielding only a single isolate were removed. Effective habitat

factors remaining for the subset included three sample size fractions (W, P, Z), three sampling sites (H, S, K), and seven sampling rounds (4-10). See Table 2.4 for results of the analysis. Lum and *luxA* showed significant association with season (ROUND). Lum also

Table 2.4. Significance of association between habitat and *V. cholerae* luminescence (Group II) by log-linear regression.

		Saturated Model[a]			Best Model[b]						
		Tests of partial association (P) with			Significance (P) of interaction with[c]						
	Level s	RD.	SITE	FRAC.	RD.	SITE	FRAC.	RD.* FRAC.	RD.* SITE	SITE* FRAC.	Goodness-of=fit Test (P)
luxA	2	<0.001	0.010	0.200	<0.001	0.001					0.26
Lum	2	<0.001	0.127	0.038				0.047			0.67

a frequency was regressed by ROUND, SITE, FRACTION, the trait in each row of the table and interaction among the four variables.

b Backward elimination (P = 0.05)

c asterik (*) means the interaction term by the factors at the left and right of it. Only significant values (P<0.05) are shown while blank means insignificant.

showed significant association with FRACTION. Lum association with the different size fractions was, however, dependent on season (ROUND), because the best model obtained was by ROUND*FRACTION*Lum interaction not by FRACTION*Lum interaction. In the case of *luxA*, there was significant correlation with location of sampling (SITE). Taken together, certain sites yielded isolates possessing *luxA*, while luminescent isolates were associated with certain size fractions.

2.4 Discussion

West et al. (1986) identified 10% of *V. cholerae* isolates as luminescent by visualization of agar plates in the dark. When several of the "non-luminescent" strains from

this study were re-evaluated by Palmer and Colwell (1991), an additional 21% were found to be dimly luminescent. Ramaiah et al. (2000) screened 7,801 environmental isolates from the Chesapeake Bay for luminescence by the visual examination method. All were non-luminescent; however, 136 of the 7,801 (1.7%) isolates hybridized with a *luxA* probe designed from a *V. harveyi* sequence. These results indicate that dark-adjusted visualization of colonies on agar plates is not a reliable method for detection of luminescent bacteria.

In this study, we developed a method for determining expression of bioluminescence by *Vibrio cholerae,* using broth cultures. Five media were tested and Marine broth 2216 was found to be superior in stimulating luminescence (Figures 2.2A-E). In all cases, it allowed greater expression of luminescence than the other four media. When 47 of 62 previously tested strains of *V. cholerae* (Palmer and Colwell, 1991) were re-examined using our bioassay, we found that approximately half of the luminescent strains were not detected when LB, instead of MB, was used as the culture medium (Table 2.1). In addition, the level of luminescence expression in LB was greatly reduced, compared to MB.

Not surprisingly, we found that luminescence expression by the reference strains (*V. cholerae* UM4102, UM4086, and UM4057) was significantly stimulated by aeration. By varying broth culture volume in 150-ml flasks and 16 x 125 mm test tubes, we found maximal expression of luminescence when the air to sample ratio was 5:1 (tubes) - 6:1 (flask). Interestingly, luminescence expression decreased at ratios promoting the highest growth rate (15:1 and 10:1). Bacterial growth increased with increasing air to sample ratio, over all of the ratios tested.

In Figure 2.2, it can be observed that late-exponential phase broth cultures are preferred for assaying luminescence. Four of the five strains (*V. cholerae* UM4057, 4086,

4089, and 4102) showed peak luminescence at 4-6 hr post dilution (Figure 2.2A-D). For *V. cholerae* UM4157 (Figure 2.2E), luminescence expression peaked at 8 hr post dilution.

Two previous studies (Palmer and Colwell, 1991; Bassler et al., 1997) employed liquid scintillation counting. The luminometer used in this study gave a positive value range of 20 to 2,000,000, with a background of 20 (mean luminometer reading of 25 or higher was considered positive). From Table 2.1, it can be seen that the sensitivity of the luminometer is much greater than liquid scintillation, for which the positive value range was 6.2, with a minimum value of 5.6 (Palmer and Colwell, 1991).

The luminescence bioassay developed in this study was proven to be more sensitive and specific with 13/47 strains of Group I found to emit light. According to *luxA* PCR verification, the 5 additional strains identified as luminescent in the previous study (Palmer and Colwell, 1991) are concluded to be false positives, attributable to differences in growth medium and instrumentation.

In this study, greater occurrence of luminescent *V. cholerae* in the estuarine environment was observed (Group II). Previously, the occurrence of luminescent *V. cholerae* was reported to be 10% (West et al., 1986). The higher incidence of luminescent *V. cholerae* in Chesapeake Bay observed in this study suggests environmental selection for luminescence. Also, sensitivity of the detection assay is significantly greater. Finally, the strains assembled for assay were selected differently. For Group II (and V), environmental samples were collected throughout the year at regular intervals to isolate all *V. cholerae* present in the samples, namely a census-style survey of *V. cholerae* populations for this environment. In contrast, the 47 strains of Group I include 27 isolates from Louisiana, 6

from Chesapeake Bay, 12 from Tilamook Bay, OR, 1 from FL, and 1 from England; a geographically diverse set of strains.

Luminescence and the *lux* operon were not detected in the *Vibrio cholerae* O1 and O139 strains examined in this study, which included clinical strains from geographically diverse sources (Groups III, IV), and environmental strains (Group V) of *V. cholerae* O1 and O139. In addition, a lower incidence of luminescent *V. cholerae* isolates from a cholera endemic area (Bangladesh, Group V, 6.3%) was observed, as opposed to a cholera non-endemic area (Chesapeake Bay, Group II, 52%).

Verification of luminescence for Group I by *luxA* screening (PCR) revealed that some strains of *V. cholerae* possess *luxA*, yet are not luminescent. Analysis of the 47 strains examined by Palmer and Colwell (1991) showed that 8.5% (4/47) were non-luminescent, yet testing positive for presence of *luxA*. This phenomenon was observed for Groups II (6.3 %) and V (4.2%), as well, suggesting altered or defective regulation or naturally occurring K variants. The $luxA^{+}$/Lum^{-} strains were non-luminescent, even after retesting. Various formulations of Marine broth 2216, as well as several inducers, were tested for stimulation of luminescence for these strains, but were unsuccessful (results not shown). As successful as the bioassay has been shown to be, it is not sufficient to detect luminescence in all lux^{+} strains.

Analysis of luminescent *V. cholerae* strains from Group II, with respect to environmental parameters and phenotypic and genotypic traits (Table 2.2), revealed significant correlation of luminescence and *ompU, stn, ompW*, *toxR,* and temperature of the water at time of sampling. Results of the McNemar and marginal homogeneity tests for *stn*

and *ompU* are explained by examining frequency distribution of the traits (Tables 2.5 and 2.6, respectively). For correlation of luminescence with *stn*, Table 2.5 shows many isolates

Table 2.5. Frequency distribution of luminescence (Lum, *luxA*) and heat-stable enterotoxin (*stn*) for *V. cholerae* isolated from Chesapeake Bay.

		stn	
		-	+
luxA	-	90	4
	+	88	42
Lum	-	104	4
	+	74	42

were luminescent (Lum, +; *luxA*, +), but did not possess the enterotoxin gene (stn^-). However, except for four isolates, strains possessing the enterotoxin gene (stn^+) were luminescent (91%, or 42/46). The frequency distribution of *ompU* and luminescence, shown in Table 2.6, suggests a strong preference of luminescent isolates with high *ompU* probe homology (*ompU*, ++++). This was weaker for non-luminescent strains, which leads to the conclusion of a heterogeneous population.

Statistically, luminescent *V. cholerae* were distributed throughout the year in the Chesapeake Bay, although significantly higher numbers of strains were isolated in the late summer (July and August) when the water temperature was higher (Figure 2.5). Luminescent *V. cholerae* occurred in all three sample size fractions over the entire study period, with slightly greater occurrence in the smallest size fraction (Table 2.3). If the two

Table 2.6. Frequency distribution of luminescence (Lum, *luxA*) and outer membrane protein U (*ompU* of *V. cholerae* N16961) by dot-blot hybridization for *V. cholerae* isolated from Chesapeake Bay.

		*ompU**				
		-	+	++	+++	++++
luxA	-	0	2	25	26	41
	+	3	0	3	17	107
Lum	-	0	2	25	30	51
	+	3	0	3	13	97

*response level (+s) determined by densitometer

larger size fractions are combined, the overall incidence of these strains becomes approximately 1:1. Log-linear analysis revealed a more complex distribution pattern. That is, for the year 1998, luminescent isolates are rare early in the year, before summer, (<10% in all fractions) (Figure 2.5). The proportion of luminescent isolates increased in early summer, notably in the smallest size fraction. This changed to the larger size fraction (plankton), in late summer. An increase in the proportion of luminescent isolates in the largest size fraction (most likely zooplankton) in round 9 of 1998 was observed; however this may not be a valid conclusion since only 7 isolates were obtained from this round.

The generally high incidence of luminescence in non-symbiotic, environmental *V. cholerae* isolates, Group II in Chesapeake Bay, indicates a selective advantage for this phenotype in this environment. Czyz *et al*. (2003) reported that *lux*$^-$ strains become dominant over *lux*$^+$ strains of *V. harveyi* under normal growth conditions, in laboratory experiments. Conversely, *lux*$^+$ strains of *V. harveyi* became dominant over *lux*$^-$ strains in cultures exposed to stress and damage incurred from low levels of UV irradiation (Czyz *et*

al., 2003). It has been proposed that expression of the luciferase gene may facilitate bacterial DNA repair by providing an internal light source for photoreactivation, the photo-mediated repair mechanism (Cryz et al., 2003; Cryz et al., 2000; Szpilewska et al., 2003). Thus, a free-living bacterium in the aquatic habitat, exposed to DNA damage via UV exposure from increased sunlight, may use luminescence as a protective mechanism. When attached to larger particles, such as detritus or plankton, the bacterium may be afforded some protection from UV damage, so more than one selective mechanism may occur in the estuarine and marine environment.

Chapter 3 COLONY BLOT HYBRIDIZATION OF TOTAL *VIBRIO CHOLERAE* POPULATIONS

3.1 Introduction

Vibrio cholerae is an autochthonous marine and estuarine bacterium, some strains of which produce an enterotoxin and are capable of causing the epidemic diarrheal disease cholera. Strains of *V. cholerae* are classified by their O antigen serogroup, with over 210 serogroups recognized to date. The seven pandemics of cholera that have occurred throughout recorded human history were caused by serogroup O1 and epidemics more recently by O139. However, toxigenic non-O1/non-O139 *V. cholerae* have caused outbreaks of cholera and, conversely, strains of *V. cholerae* O1 that do not produce cholera toxin or possess the cholera toxin genes have been isolated. The genes responsible for cholera toxin, *ctxAB*, and other pathogenic factors have been shown to reside in a mobile genetic element of phage origin, designated CTXΦ (Waldor and Mekalanos, 1996), which can be transferred from pathogenic strains to non-pathogenic strains that possess the toxin co-regulated pilus (TCP). The cholera toxin (CTX) prophage was recently discovered in clinical strains of *V. mimicus* and it has been proposed that *V. mimicus* is a natural reservoir for CTXΦ (Boyd et al., 2000). It is becoming clear that *V. cholerae* populations in endemic areas may be more dynamic than previously thought.

Detection of *V. cholerae* present in natural waters relies mostly on an isolation method originally developed for clinical diagnosis, namely enrichment in alkaline peptone water, followed by subculture on selective media, and confirmation using selected biochemical and immunological tests. The alkaline nature of the enrichment broth allows differential multiplication of *Vibrio* species. The selective media, thiosulfate citrate bile

sucrose agar or vibrio selective agar (TCBS) and taurocholate-tellurite-gelatin agar (TTGA), offer some advantage to growth of *Vibrio* species and aid in the presumptive identification of *V. cholerae*. Identification of presumptive colonies is confirmed by biochemical tests designed to differentiate *V. cholerae* from other species of *Vibrionaceae* and *Enterobacteriaceae*. Confirmed *V. cholerae* strains are then serogrouped as O1 or O139 by simple slide agglutination with antisera. This method is appropriate for detection but not enumeration, because of the artificial multiplication step (APW enrichment).

Recently, PCR methods and oligonucleotide hybridization have shown promise in detecting and enumerating toxigenic *V. cholerae* (Koch et al., 1993; Wright et al., 1992). These methods rely on amplification of, or hybridization to, *ctx* DNA sequence. As discussed above, many environmental *V. cholerae* O1 strains isolated from endemic areas do not harbor the *ctx* genes (Kaper et al., 1991) and it has been shown that CTXΦ is capable of lysogenic conversion of strains that are $CTX\Phi^-$ (Waldor and Mekalanos, 1996). Enumeration based on *ctx* genes will not detect nontoxigenic *V. cholerae* that may be converted to CTX^+ by phage infection. Additionally, *V. mimicus*, which has high sequence homology for *ctx* in its gene encoding for cholera toxin-like toxin, can cross-react with many *ctx* probes.

These problems underline the need for a method of detection of the total number of *V. cholerae* present in a sample, by targeting a conserved region in the *V. cholerae* genome that is present, regardless of serogroup. The 16S rRNA is an appropriate target; however, these sequences from *V. cholerae* and *V. mimicus* differ by only 6 of 1456 nucleotides (Ruimy et al., 1994), evidence considered by some investigators to indicate that *V. cholerae* and *V. mimicus* are the same species. PCR primers specific for *V. cholerae* based on the intergenic spacer region (ISR) between the 16S and 23S rRNA genes have been reported

(Chun et al. 1999). For this primer pair, the left primer imparts differentiation between *V. cholerae* and *V. mimicus,* while the right primer imparts species specificity for *V. cholerae/mimicus* compared with other *Vibrio* sp.

For use in colony blot hybridization for detection and enumeration of total *V. cholerae,* an oligonucleotide probe, pVCITS, based on the sequence of one of the ISR primers above, was developed. The sequence of the left primer, pVC-F, was shortened to 18-bp, by removing the first 4 nucleotides, and modified at one base (#9, R to G), to produce the probe. A second probe, Vchomim1276, previously described by Heidelberg (1997), was also employed in a colony blot hybridization protocol targeting rRNA. The specificity of the two probes was tested using laboratory reference strains, as well as numerous environmental and clinical isolates. The sensitivity of the probes was evaluated by colony blot hybridization of seawater and natural water samples to which *V. cholerae* and/or other *Vibrio* spp. were added.

3.2 Materials and Methods

Two colony blot hybridization methods were developed and evaluated. The first method involved the use of a radiolabeled oligonucleotide probe specific for total *V. cholerae*, based on 16S – 23S intergenic spacer region, to detect DNA immobilized onto membranes. The second method, developed as an alternative to methods involving radioisotopes, employs a fluorescently labeled *V. cholerae* and *V. mimicus*-specific 16S rDNA probe to detect RNA immobilized onto nylon membranes.

3.2.1 Bacterial strains and media

Bacterial strains used in this study are listed in Table 3.1 (DNA colony blot hybridization) and Table 3.2 (RNA colony blot hybridization). Strains used included *V.*

Table 3.1. Bacterial strains used to develop and evaluate specificity of the DNA colony blot hybridization method using probe pVCITS.

Strain	Hybridization
V. cholerae O1 classical ATCC14035	+
V. cholerae O1 classical ATCC11623	+
V. cholerae O1 El Tor Inaba ATCC39315	+
V. cholerae non-O1/non-O139; Ches. Bay, MD (10)	+
V. cholerae O1 El Tor Ogawa; Bangladesh (33)	+
V. cholerae O1 El Tor Inaba; Bangladesh (9)	+
V. cholerae O139; Bangladesh (5)	+
V. cholerae non-O1/non-O139; Bangladesh (4)	+
V. cholerae non-O1/non-O139; Ecuador shrimp farm	+
V. cholerae O139 (17)	+
V. cholerae O1 El Tor Inaba (12)	+
V. cholerae O1 (20)	+
V. cholerae O1 El Tor Ogawa (6)	+
V. cholerae non-O1/non-O139; Mexico (45)	+
V. cholerae non-O1/non-O139; Louisiana (17)	+
V. cholerae non-O1/non-O139; Oregon (8)	+
V. cholerae non-O1/non-O139; Florida (9)	+
V. cholerae non-O1/non-O139; England	+
V. cholerae non-O1/non-O139; human gastroenteritis (2)	+
V. mimicus ATCC33653	-
V. mimicus (6)	-
V. vulnificus ATCC33816	-
V. vulnificus ATCC27562	-
V. aestuariamus ATCC35048	-
V. alginolyticus ATCC17749	-
V. fluvialis ATCC 33809	-
V. furnissii ATCC35016	-
V. proteolyticus ATCC15338	-
V. diazotrophicus ATCC33466	-
V. pelagius ATCC25916	-
V. campbellii ATCC25920	-
V. anguillarum ATCC19264	-
V. carchariae ATCC35084	-
V. tubiashii ATCC19109	-
V. orientalis ATCC33934	-
V. nigropulchritudo ATCC27043	-
V. costicola ATCC33508	-
V. fischeri ATCC7744	-
V. natriegens ATCC14048	-
V. salmonicida ATCC43839	-
V. nereis ATCC25917	-
V. splendidus ATCC33125	-
V. hollisae ATCC33564	-
V. damsela	-
V. carchariae	-
Aeromonas hydrophila	-
Aeromonas jandaei	-
Aeromonas spp. (3)	-
Escherichia coli (4)	-
Klebsiella pneumoniae ATCC13883	-

Table 3.2. Bacterial strains used to develop and evaluate specificity of the RNA colony blot hybridization method using probe Vchomim1276.

Strain	Hybridization
V. cholerae O1 classical ATCC14035	+
V. cholerae O139 AI1877	+
V. cholerae O1 El Tor N16961	+
V. cholerae non-O1/non-O139 CB98-203	+
V. cholerae non-O1/non-O139 CB99-18	+
V. mimicus ATCC33563	+
V. mimicus UM4198	+
A. caviae ATCC15468	-
E. coli K12	-
V. vulnificus ATCC27562	-
V. anguillarum ATCC19264	-
V. hollisae ATCC33564	-*
V. fluvialis CB99-14	-

*weak cross-reactivity

cholerae O1 Classical, O1 El Tor, O139 and non-O1/non-O139 collected from clinical and environmental sources in the United States, Mexico, Bangladesh, England and Ecuador. Three *V. cholerae* and 23 non-*V. cholerae* species strains obtained from the American Type Culture Collection (Manassas, VA) were also included in this study. Unless specified, all strains were grown on LB-30 agar, comprising LB amended with NaCl to a final concentration of 3%. The bacterial cultures were maintained at -80°C in LB broth to which 25% glycerol had been added and on LB-30 agar slants at room temperature.

3.2.2 *V. cholerae*-specific probes

The intergenic spacer region, ISR, from the 3' end of the 16S rRNA to the 5' end of the 23S rRNA genes, of *Vibrio cholerae* O1 classical (ATCC 14035) had been previously amplified and sequenced (Chun et al., 1999). A stretch of 18 nucleotides in this region was found to be conserved within *V. cholerae,* but variable among other *Vibrio* and related

species, especially the closely related *V. mimicus*. This sequence, GCS TTT TCG CTG AGA ATG, was designated pVCITS. For use in traditional DNA colony blot hybridization, pVCITS (5 μM, final concentration) was radiolabeled with 80 μCi ^{32}P-ATP using T4 kinase. Alternatively, a previously described 16S rDNA probe, Vchomim1276, specific for *V. cholerae* and *V. mimicus* (Heidelberg, 1997), was used for RNA colony blots. This probe, ACT TTG TGA GAT TCG CTC CAC CTC G, was 5' end-labeled with either fluorescein or Cy3 at the time of manufacture (Sigma-Genosys).

3.2.3 *V. cholerae* colony blot lift

Strains were inoculated onto LB-30 agar and grown overnight at room temperature to a colony size no larger than 5 mm in diameter. Four methods of colony blot lift were used in this study and are numbered for clarity. Methods 1 and 2 are traditional DNA colony blot preparations and Methods 3 and 4 are previously reported methods for RNA colony blot preparation. Method 1 was used to prepare DNA colony blots of control strains for evaluation of radiolabeled pVCITS, using Whatman 541 filter paper. For evaluation of fluorescently labeled probe Vchomim1276, colonies were grown as above and 85-mm nylon (MagnaLift, GE Osmonics) and/or nitrocellulose (NitroPure, GE Osmonics) membranes were overlaid for 30 minutes. When necessary, the colony blot was replicated. The nucleic acid was liberated, denatured, and immobilized to the membrane using Method 2-4. Because of its simplicity, Method 1 was also evaluated using probe Vchomim1276.

3.2.4 Colony blot lift Method 1 (Wright *et al.*, 1992)

Whatman 541 filters (85mm) were overlaid on the plates for 1 hr. Filters were transferred, colony side up, onto alkaline buffer (1.5M NaCl, 0.5M NaOH) soaked blotting paper and microwaved for 1-2 minutes. Filters were then neutralized in 2M ammonium

acetate for 5 minutes and washed twice in 1X SSC/ p.H.7.0 for 10 minutes and allowed to air dry. Blots were stored at -20°C until hybridization.

3.2.5 Colony blot lift Method 2 (GE Osmonics)

Membranes were laid, colony-side up, onto Whatman #1 filter paper saturated with 0.5 N NaOH for 10 minutes at room temperature to lyse the cells and denature the nucleic acid. The membranes were transferred to fresh filter paper to absorb excess moisture. The DNA was immobilized onto the membranes by baking at 80°C (nitrocellulose) or UV crosslinking (nylon) using a model FB-UVXL-1000 UV crosslinker (FisherBiotech, Pittsburgh, PA). The membranes were neutralized in a pool of 0.5 M Tris-HCl/pH 8.0, 0.5 M NaCl for 2 minutes and transferred to fresh filter paper to absorb excess moisture. The membranes were rinsed in 2X SSC and air dried.

3.2.6 Colony blot lift Method 3 (Kaufmann et al., 1997)

Colonies were lysed by incubating the membranes on 3MM filter paper (Whatman) saturated with 10 mM Tris-HCl/pH 7.5, 250 mM sucrose, 5 mg/ml lysozyme, for 1 hour at 37°C. The nucleic acid was denatured by incubating the membranes on filter paper saturated with 1 M NaCl, neutralizing on filter paper saturated with 1 M Tris-HCl/pH 8.0, and washing on filter paper saturated with 2X SSC. Each step was run for four minutes at room temperature. DNA was fixed to the membranes by baking at 80°C for 10 minutes followed by UV cross-linking.

3.2.7 Colony blot lift Method 4 (Ivanov and Gigova, 1986)

Membranes were incubated on filter paper, pre-wetted, but not saturated, with 10% SDS to lyse the colonies. The membranes were transferred to filter paper pre-wetted with 3X SSC and incubated at 65°C for 15 minutes to denature the nucleic acid. The membranes

were dried at 37°C for 10 minutes and the nucleic acid was fixed to the membrane by baking at 70°C for 15 minutes. For hybridization involving the fluorescently labeled Vchomim1276 probe, Methods 1 - 4 were evaluated.

3.2.8 DNA colony blot hybridization

Colony blots prepared by Method 1 were washed in 3X SSC, 0.1% SDS, three times for 10 minutes each, at room temperature, and once at 65°C for 2 hrs, to remove cellular debris that could interfere with the hybridization. Blot preparations were pre-hybridized for 1 hr at 37°C in 6X SSC containing 5X Denhardt's solution, 0.05% sodium pyrophosphate, 100 μg/ml boiled salmon sperm DNA, and 0.5% SDS. Blots were hybridized overnight at 42°C in 10 ml of 6X SSC, 1X Denhardt's solution, and 0.05% sodium pyrophosphate, with 40 μl of ^{32}P-labelled pVCITS (5 μm) probe. Blots were washed three times, for 15 min each, with 6X SSC, 0.05% sodium pyrophosphate. Blots were stringently washed for 30 min at 55°C, sealed in plastic bags, and visualized by autoradiography or phosphorimaging. Autoradiography was performed overnight, at -80°C, on Biomax (Kodak) X-ray film. Phosphorimaging was performed for two hours at room temperature and the storage phosphor screen was scanned using a Storm PhosphorImager (GE Healthcare).

3.2.9 RNase treatment

Solutions used for RNA colony blot lift and hybridization were treated with diethyl pyrocarbonate (DEPC) to eliminate RNase contamination. Briefly, DEPC was added to solutions at a final concentration of 0.1% (vol/vol), stirred thoroughly to bring the chemical into solution, and incubated at 37C for 12-16 hours (overnight). The treated solution was autoclaved (15 minutes at 121 psi) to deactivate DEPC.

3.2.10 Methylene blue staining

Methylene blue was used to qualitatively examine RNA and/or DNA immobilized onto membranes. The method allows qualitative detection of RNA (or DNA) at concentrations above 100 ng and provides a safe alternative to ethidium bromide staining. Membranes were soaked in methylene blue solution, 0.025% (wt/vol) methylene blue in distilled water, for 10 minutes, after which the membranes were washed in distilled water three times, for two minutes each. Membranes were destained in 70% ethanol (two times, 10 minutes each), and washed in distilled water. This protocol was used to determine the quality of the colony blot preparations (Methods 1-4) for use with probe Vchomim1276.

3.2.11 RNA colony blot hybridization

Membranes were pre-washed in 3X SSC, 0.1% SDS, as described above for DNA colony blot hybridization. Membranes prepared by Methods 1-4 were prehybridized at two temperatures, 35°C and 60°C, for 30 minutes in hybrization solution base (10 ml per 100 cm^2 membrane), 0.9 M NaCl, 50 mM sodium phosphate/pH 7.0, 5 mM EDTA, 0.5% SDS in DEPC-treated water. Hybridizations were performed overnight (approximately 16 hours), using hybridization solution base (10 ml per 100 cm^2 membrane) containing fluorescently labeled Vchomim1276 probe (80 ng/ml). Two blocking agents, salmon sperm (SS) DNA (0.5 mg/ml, denatured by boiling for 5 minutes followed by rapidly cooling on ice) and Denhardt's solution (10X), were evaluated for ability to improve signal to noise ratio and reduce non-specific binding. After hybridization, membranes were washed in washing solution, 1% SDS, 1X SSC in DEPC-treated water, at hybridization temperature for 30 minutes. Membranes were wrapped in plastic wrap or placed in a hybrization bag and visualized using a Typhoon (GE Healthcare, Piscataway, NJ) variable mode imager. Colony

blots hybridized with Cy3-labeled Vchomim1276 were excited using a green laser at 532 nm and detected using an emission filter at 580 nm with a band pass of 30. For fluorescein-labeled probe hybridization, excitation was identical to Cy3, but the detection was done using a single pass filter at 526 nm. The PMT setting ranged from 400 to 475, as necessary. As an alternative, colony blots hybridized with Cy3- or fluorescein-labeled Vchomim1276 were visualized with a Dark Reader hand lamp (Clare Chemical).

3.2.12 Spiking experiments

Several experiments were done whereby natural water samples were amended with laboratory cultures of *V. cholerae*, to evaluate the *V. cholerae* colony blot hybridization protocols.

pVCITS evalutation

To test probe pVCITS, two 500 ml water samples collected from each of three sites in the Chesapeake Bay (**H**orn Point Environmental Research Laboratory, **K**ent Island, and **S**mithsonian Environmental Research Center, Edgewater, MD; see Figure 2.1) were spiked with *V. cholerae* non-O1/non-O139 strain TMA21, originally isolated from seawater from Brazil, to obtain *V. cholerae* concentrations of approximately 1 and 0.1 cell per ml of sample. One-hundred and fifty ml aliquots were filtered onto 0.2 μm pore size, 85mm Magna Lift (GE Osmonics) nylon membranes. The membranes were placed onto three different media: marine agar (Difco), TCBS agar (Oxoid) and 1% alkaline peptone agar/pH 8.6 to determine optimal recovery. Plates were incubated for 1-2 days at room temperature. For negative controls, Chesapeake Bay water without *V. cholerae* added was tested as above. For a positive control, Chesapeake Bay water was first filtered (0.22 μm) to remove bacteria and large particles, after which *V. cholerae* was added and the method tested. Colony blot lifts

were prepared following manufacturer's instructions (GE Osmnics, Method 2 above). Membranes were hybridized with ^{32}P-labeled pVCITS in a 5X SSPE solution containing 50% formamide, 0.1%SDS, and 2X Denhardt's solution, at 42°C. The membranes were washed under medium stringency conditions, 30 minutes at 65°C in 2X SSC, 0.5% SDS. Autoradiography was performed at -80°C for 2 or more days. The experiment was replicated 5 times.

Vchomim1276 evaluation

To test probe Vchomim1276, aliquots from a fresh (Lake Artemesia, MD) and estuarine (Baltimore Harbor, MD) water samples were amended with luminescent *V. cholerae* non-O1/non-O139 strain CB99-18 that had been isolated from Chesapeake Bay, MD (Louis et al., 2003). Appropriate dilutions were made to obtain approximately 150 total colonies, including approximately 40 *V. cholerae* colonies, per spread plate using LB-30 agar and heterotrophic plate count agar. Plates were incubated for 1-2 days at room temperature, after which RNA colony blots were prepared using Method 4 above. Membranes were hybridized with Cy3-labeled Vchomim1276.

3.2.13 Field trial evaluation

pVCITS

The probe, pVCITS, and corresponding DNA colony blot hybridization method were tested using water samples from a cholera non-endemic area, Chesapeake Bay, MD, and a cholera-endemic area, Bangladesh, to evaluate performance with environmental samples. A two year study was conducted to monitor *V. cholerae* population in Chesapeake Bay, MD at four sites, in which the primary means of isolation was APW enrichment (Louis et al., 2003). In an effort to improve isolation success for *V. cholerae* and to evaluate performance, the

DNA colony blot hybridization method using probe, pVCITS, was employed, in parallel with the enrichment method. A total of 14 samples were analyzed (Choopun, 2004). Additionally, colony blots of water samples collected in urban and rural provinces from Bangladesh during the months of July and August were prepared and hybridized with probe pVCITS at the University of Maryland

Vchomim1276

The probe, Vchomim1276, and the RNA colony blot hybridization protocol (Method 4) were evaluated with water samples collected from a cholera-endemic area, Bangladesh. RNA colony blot lifts from spread plates of water samples collected from 14 sites over two widely separated geographical areas, Mathbaria and Bakerganj provinces, Bangladesh were prepared and hybridized using the probe, Vchomim1276. A total of 40 samples were tested. For water samples from Bangladesh, the colony blots were prepared in Bangladesh and the hybridizations were performed at the University of Maryland.

3.3 Results

3.3.1 DNA colony blot hybridization

The DNA colony blot hybridization protocol was developed to isolate and enumerate *V. cholerae* in natural water samples. A total of 11 laboratory reference strains of *Vibrio cholerae* O1, O139 and non-O1/non-O139, eight *V. mimicus* (clinical and environmental), three *Aeromonas* spp., two *Vibrio vulnificus*, and one *Klebsiella pneumoniae* strain were used to optimize the DNA colony blot hybridization protocol (Method 1) employing the oligonucleotide probe, pVCITS. The theoretical T_m of the probe was determined to be 43°C (Gibco BRL). Combinations of three hybridization temperatures, 39°C, 42°C, and 45°C, and two stringent wash temperatures, 55°C and 60°C were tested. The optimal hybridization

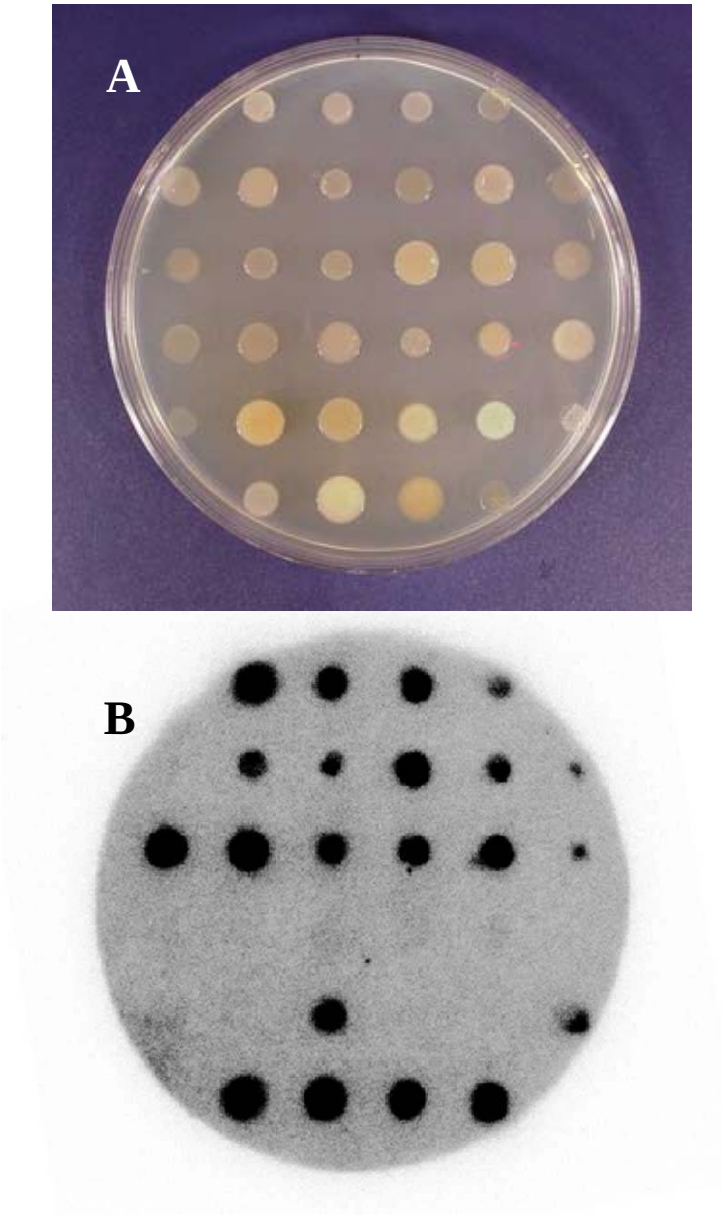


Figure 3.1. Specificity of probe pVCITS, using DNA colony blot lift method 1, Whatman 541 filter paper overlay (Wright et al., 1992; 1993). A. LB plate prior to filter overlay. B. Autoradiograph of 541 colony blot lift after hybridization with pVCITS. Colony key: Row 1 (from left to right): Chesapeake Bay *V. cholerae* isolates; CB98-5, -2, -11, -14; Row 2: *A. jandaei*, *V. cholerae* ATCC14035, *V. cholerae* ATCC11623, *V. cholerae* AI1877 O139, *V. cholerae* O139, *V. cholerae*; Row 3: *V. cholerae* O1, *V. cholerae* O1 CVD15, *V. cholerae* O139 (2), *V. cholerae* N16961, *V. cholerae* O1; Row 4: *V. mimicus* (6); Row 5: *V. mimicus* (2), *V. cholerae* non-O1/non-O139, *V. vulnificus* (2), Bangladesh *V. cholerae* isolate 20-01; Row 6: Bangladesh *V. cholerae* isolates: 20-08, -27, -40, -61.

temperature for the probe, using the above reference strains and colony blot lift Method 1, was 42°C, and optimal stringent wash temperature was 55°C for 30 minutes.

3.3.2 Specificity of pVCITS

With optimum conditions determined for DNA colony blot hybridization using pVCITS, several laboratory collection strains were used to determine specificity of the probe under these conditions (Figure 3.1). pVCITS hybridized with all strains of *V. cholerae*, including non-O1/non-O139 (97/97), *V. cholerae* O1 (83/83), and *V. cholerae* O139 (22/22) (Table 3.1). The probe was tested against several *Vibrio* spp., other than *V. cholerae*, as well as related Gram-negative bacteria. pVCITS did not hybridize with DNA from 31 of the non-*V. cholerae Vibrio* or with DNA from 10 related non-*Vibrio* strains (Table 3.1).

3.3.3 Sensitivity of pVCITS

To determine sensitivity of the DNA colony hybridization protocol, using radiolabeled pVCITS, experiments were done using water samples collected from Chesapeake Bay, MD. To simulate conditions in which *V. cholerae* would be present in low numbers, a DNA colony blot preparation incorporating concentration by filtration step was used. Figures 3.2 and 3.3 show typical results of experiments whereby *V. cholerae* was added. From the results, it was concluded that inhibitors in natural water interfering with colony blot hybridization would not be a problem (Figure 3.2F, 3.3C, 3.3F). However, the presence of competing naturally occurring bacteria affected the hybridization signal significantly (Figure 3.3B and 3.3E). Only colony blot lifts incubated on TCBS gave clear positive signals (Figure 3.2E).

In addition to sensitivity of probe pVCITS, media used for the filtration colony blot were also tested. Results shown in Figures 3.2 and 3.3, TCBS was found to be superior to

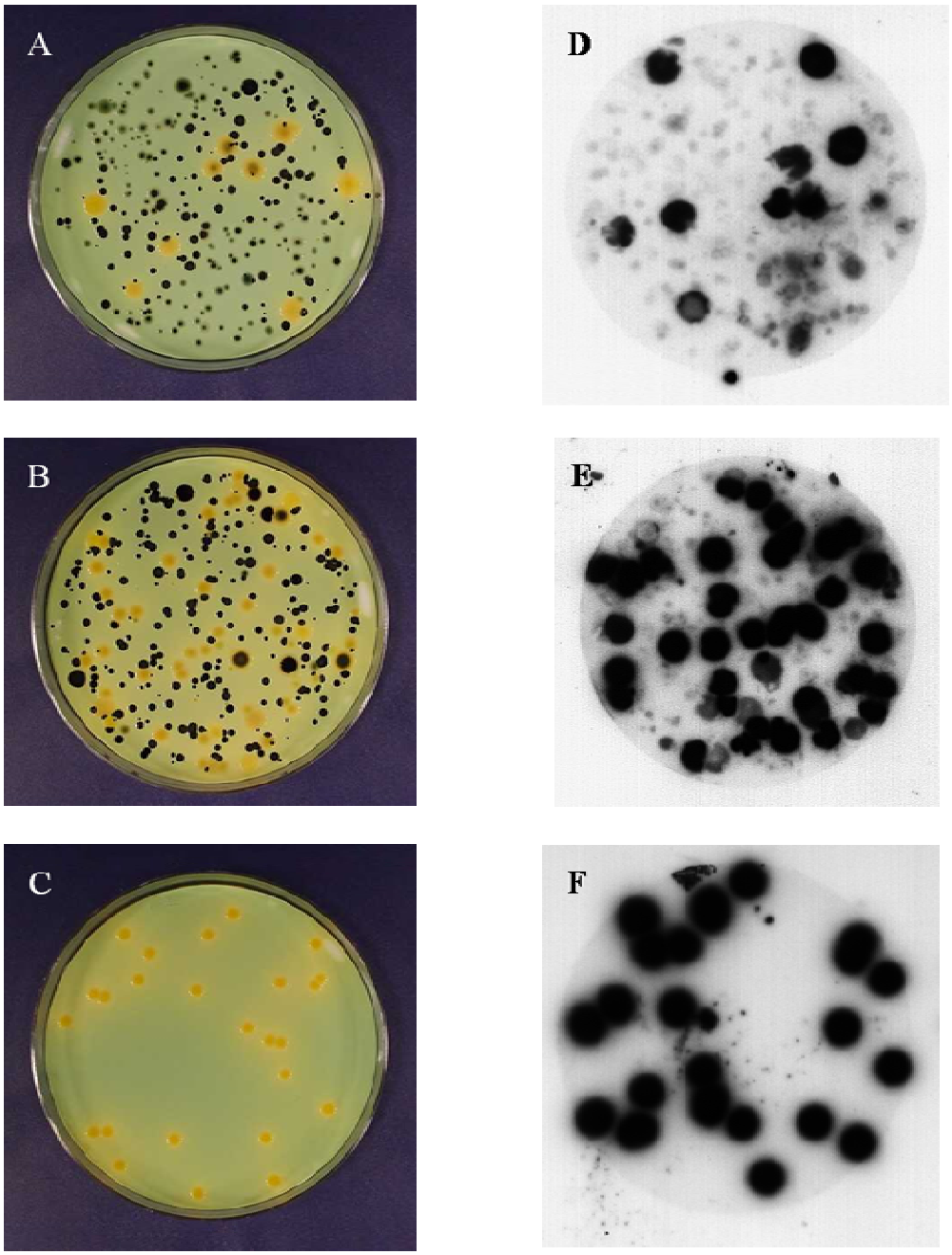


Figure 3.2. Results of nylon membrane filtration colony blot hybridization with probe pVCITS of Chesapeake Bay K site water with and without *V. cholerae* TMA21 overlaid on TCBS agar plates. A, bay water without *V. cholerae*; B, bay water with *V. cholerae*; C, 0.22 μm filtered bay water with *V. cholerae*; D-F, autoradiographs of A-C, respectively.

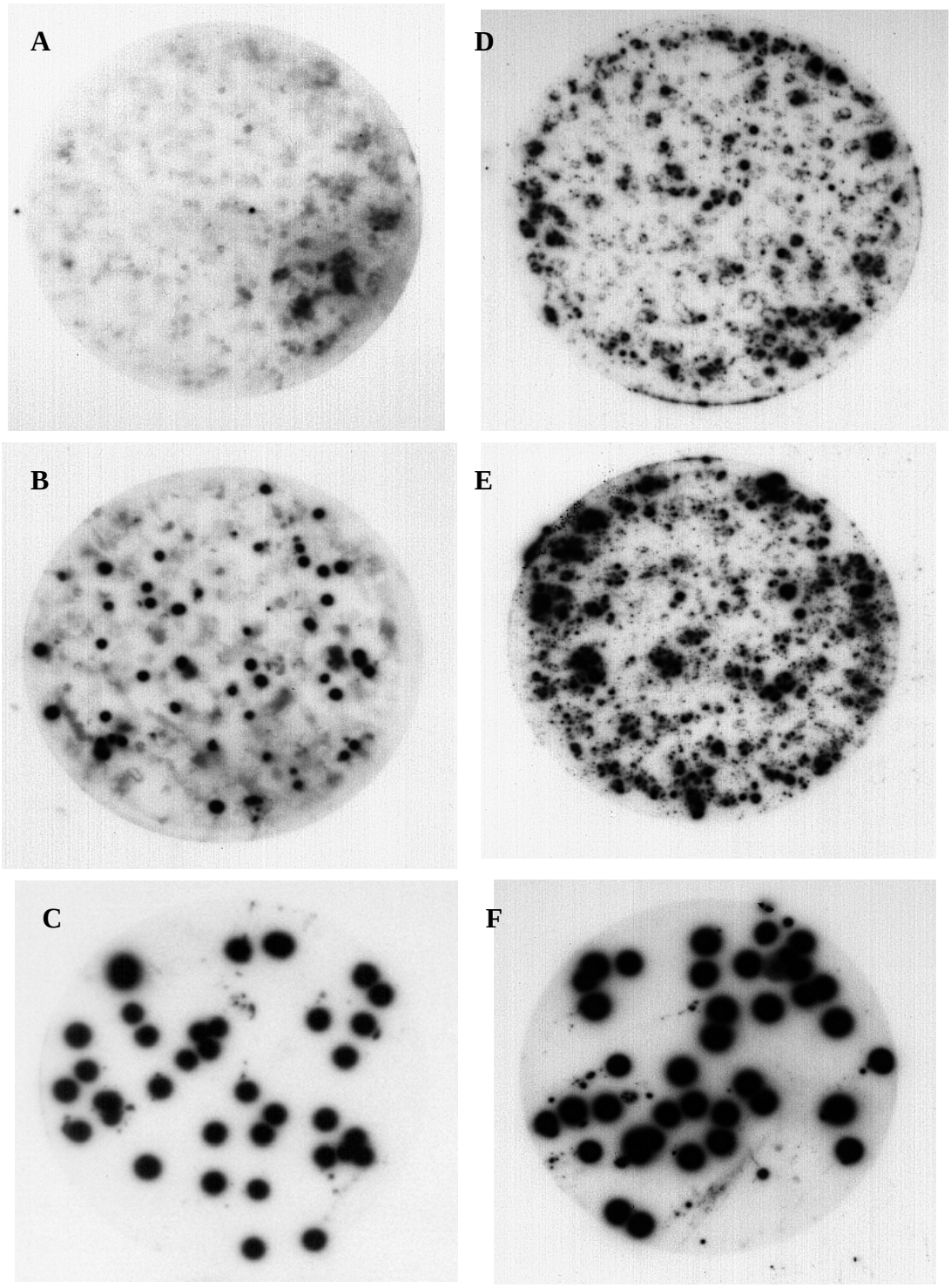


Figure 3.3. Autoradiographs of nylon membrane filtration colony blot hybridization with probe pVCITS of Chesapeake Bay K site water with and without *V. cholerae* TMA21 overlaid on AP agar (A-C) and Marine agar 2216 (Difco, D-F). A and D, bay water without *V. cholerae*; B and E, bay water with *V. cholerae*; C and F, 0.22 μm filtered bay water with *V. cholerae*.

APA or MA when *V. cholerae* was added to the water samples. However, recovery of *V. cholerae* from filter-sterilized Chesapeake Bay water (Figure 3.2F, 3.3C, and 3.3F), sterile artificial seawater, or sterile PBS was less effective when TCBS was used (33%), compared with APA (62%) or MA (66%). Total recovery was calculated by plate counts on LB-30 agar and direct counts using acridine orange. Interestingly, results presented in Figure 3.2 and 3.3, showed that the filtration colony blot of Chesapeake Bay water without *V. cholerae* TMA21 added and incubated on TCBS agar yielded nine positive signals. The filtration colony blot method may underestimate the number of *V. cholerae*; however, as suggested by the observation reported by Chaiyanan et al. (2006) that coccoid cells of *V. cholerae* in environmental samples can pass through 0.2 μm filters.

3.3.4 Field testing of pVCITS

The DNA colony blot protocol (Method 1), employing the pVCITS probe, was evaluated using water and plankton samples collected from Chesapeake Bay, MD and Bangladesh. Figure 3.4 shows blots producing positive signals. Of 14 samples collected from Chesapeake Bay, five (36%) were positive by colony blot hybridization, yielding 34 isolates (Choopun, 2004). Colonies producing positive signals were subcultured and confirmed to be *V. cholerae* at a rate of 91% (Choopun, 2004). For purposes of comparison, with traditional enrichment method (Huq et al., 2006)10 samples were positive for *V. cholerae*, with 23 isolates confirmed to be *V. cholerae* (Choopun, 2004). Figure 3.5 shows colony blots prepared with water samples collected in Bangladesh. As expected, the blots contained more positive signals than the Chesapeake Bay water samples, since the water samples were collected from a cholera endemic area and under environmental conditions favoring higher densities of *V. cholerae*.

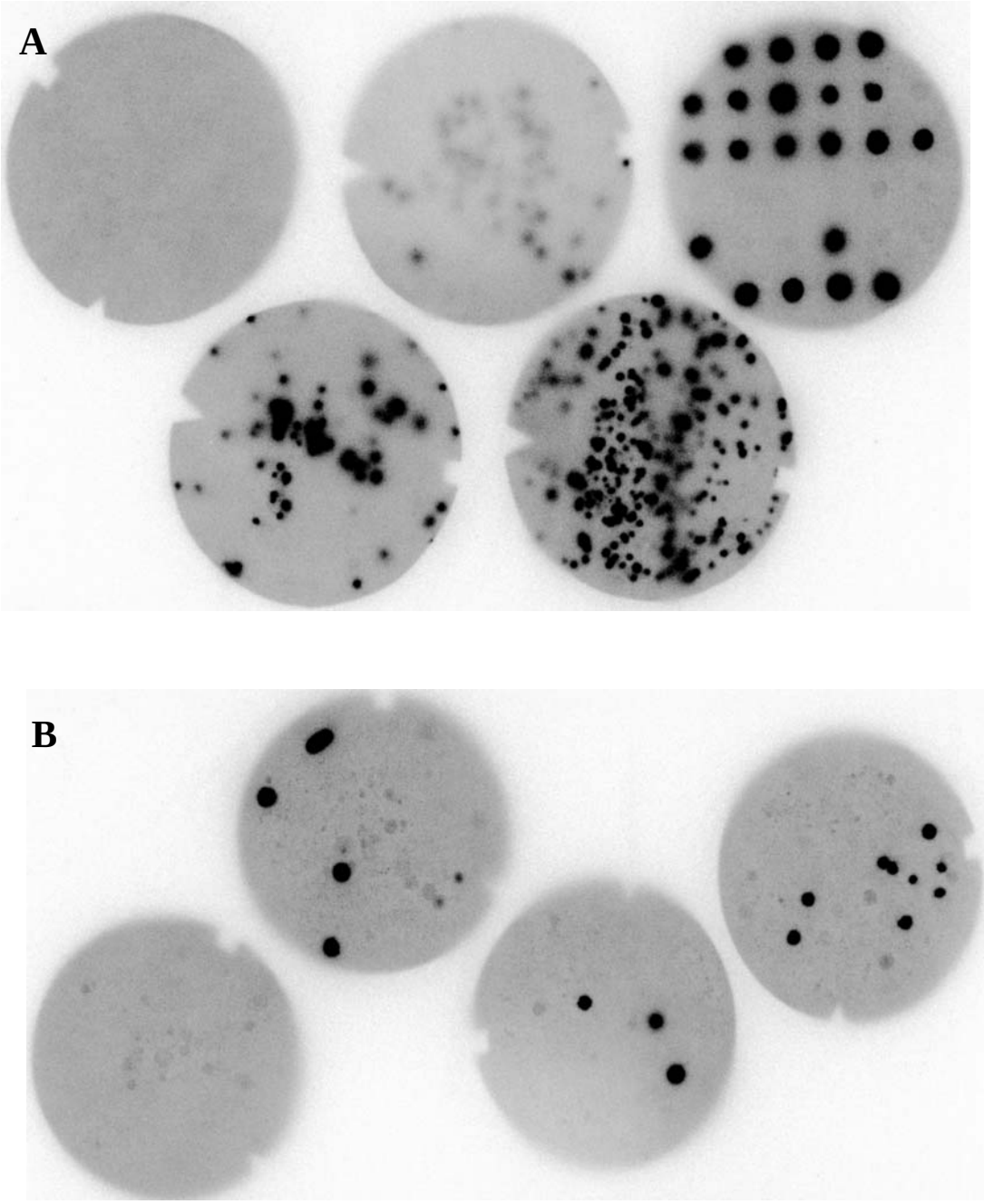


Figure 3.4. Autoradiographs of 541 filter paper colony blot lifts hybridized with probe pVCITS for Chesapeake Bay water samples: A, July, 1998, with positive/negative control blot (top-right, see Figure 3.1); B, August, 1998.

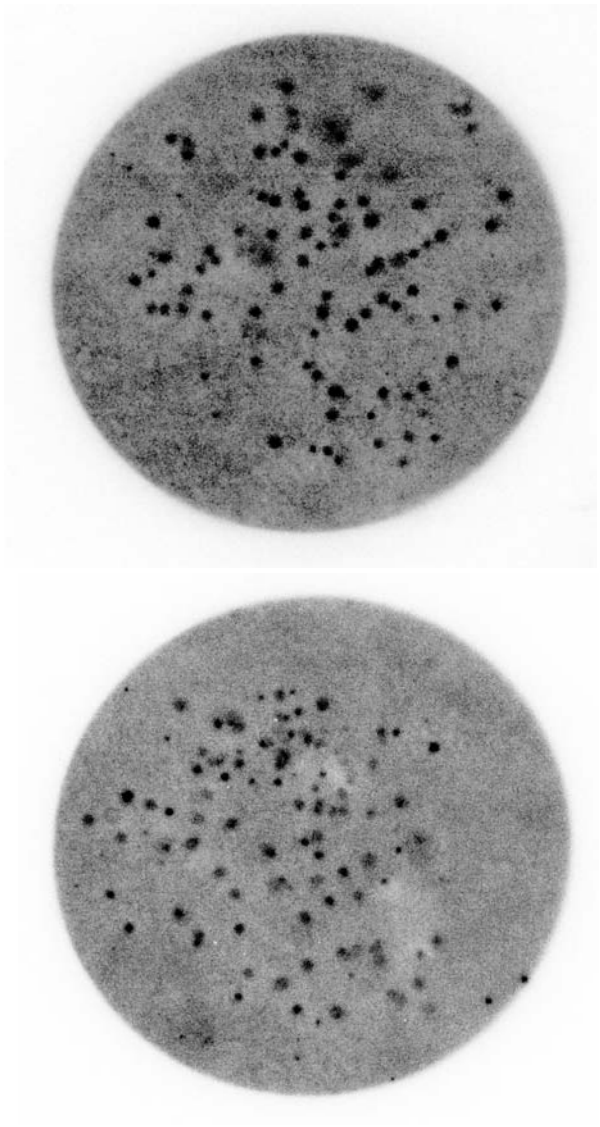


Figure 3.5. Autoradiographs of (2) 541 filter paper colony blot lifts hybridized with probe pVCITS for Bangladesh water samples.

3.3.5 RNA colony blot hybridization

As an alternative method, avoiding the use of radioisotopes, the RNA colony blot hybridization method, using a fluorescent-labeled *V. cholerae*-specific 16S rDNA oligonucleotide probe, was developed. Since the fluorescent label is not as intense as a radiolabel (but much safer for field use), four methods that employed the 16S rDNA-based

probe were evaluated. Spread plates of luminescent *V. cholerae* non-O1/non-O139 strain CB99-18 were prepared on LB and HPC agar plates and incubated overnight at 37°C. Colony blot lifts, using nylon membranes and 541 filter paper, were prepared employing each of the four methods. To assess the amount of nucleic acid immobilized onto membranes using each method, the blots were stained with methylene blue and destained in alcohol (Figure 3.6). Two successive staining steps were required for the two RNA colony blot methods (Methods 3 and 4). All four methods were effective in liberating, denaturing, and immobilizing nucleic acid to the nylon membrane. However, Method 1 gave significantly smaller signals, compared to the other three, while Method 4 gave very large signals and low background. The nylon membranes were then cut into quarters and hybridized with Cy3-labeled probe Vchomim1276.

3.3.6 Optimization of RNA colony blot hybridization protocol

In addition to the colony blot lift method, several steps in the colony blot hybridization were optimized, including hybridization temperature, and presence/absence of pre-hybridization washing, blocking agents used, and RNase treatment of solutions. Optimization was carried out in two steps, first with spread plates of *V. cholerae* CB99-18 (Figures 3.7), and then with colony dot blots prepared with four strains of *V. cholerae* and eight strains of the other *Vibrio* spp. and related bacteria included in this study (Figure 3.8). Figure 3.7 shows results of an optimization experiment involving the colony blot method (1-4), hybridization temperature (35° and 60°C), blot material (541 filter paper and nylon membrane), and presence/absence of pre-hybridization washing. Based on the results obtained (See Figure 3.7 panels A-D), Method 4 consistently gave strong signals, relative to background, when Cy3 labeled Vchomim1276 probe was employed. As expected, nylon

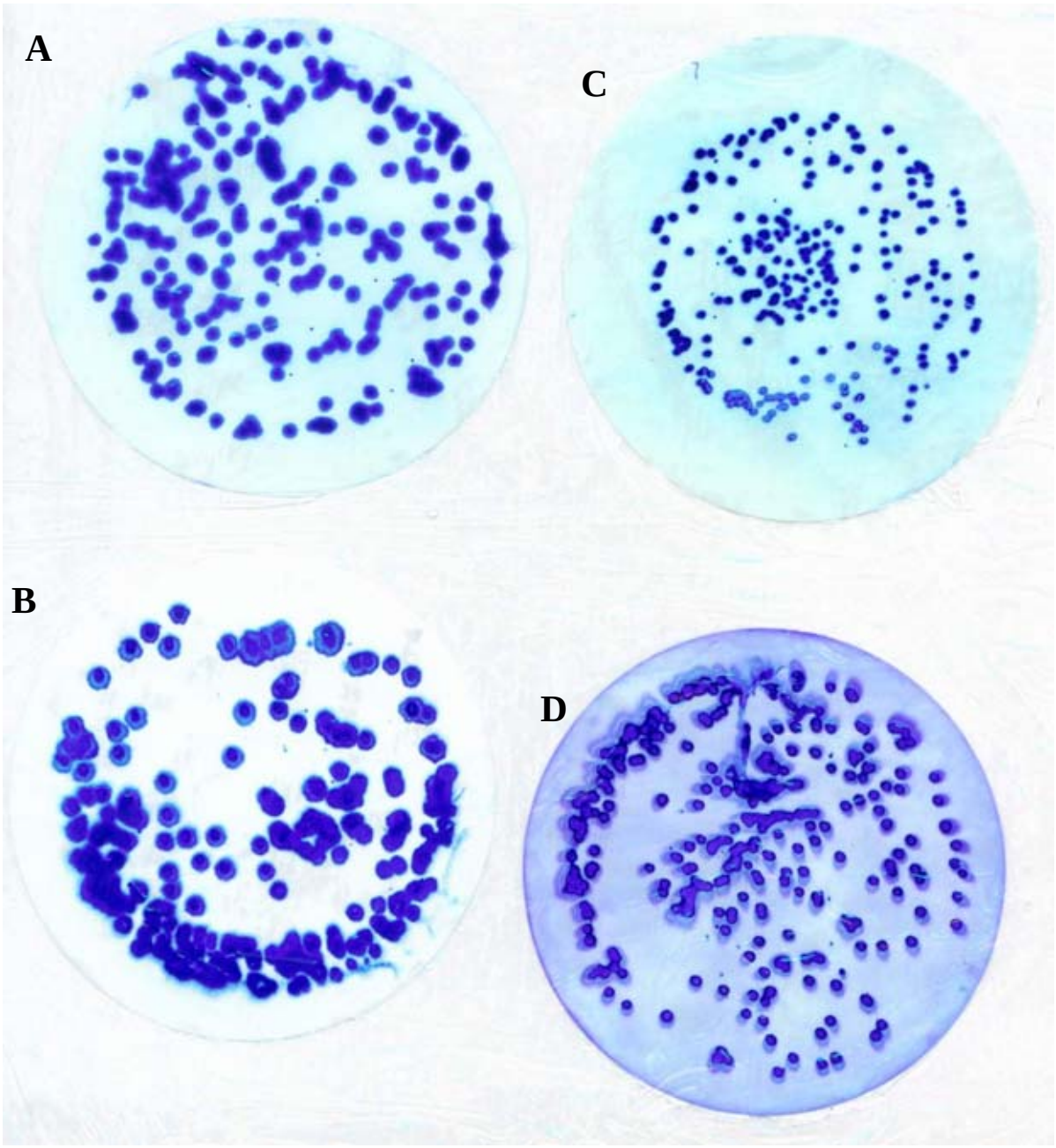


Figure 3.6. Methylene blue staining of colony blot lifts on nylon membranes prepared using four different methods. Spread plates are of *V. cholerae* CB99-18 on LB agar. A, Method 2; B, Method 4; C, Method 1; D, Method 3.

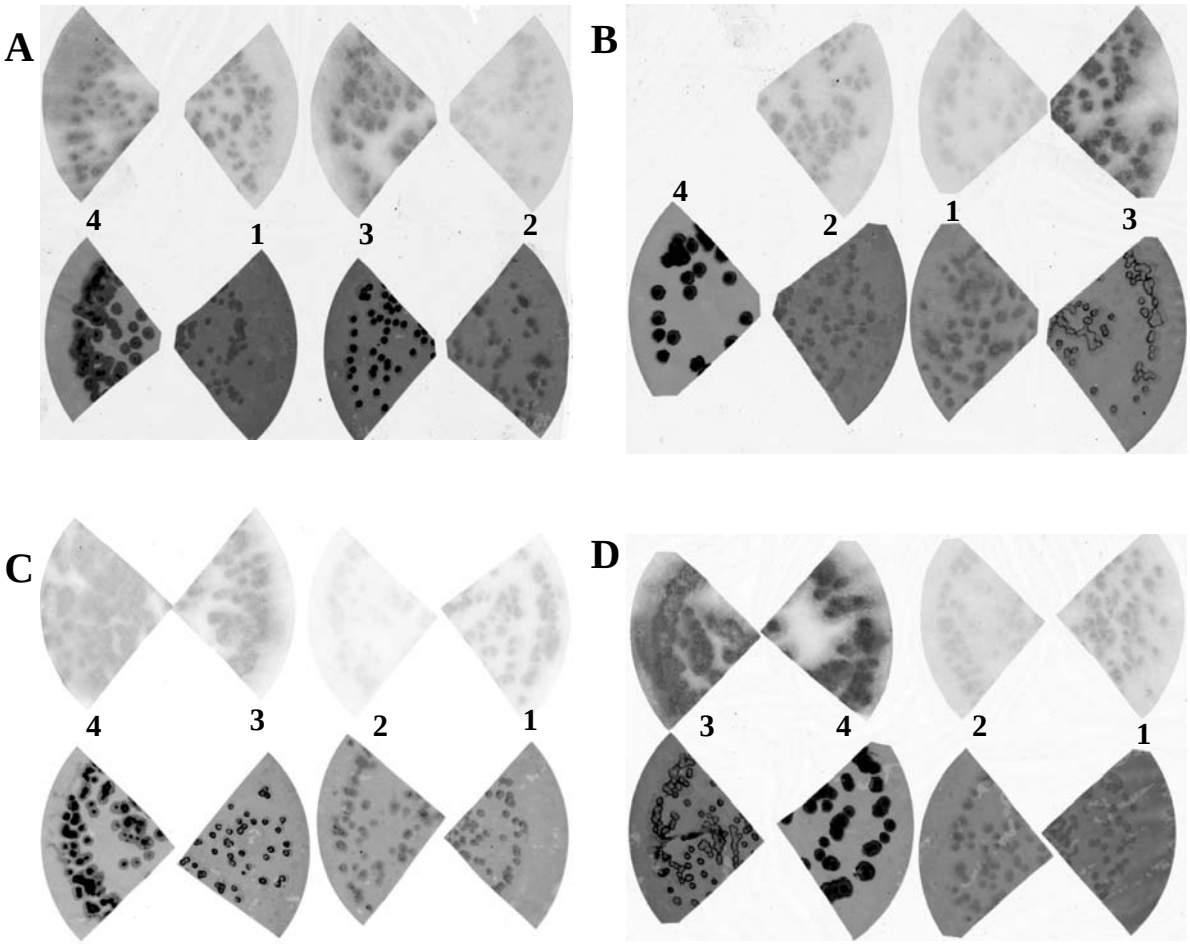


Figure 3.7. Typhoon images of colony blots from Figure 3.6, cut into quarters and hybridized with Cy3-labeled probe Vchomim1276. A, hybridization temperature of 35°C, no prehybridization wash; B, hybridization temperature of 35°C, prehybridization wash; C, hybridization temperature of 60°C, no prehybridization wash; B, hybridization temperature of 60°C, prehybridization wash. Numbers by blots correspond to Method #. Top row, 541 Whatman filter paper used for colony blot lift; Bottom row, Magna nylon membranes used for colony blot lift.

membranes gave a significantly better signal than 541 filter paper. Pre-hybridization washing was effective in improving the hybridization signal (Figure 3.7B and D versus A and C).

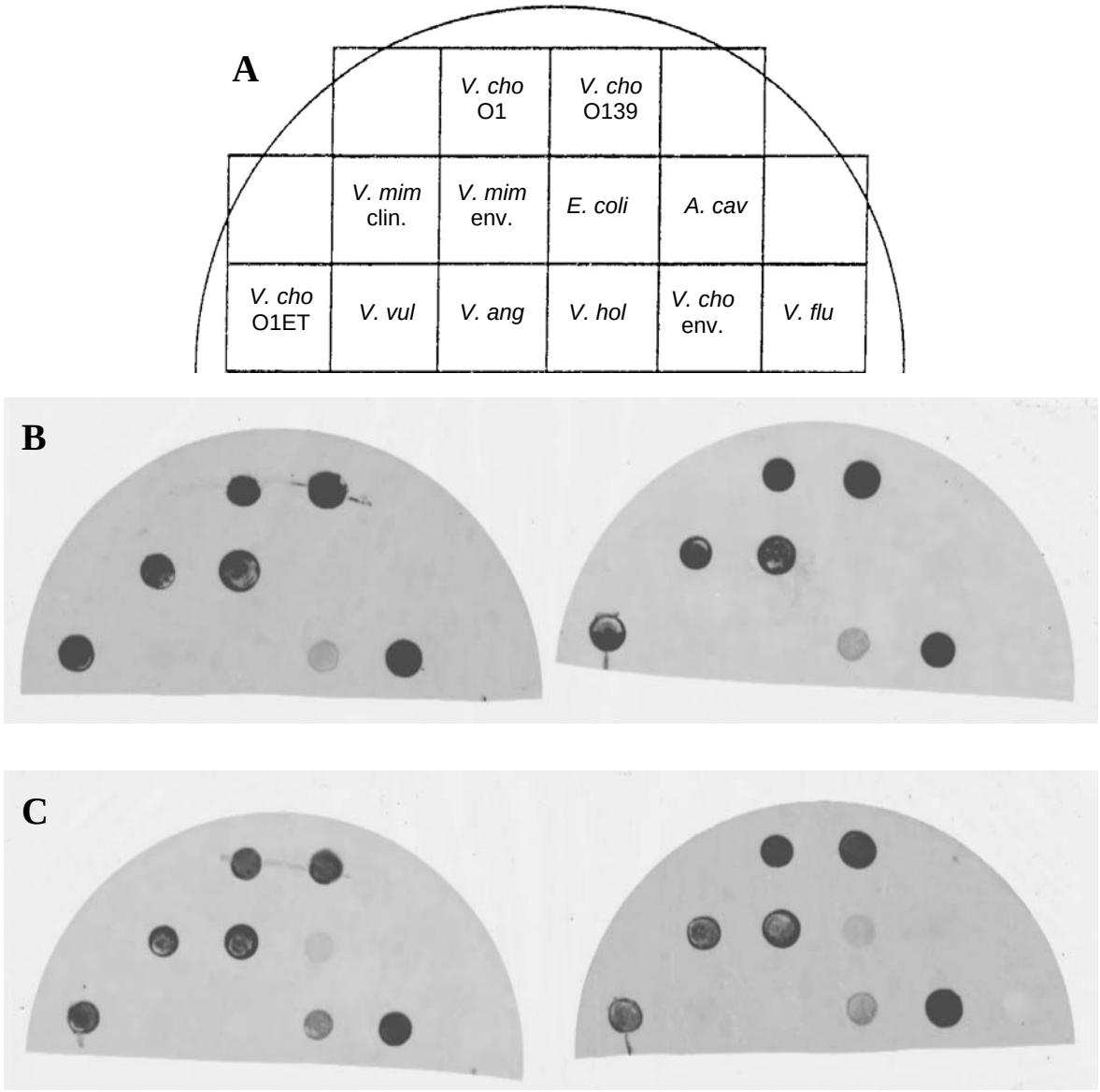


Figure 3.8. Typhoon images of RNA colony blot hybridization with Cy3-labeled Vchomim1276 to determine specificity of probe, while optimizing temperature and membrane material. A, schematic representation of the bacterial strains used to make colony dot blot; B, Typhoon image of RNA colony blot lift (Method 4) using nylon and nitrocellulose membranes with hybridization performed at 60°C; C, Typhoon image of RNA colony blot lift (Method 4) using nylon and nitrocellulose membranes with hybridization performed at 35°C.

RNase treatment of the hybridization solutions was also tested and Figure 3.9 shows results indicating that the use of DEPC-treated solutions are necessary. The T_m of the probe was determined to be 72°C at the time of manufacture (Sigma Genosys, TX), but results given in Figure 3.7 with *V. cholerae* (essentially a positive control), demonstrated that there was only a very small effect related to hybridization temperature (Figure 3.7A and B versus C and D). Results of the RNA colony blot lifts when closely related strains (negative controls) were used showed that the higher temperature is needed to prevent cross-reaction (Figure 3.9). However, all cross-reactions were not eliminated. Also, the results shown in

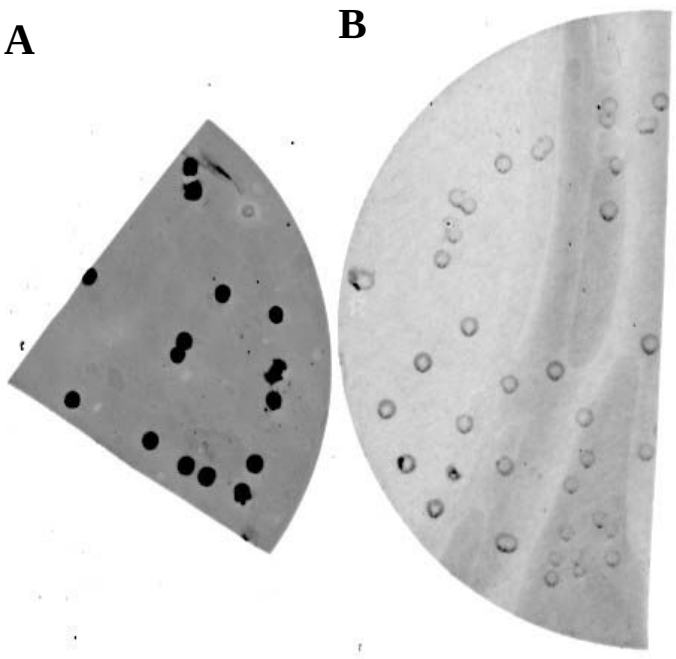


Figure 3.9. Effect of RNase treatment of RNA colony blot lift and hybridization solutions with DEPC on signal. A, All solutions treated with DEPC; B, no RNase treatment.

Figure 3.9 demonstrate that there was little difference in signal intensity when nylon or nitrocellulose membranes were used. Results for the two blocking agents, denatured salmon

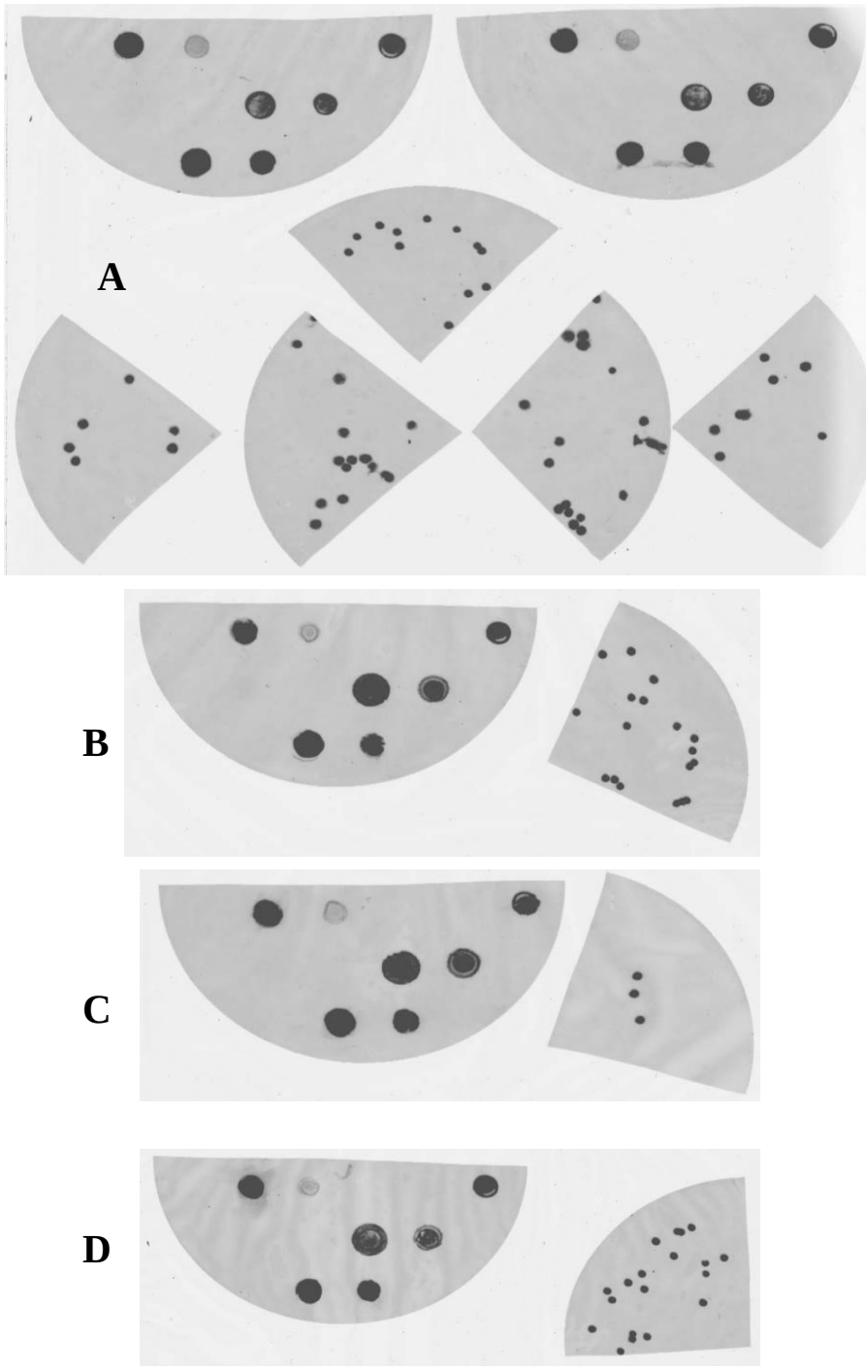


Figure 3.10. Effect of blocking agents on RNA colony blot lift and hybridization with Cy3-labeled probe Vchomim1276. A, with SS DNA (0.5 mg/ml) and Denhardt's solution (10X); B, without both; C, with SS DNA only; D, with Denhardt's solution only. Parallel blots of the membranes used in Figure 3.8 and Figure 3.6 were both used.

sperm (SS) DNA and Denhardt's solution, showed that neither blocking agent was required for high signal sensitivity (Figure 3.10).

3.3.7 Evaluation of RNA colony blot hybridization

Fluorescent-labeled probe Vchomim1276, employing RNA colony blot hybridization (Method 4) and addition of *V. cholerae* cells to the samples yielded results as follows. Luminescent *V. cholerae* RC488 added to Lake Artemesia pond and Baltimore Harbor water samples and spread plated, using 10-fold serial dilutions, gave results shown in Figure 3.11. A total of 58 *V. cholerae* colonies were enumerated on this membrane by the probe. Plate counts done prior to RNA colony blot lift and hybridization, yielded a total of 187 colonies.

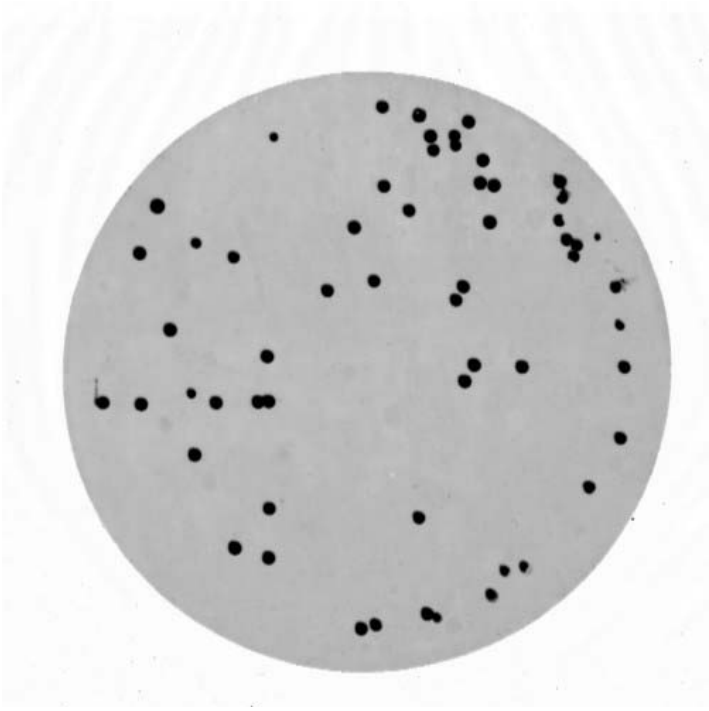


Figure 3.11. Typhoon image of RNA colony blot of pond water to which *V. cholerae* CB99-18 had been added, hybridized with Cy3-labeled probe Vchomim1276.

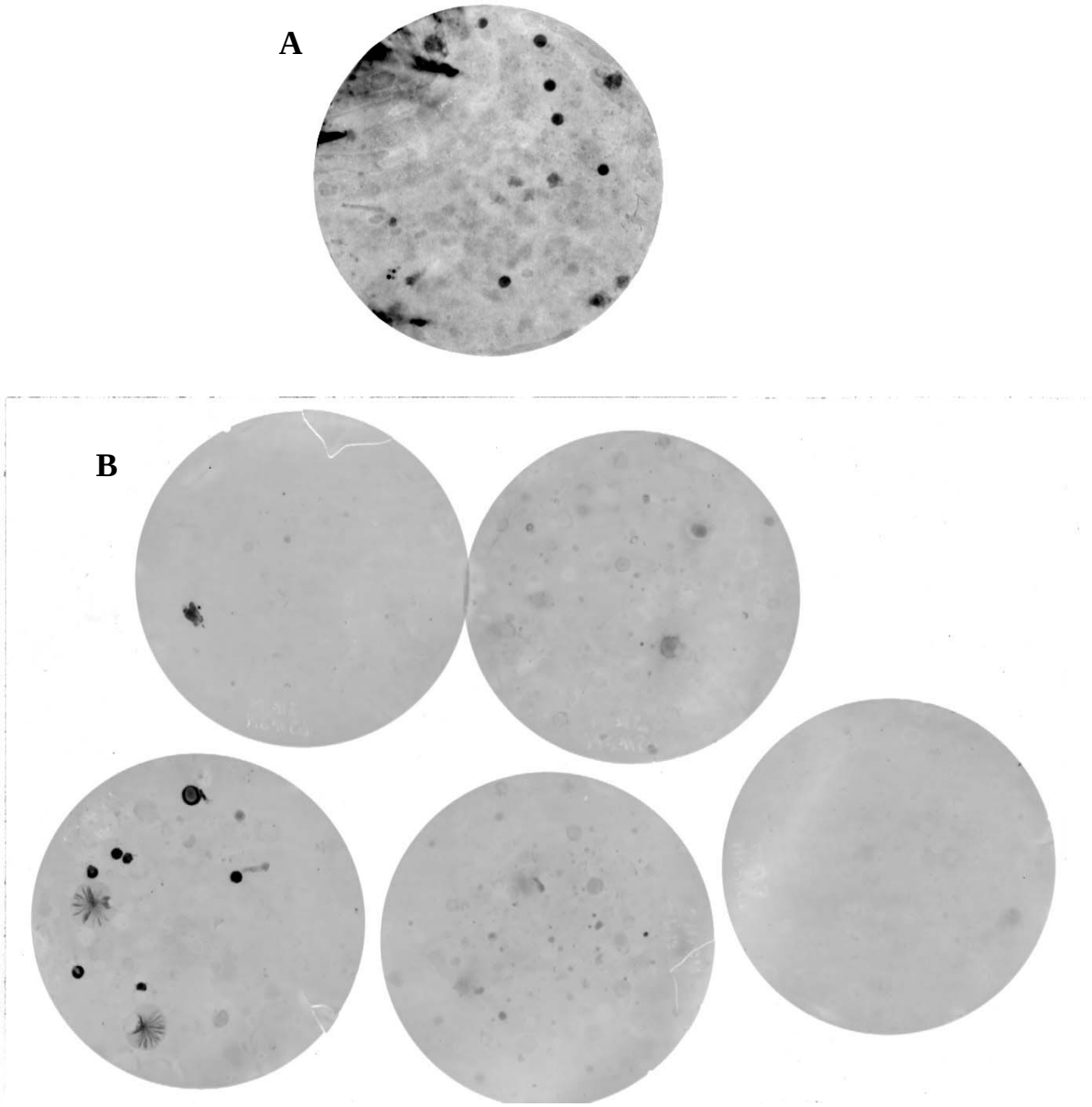


Figure 3.12. Typhoon images of RNA colony blots produced in Bangladesh from water sample spread plates on L agar and hybridized with Cy3-labeled Vchomim1276. A, positive blot from 2 initial test blots; B, Blots from 5 sites from the Mathbaria province, Site 1, 5, 3, 4 and 2 (clockwise, from top left).

There was no difference in the concentration of *V. cholerae,* determined by RNA colony blot hybridization, between natural water samples to which *V. cholerae* had been

added and water samples which had naturally occuring bacteria and particles removed by filtration prior to addition of *V. cholerae*.

3.3.8 Field trial in Bangladesh

To assess practicality of the RNA colony blot lift and hybridization method, water samples collected from a cholera endemic area were tested. Forty blots prepared from samples collected at 14 sites in Bangladesh were hybridized with the Cy3-labeled Vchomim1276 probe. Results showed five of the 40 (12.5%) samples were positive. Figure 3.12 shows results for two of the positive blots prepared using water collected from the Mathbaria province, Bangladesh (site 1 and site 2).

3.4 Discussion

In the study reported here, two colony blot hybridization methods were developed that were designed to improve detection, isolation and enumeration of *V. cholerae* from environmental water samples. In its simplest form, the method can be used to detect total V. cholerae. In this procedure, the ability to quickly determine the identity of suspected isolates from TCBS agar by DNA-DNA or DNA-RNA hybridization is preferred compared to the use of the tedious and labor-intensive battery of biochemical tests used in the traditonal culture method. The method can also be adapted for use in isolation of the organism (Huq et al., 2006). If the colony blot lift is made from a spread-plate, then the master plate can be stored at 15°C, until the hybridization is complete, at which time the positive signals can be correlated to colonies and then subcultured. If the colony blot is made by direct filtering of a water sample onto the membrane (and then overlaying onto an agar plate), then the subsequent growth on the membrane can be replica-plated onto a fresh agar plate prior to colony lysing. Again, positive signals can then be correlated to colonies and then

subcultured and confirmed. Regardless of which probe method was used, the procedure is limited because each relies on culture. Thus, for routine monitoring, this approach is inferior to PCR-based methods, which are also usually more sensitive. These two methods were developed for *V. cholerae* population studies in the Chesapeake Bay, MD, an estuary, and Bangladesh, a freshwater system with tidal input, for which isolation of the bacterium, followed by characterization, was necessary; i.e. culture isolates were needed. Resultant isolates were tested for ability to express luminescence and for presence of *lux* genes. For this purpose, this method is preferred over traditional enrichment/selective medium isolation methods, because the necessity for extensive and laborious biochemical tests can be avoided. The colony blot hybridization methods can also be used for enumeration of *V. cholerae*, since no enrichment step is required, but several replicates of multiple dilutions or sample volumes are required in order to obtain reliable results.

The single major influence for each probe, was the label that was used. The DNA colony blot hybridization method was designed for use with a ^{32}P-labeled *V. cholerae*-specific ITS region probe. Labelling with radioisotope gave an intense signal and can be used to detect single- and low-copy number genes. The 18-bp inter-specific variable region was present in the majority of the nine *V. cholerae* O1 and O139 and ten non-O1/non-O139 *rrn* operons of *V. cholerae*. Target abundance, relative to a single-copy gene, became obvious from the strong signal, as shown in Figures 3.1-3.5. Over the past 10-15 years, alternatives to radioisotope labeling of nucleic acid probes have become available. These alternatives, such as alkaline phosphatase, DIG (Roche), and fluorochromes, have advantages, compared with radioisotopes, with respect to laboratory personnel safety and operational regulations. In most third world countries, avoidance of radioisotope use is

preferred. Therfore an alternative labeling method was desirable and thus fluorochrome-labeling was employed for colony blot hybridization in Bangladesh. Fluorescent labeled probes are safer, compared with radioisotope labeling. However, the signal, relative to background, is less strong, when a single- or low-copy gene (DNA) is the target, in the case of fluorescent labeled probes.

In addition to a different probe label, a new probe sequence was employed in the second colony blot hybridization method. The first probe (pVCITS)-DNA colony blot hybridization protocol was developed chronologically earlier than the second method (Cy3-labeled Vchomim1276 – RNA colony blot hybridization). Further analysis of the 18-bp interspecific variable ISR region of many *V. cholerae* isolates revealed that this probe would not hybridize to strains of a small phylogenetic clade of *V. cholerae*, under conditions inhibiting inter-species cross-reaction (Y. Zo, personal communication). Therefore, a previously described *V. cholerae*/*mimicus*-specific 16S rDNA probe (Heidelberg, 1997) was employed for fluorescent label/RNA colony blot hybridization. Inclusion of *V. mimicus* in the RNA colony blot hybridization method was deemed acceptable, since *V. mimicus* is a reservoir for CTX Φ (Boyd et al., 2000), and is considered to be the same species as *V. cholerae*, at least by some investigators. Also, it can be readily distinguished when green, sucrose-negative, colonies are subcultured onto TCBS. To increase signal intensity, an RNA colony blot lift and hybridization method was developed, using fluorescent labeled *V. cholerae*-specific 16S probe. This method takes advantage of the fact that during early growth (16 hours to 2 days) on complex or rich agar medium, growth is vigorous. Under these conditions, bacterial colonies have a high mRNA content. This condition changes the target from low-copy number (DNA) to high copy number (mRNA).

The pVCITS-DNA colony blot hybridization required optimization of hybridization temperature and washing conditions, which was accomplished using a 52 dot colony blot containing 15 *V. cholerae* strains and 37 non-*V. cholerae* strains. Six replicate blots were made, and these were tested in a 3 x 2, hybridization x washing temperature, design. Blots washed at 60°C produced smaller positive colony signals, compared to 55°C, with some positive signals missing. The lowest hybridization temperature to not allow cross-reactivity was 42°C (data not shown).

The Vchomim1276-RNA colony blot hybridization method required more extensive optimization. A review of the literature revealed that few attempts had been made to capitalize on the abundance of 16S rRNA content for colony blot hybridization. As a consequence, there is no consensus RNA colony blot lift and hybridization method. Four colony blot lift methods were tested based on rRNA liberation, denaturation and immobilization onto membranes. Two RNA colony blot hybridization methods were found to be superior to two DNA colony blot hybridization methods, with respect to content of immobilized RNA content. However, all four methods immobilized adequate amounts of nucleic acid (Figure 3.6 and 3.7). Additional optimization revealed that it is essential that all measures be taken to minimize contamination with RNase (Figure 3.9). These ubiquitous enzymes are extremely stable and do not require cofactors to function. To minimize RNase contamination, sterile, disposable plasticware was used, solutions were treated with DEPC, and gloves were changed frequently. Prehybridization washing greatly improved signal quality (Figure 3.7), most likely as a result of removing cellular debris and thereby improving probe access to target nucleic acid. Nylon or nitrocellulose membranes can be used with this

method, but less expensive filter paper cannot (Figure 3.7 and 3.8). Furthermore, the traditional DNA hybridization blocking agents are not necessary (Figure 3.10).

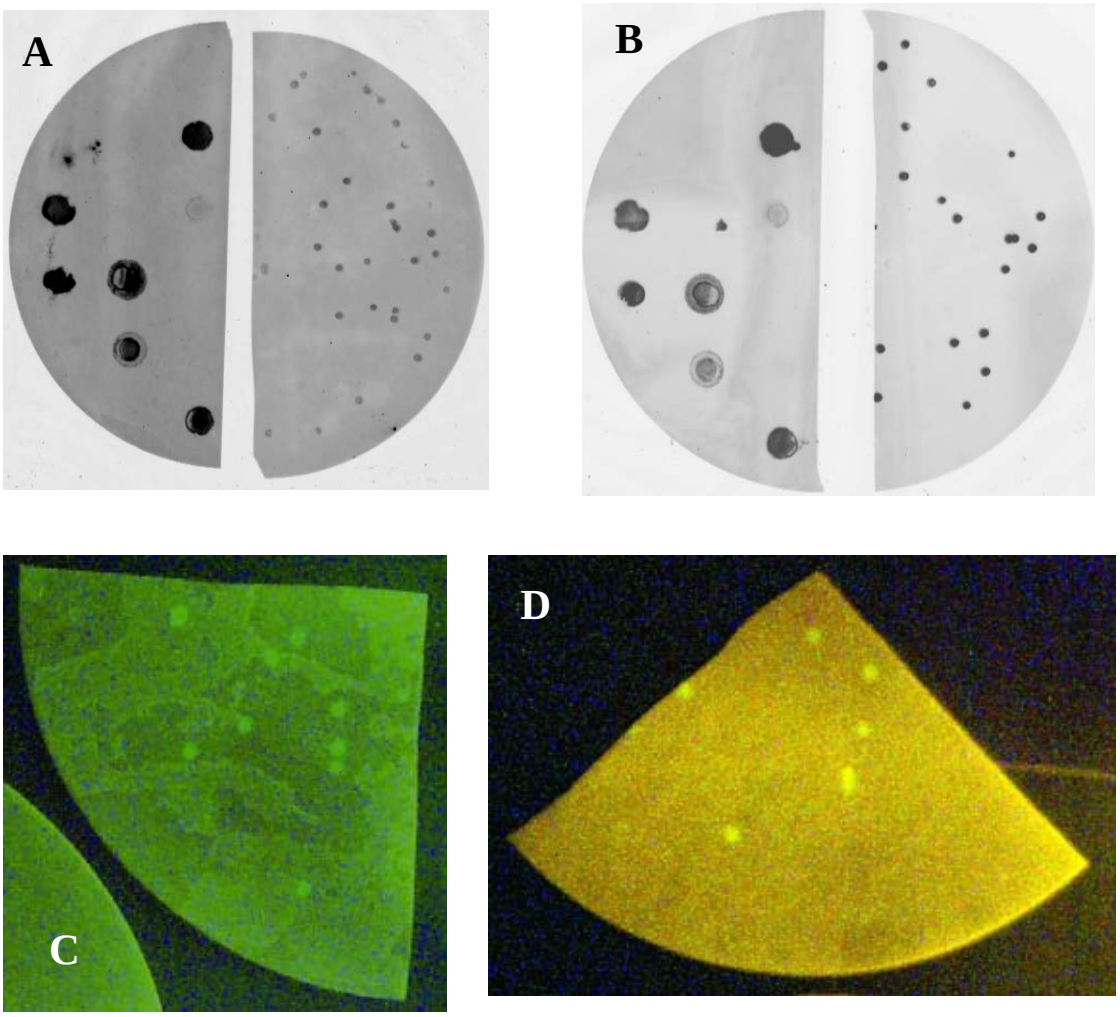


Figure 3.13. Comparison of Cy3- and fluorescein-labeled probe Vchomim1276 using *V. cholerae* spread plate colony blots and optimization colony dot blots (containing positive and negative controls). Dark Reader images were produced using a digital camera equipped with appropriate amber lens. A, fluorescein-labeled probe, Typhoon image; B, Cy3-labeled probe, Typhoon image; C, fluorescein-labeled probe, Dark reader image; D, Cy3-labeled probe, Dark Reader image.

In addition to colony blot hybridization optimization, two fluorochromes were evaluated for use with the Dark Reader hand lamp (Clare Chemical, Inc, Dolores, CO), an inexpensive device. The Dark Reader lamp allows DNA to be visualized by visible light when two color filters are used, a blue filter placed between the lamp and sample and an amber filter placed between the sample and observer (eyeglasses or camera lens). The hand lamp is generally equally sensitive as a UV light, without the health risk. Both Cy3 and fluorescein-labeled Vchomim1276 were evaluated with both detection methods. The Cy3 label was slightly more sensitive than the fluorescein label, although both were acceptable (Figure 3.13).

Each of the probes used with corresponding optimized colony blot lift and hybridization conditions employed was satisfactory in specificity. More than 200 strains of *V. cholerae* were tested using the probe, pVCITS (Table 3.1). In addition, forty closely related bacteria were tested for cross-reactivity. The probe hybridized only with *V. cholerae* DNA, with no cross-reactivity, not even weak, with any of the non-*cholerae Vibrio* sp. and related strains observed (Figure 3.1). The probe proved to be completely specific for the strains of *V. cholerae* and other bacterial species tested. Probe Vchomim1276 was also highly specific for *V. cholerae,* as well as *V. mimicus* (Figure 3.9). Fewer strains were used to test specificity of the Vchomim1276 probe (Table 3.2) because this probe had been extensively evaluated for specificity previously (Heidelberg, 1997). It did show some weak cross-reaction with rRNA from *V. hollisae* ATCC33564. However, the signal could be easily distinguished from that of *V. cholerae* when conditions were optimized (Figure 3.9B). .

Results of experiments, in which *V. cholerae* was added to the samples, and field trials demonstrate that both probe-colony blot hybridization methods can be used to detect,

isolate, and enumerate *V. cholerae* in environmental water samples. Earlier experiments using the pVCITS probe revealed that the major obstacle to optimal performance when natural water samples were tested is inter-specific competition on agar plates. Just as colony size is reduced when bacterial density is high on an agar surface, nucleic acid content is correspondingly less (Figure 3.3B and E). Colony blots incubated on TCBS (Figure 3.2E) were easier to interpret, as expected, because of the selective nature of the medium. However, the recovery rate was less, compared with non-selective media, APA and MA (Figure 3.2F versus Figure 3.3C and F). In experiments with *V. cholerae*, the filtering method yielded 25.5%, 50.5% and 47.0% of the bacterial counts (determined by plate counts on LB agar) from the inoculum on TCBS, marine agar and alkaline peptone agar, respectively. Five replicates for each medium were done. The same results were obtained when the *V. cholerae* strain was added to filtered (0.2 μm) Chesapeake Bay water (Figure 3.2F, 3.3C and F).

Results of experiments in which *V. cholerae* was added, using Vchomim1276-RNA colony blot hybridization, gave more easily interpretable results, compared to pVCITS colony blot hybridization, but was largely due to improved experimental design; i.e., the method was designed for use with water samples from cholera-endemic areas, where the numbers of *V. cholerae* would be expected to be high. Therefore, spread plating method was used, along with a high concentration of *V. cholerae* cells. Resutls of these experiements demonstrated that when *V. cholerae* is present in high numbers and an appropriate spread-plate dilution is employed, detection, isolation, and, most importantly, enumeration of total *V. cholerae* can be achieved (Figure 3.11).

Results of field trials with the two colony blot hybridization methods supported the results obtained from experiments where known quantities of *V. cholerae* had been added. For the probe pVCITS-DNA colony blot hybridization protocol, the field trials were done using spread plating on APA of water samples collected from Chesapeake Bay, MD, during July and August, when chances of detecting *V. cholerae* would be highest. Results shown in Figure 3.4 demonstrate that even in an area where cholera cases are not reported, the method is sufficiently sensitive to detect *V. cholerae,* present in relatively low numbers. All of the isolates from the blots were non-O1/non-O139 serogroup *V. cholerae*. DNA colony blots of water samples from ponds in Bangladesh, hybridized with probe pVCITS (Figure 5), showed that the total number of *V. cholerae* in these water samples were significantly higher than in the Chesapeake Bay, MD, as expected. Results of the field trials using the Vchomim1276 probe and RNA colony blot hybridization showed that the colony blot hybridization method is sufficiently sensitive for routine use of detecting, isolating or enumerating *V. cholerae* in environmental samples (Figure 3.12).

Results of the experiments in which *V. cholerae* cells were added and field trials indicate the limit of sensitivity of the colony blot hybridization protocol. For samples in which the expected number of *V. cholerae* is low, such as Chesapeak Bay, MD or during winter months in both Chesapeake Bay and Bangladesh, filtration can be used to concentrate samples directly onto the membrane. For experiments performed in this study, 85-mm nylon membranes were used to filter 50-150 ml water samples. The sample volume was adjusted to yield well separated colonies of sufficient size to give a reliable signal (~2-3 mm in diameter). Larger membranes (137 mm) can be used to sample even larger volumes of water (200 to 500-ml), when the number of *V. cholerae* cells is extremely low. When filtration is

used, a selective medium such as TCBS should be used to inhibit growth of non-*V. cholerae*. Based on results from our study, interspecific competition for space on filters must be minimized to obtain reliable enumeration (Figure 3.2E). Even though TCBS yielded the lowest recovery of *V. cholerae* in the control experiments, it was the only medium that recovered *V. cholerae* from samples to which *V. cholerae* cells had not been added (Figure 3.2D).

For samples containing high numbers of *V. cholerae*, colony blot lifts from spread plates are satisfactory for enumeration of *V. cholerae*. For the pVCITS probe, used to test late summer water samples from Chesapeake Bay, conditions favoring a higher density of *V. cholerae*, spread plates on AP agar were used. AP agar is based on the traditional *V. cholerae* enrichment broth, APW, which allows growth of vibrios that multiply under alkaline conditions, while suppressing growth of non-*Vibrio* bacteria. For the Vchomim1276 probe, RNA colony blot lifts were prepared from spread plates on L agar. For this probe, a complex medium was more effective in ensuring a high rRNA content of the cells. For spread plates and the filtration method, a series of dilution plates must be prepared to ensure countable blots.

In conclusion, the colony blot methods developed in this study have many advantages in detection and isolation of *V. cholerae*. Detection and isolation is less tedious than traditional culture methods and results are achieved in less time. Enumeration is also possible, whereas traditional culture methods involve enrichment. The method has high specificity, as is the case with other molecular methods, such as PCR. Environmental microbiological methods generally have low sensitivity, but this shortcoming can be addressed by including sample concentration by filtration.

Chapter 4 SEQUENCE OF THE *LUX* OPERON OF *VIBRIO CHOLERAE*, ITS PHYLOGENY AND ORIGIN

4.1 Introduction

Bioluminescence is a trait encoded by five genes arranged in a polycistronic operon: *luxCDABE*. Additional *lux* genes have been identified, including *luxF* (*P. phosphoreum* and certain strains of *Photobacterium leiognathi*; Soly et al., 1988), *luxG*, *luxH* (*Vibrio harveyi*; Swartzman et al., 1990), and the operon-linked *luxI*-*luxR* regulatory pair (*Vibrio fischeri*; Engebrecht and Silverman, 1987). Mutational (transposon insertion) analysis has determined that neither *luxG* (Engebrecht and Silverman, 1987) nor *luxH* (Martin et al., 1989) are directly required for bioluminescence. The case of *luxI*-*luxR* in the *lux* operon of *V. fischeri* most likely represents additional transcriptional regulation required for a successful host specific symbiotic lifestyle.

The simplicity of the genetics of this process makes it somewhat surprising that bioluminescence is not more widespread among bacteria. However, bacterial bioluminescence, as currently known, is restricted to a relative handful of species within only four genera, the most prevalent (by far) being members of the genus *Vibrio* and its close sister genus, *Photobacterium,* as well as *Shewanella*. The one additional genus of bioluminescent bacteria, *Photorhabdus*, is, most likely, a case of lateral gene transfer.

Luminescence and the *lux* operon have been well studied in *V. fischeri, V. harveyi, P. leiognathi, Photobacterium phosphoreum* and even *Photorhabdus luminescens.* However, very little is known about the growing list of *Vibrio* spp. containing luminescent strains. For example, only sequence data for *luxA* has been reported for *V. cholerae*, and most is only partial sequence (Ramaiah et al., 2000; Palmer and Colwell, 1991). Clearly, a survey of *lux*

genes is needed for *V. cholerae*. The finding of dimly luminescent strains by Palmer and Colwell (1991) and the apparently different levels of luminescence expression, independent of cell density, found among Chesapeake Bay isolates (discussed in Chapter 5) can be explained by missing or altered structural genes or, more likely, altered regulation. Sequences of the *lux* operon of non-pathogenic *V. cholerae*, including flanking regions, would provide information that could then be used to probe dark and dimly luminescent strains of *V. cholerae*, by PCR or probe hybridization, to determine the genetic basis of the phenotypes of these strains. This sequence information can also be used to determine chromosomal location and origin of the *lux* operon.

With the advent of whole genome sequencing, it is now possible to estimate whether certain genes present in a bacterial genome were introduced by horizontal gene transfer or vertical transmission. With the obvious case of lateral gene transfer of *lux* genes into *Photorhabdus luminescens*, the origin of *lux* genes in *V. cholerae* is unclear. This uncertainty is supported by the fact that not all species of the genus have luminescent strains. This could be explained by not enough strains of environmental isolates of nonluminescent *Vibrio* sp. having been surveyed or by partial or complete loss of *lux* genes. Origin from a common ancestor would be more plausible if the phylogeny of luminescent *Vibrio* sp. were compatible with DNA-DNA hybridization or 16S rDNA phylogeny, but this is not the case. To test whether *lux* genes in *V. cholerae* were acquired via lateral gene transfer or from a common *Vibrio* sp. or *Vibrionaceae* ancestor, we can examine several characteristics of the *lux* DNA of *V. cholerae* and compare the results with other luminescent species. Sequence homology is the clearest indicator of horizontal transfer and sequences of *lux* structural genes from *V. cholerae* non-O1 can be compared to published sequences of *V. harveyi, V. fischeri,*

P. phosphoreum, *P. leiognathi*, *P. luminescens, Shewanella hanedai, Vibrio salmonicida*, and other species for which the *lux* structural genes have been sequenced. From these sequences, a phylogenetic tree can be constructed using the neighbor joining tree function in ClustalX or PHYLIP software.

It is often difficult to answer the question of the origin of a gene cluster by any single analysis. Two methods for determining lateral acquisition of genes is analysis of G+C content and codon usage (Lawrence and Ochman, 1997). Analyzing G+C content is useful tool the species examined are distantly related or have quite different molar G+C% of their genomic DNA, but is difficult to interpret the data when species have similar genome G+C%. Additionally, after foreign DNA is incorporated into a recipient genome, amelioration takes place through mutation so that the G+C% content changes from that of the donor to that of the recipient. Lawrence and Ochman (1998) used a G+C% content comparison in which they examined only the first and third codon positions. Through this modification, G+C content may be useful in discerning the origin of *lux* genes in *V. cholerae*.

Codon usage patterns allow analysis of genes in which the base composition may be very similar but the patterns of codon usage are different (Karlin et al., 1998). Often, two such indices are determined, one for highly expressed ribosomal genes and one for all other genes. In the case of *V. cholerae,* its genome can be surveyed to determine the codon usage pattern. Then the *lux* genes can be compared to the codon usage patterns of the whole genome of *V. cholerae,* as well as that of other luminescent species

Another rather simple analysis involves examining adjacent DNA sequences of the *lux* operon. Lawrence and Roth (1996) proposed that horizontal gene transfer has mediated the clustering of genes responsible for related functions. Flanking regions of DNA around

the homologous *lux* genes can also be examined for additional homologous gene sequences from other luminescent species. In addition, Lawrence and Ochman (1998) found that many of the horizontally transferred genes in *E. coli* were situated adjacently to a tRNA gene. The tRNA genes have been shown to be potential phage insertion sites in many bacteria and this may be true for *V. cholerae*.

In addition to the above cited analyses, the *lux* DNA sequence, including the flanking regions of the *lux* operon, of *V. cholerae* can be examined for evidence of insertion sequences, such as transposases, inverted repeats, terminal direct repeats, and prophages. Over 500 prokaryotic insertion sequences have been reported (Mahillon and Chandler, 1998). A recent review by Mahillon and Chandler (1998) provides the signatures of various insertion sequences found in *V. cholerae*, which can be used to analyze the *lux* operon and *lux*-adjacent sequence of luminescent *V. cholerae*. Other signs of DNA mobility, such as ERIC consensus sequences and other repeat sequences also can provide useful information.

In the study here, two sequencing methods were used to sequence the *lux* operon of *V. cholerae* strain UM4057, PCR amplification, cloning, and sequencing, and sub-genomic cloning and sequencing using Southern hybridization to identify *lux*-containing restriction fragments. The PCR-based approach relies on known sequences that have been deposited in GenBank by other investigators, of closely related species in order to design degenerate PCR primers. Using these primers, the corresponding *lux* gene from *V. cholerae* genomic DNA is amplified using a proof-reading DNA polymerase, cloned into an appropriate sequencing vector, and sequenced. This method is only applicable to five essential *lux* operon genes, *luxCDABE*, since the organization of the *lux* operon in luminescent species is variable outside of these genes (See Introductory chapter). The *luxA* gene was targeted first to assess

the applicability of the PCR-based approach, since there are more *luxA* sequences available in GenBank, including the *V. cholerae* sequence, compared to the other *lux* genes.

Sub-genomic cloning involves digesting *V. cholerae* genomic DNA, transferring the DNA to a nylon membrane, and probing the blot to identify *lux* gene-specific fragments. Results from the Southern hybridization can be compared to gel pictures prior to blotting to determine the fragment to be excised from a parallel gel. The extracted fragment can be cloned into an appropriate vector, and sequenced. This method requires a known starting sequence, in order to design the first probe. Since the operon contains, at minimum, five genes, multiple steps were anticipated. Sub-genomic cloning is repeated, in steps, using either larger fragments from different genomic digestions and the same *luxA* probe, or smaller fragments and probes designed from sequence data from the first sequencing step. One advantage to using *luxA* as a starting point is that it is located in the middle of the conserved *lux* structural operon, which means that the sequence can be simultaneously "walked" in either direction. This method is ideal for variable regions of the *lux* operon and flanking regions.

To detect *lux* fragments on Southern blot transfer membranes without using radionuclides, the DIG chemiluminescent system was selected. The DIG system (Roche) involves incorporating a digoxigenin-labeled nucleotide, dUTP, into a nucleic acid probe, either by PCR or nick translation. Digoxigenin is a steroid hapten, found in *Digitalis* (Foxglove) plant species. After hybridization, the bound probe is detected using anti-digoxigenin alkaline phosphatase-labeled conjugates. When the blot is incubated with a suitable reagent, such as CSPD, disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2´-(5'-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate, phospatase activity is detected by a

chemiluminescence reaction, which can be detected by exposing the membrane to X-ray film.

DIG-labeled probes are generated using the DIG PCR probe synthesis kit (Roche). The kit allows the direct digoxigenin (DIG) labeling of DNA fragments generated by PCR, using a high fidelity polymerase, which ensures maximal probe yield with the highest accuracy. DIG-11-dUTP is incorporated into the dNTP mix at a concentration of ½ that of dTTP, with their combined concentration equaling that of each of the other 3 dNTPs. The incorporation of DIG-dUTP into the PCR product produces a shift in molecular weight that is dependent on the amount of nucleotide incorporated. For this reason, parallel *Taq* polymerase PCR reactions are performed to accurately size PCR products.

4.2 Materials and Methods

4.2.1 Bacterial strains and media

Luminescent *V. cholerae* strain UM4057 (Palmer and Colwell, 1991) was used to sequence the *lux* operon and flanking regions. Unless stated, Luria Bertani agar and broth were used to propagate *V. cholerae*. When necessary, kanamycin was supplemented at 50-µg/ml.

4.2.2 Genomic DNA extraction

Genomic DNA was extracted from *V. cholerae* UM4057 using the miniprep method of Wilson (1994). Briefly, 1.5 ml of overnight growth in LB was centrifuged in a microcentrifuge (Eppendorff) for 2 minutes. After discarding the supernatant, the pellet was resuspended in 567 µl TE buffer. Thirty µl of 10% SDS and 3 µl of 20 mg/ml proteinase K was added and thoroughly mixed. The suspension was incubated for 1 hour at 37°C to lyse the bacterial cells. One hundred µl of 5 M NaCl was added to the viscous, but clear solution

and mixed thoroughly. Eighty µl of CTAB/NaCl solution was added, and the solution was vortexed and incubated for 10 minutes at 65°C. An equal volume (800 µl) of choloform/isoamyl alcohol (24:1) was added. The mixture was vortexed and centrifuged for 5 minutes in a microcentrifuge to separate the CTAB-protein/polysaccharide precipitate from the DNA in aqueous solution (upper phase). Seven hundred µl of phenol/chloroform/isoamyl alcohol (25:24:1) were added to the viscous supernatant in a clean microcentrifuge tube. The mixture was vortexed and centrifuged for 5 minutes in a microcentrifuge. The supernatant was transferred to a new microcentrifuge tube and 0.6 volume of isopropanol was added to precipitate the nucleic acids. The DNA was pelleted by centrifugation for 5 minutes in a microcentrifuge. The DNA was washed with 70% ethanol to remove residual CTAB, and re-pelleted by centrifugation. The pellet was dried in a Speedvac evaporator for approximately 20 minutes. The DNA was resuspended in 50 to 100 µl TE buffer.

4.2.3 *lux* DNA PCR

Lux degenerate primers were developed using known *lux* sequences from *V. fischeri* and *V. harveyi* and partial sequences from *V. cholerae* (*luxA* only), and employing the GeneFisher (Divide and Conquer consensus method, Giegerich et al., 1996) and/or Primer3 program (Rozen and Skaletsky, 2000). Primer quality was tested using the FastPCR program (Kalendar, 2006). Employing *V. cholerae* UM4057 genomic DNA as the template and *Pfu* polymerase, amplification of *lux* gene targets was accomplished using the following general cycling conditions: initial denaturation at 94°C for 2 minutes; 30 cycles of denaturation at 94°C for 1 minute; annealing at Tm – 5°C for 1 minute; extension at 72°C for 1 minute per expected kb; and a final extension at 72°C for 10 minutes. Gel electrophoresis (2% NuSieve 3:1 gel (FMC), in 1X TAE, 1 hr at 100V in a model MPH horizontal gel apparatus (IBI) was

carried out on the PCR product, with a DNA ladder (Hyperladder IV, Bioline) to estimate size. When multiple bands were generated, the band of appropriate molecular weight was excised from the gel using a clean scalpel and the DNA extracted from the gel slice using a QIAquick gel extraction kit (Qiagen). For reactions producing a single intense band of correct size, cloning proceeded without purification. Blunt-ended *lux* fragments were cloned into vector pCR-Blunt II-TOPO (Invitrogen) using the Zero Blunt TOPO PCR cloning kit (Invitrogen) and the resulting plasmid, pCJG#, was transformed into chemically competent *E. coli* TOP10 cells (provided in the above kit). The plasmid was extracted from the host cells, using QIAprep spin miniprep kit (Qiagen). The inserted fragment was sequenced using the flanking vector M13 primer sites (forward and reverse) on an ABI 3100 automated capillary DNA sequencer.

4.2.4 Restriction endonuclease digestion

Genomic DNA from *V. cholerae* UM4057 was digested using restriction endonucleases. To determine an appropriate set of enzymes to use in the Southern hybridization and cloning sequencing, *V. cholerae* UM4057 genomic DNA was digested by several restriction endonucleases. The restriction enzymes used included *Bam*HI, *Sma*I, *Sau*3AI, *Hae*II, *Apa*I, *Kpn*I, *Mlu*I, *Nde*I, *Xba*I, *Hin*dIII, *Eco*RI, *Ssp*I, *Eco*RV, *Sac*I, *Alu*I, *Bst*XI, *Ceu*I, *Msp*AI, *Not*I, and *Sfi*I. Analytical restriction digestions (small-scale) were performed in 20 µl of 1X reaction buffer (appropriate for each enzyme), with 5 µg of DNA, 2µg of BSA, and 1.2 µl of restriction enzyme (10 U/µl). Quantitative restriction digestions (large-scale) were performed in 200 µl of 1X reaction buffer (appropriate for each enzyme), with 50 µg of DNA, 20µg of BSA, and 12 µl of restriction enzyme (10 U/µl). All digests were incubated in a 37°C water bath for 4 hours, except those for *Bst*XI (50°C) and *Sma*I

(25°C). When applicable, restriction enzymes were heat inactivated by incubation at 65°C for 15 min. Gel electrophoresis was performed on digested UM4057 genomic DNA (0.75% agarose gel, in 1X TAE, 3 hr at 100V), with a DNA ladder, Hyperladder I (Bioline) and/or DIG Molecular Weight Marker II (Roche), included in each gel. The gel was stained with ethidium bromide (1 μg/ml) for 15 minutes, de-stained in distilled water (4:1, DI water to gel volume ratio) for 30 minutes, and visualized under UV light. Gel images were prepared using Kodak 667 Polaroid film or the Kodak EDAS 290 gel documentation system.

4.2.5 Capillary DNA transfer

Transfer of digested *V. cholerae* UM4057 genomic DNA from gel to MagnaCharge nylon membranes (GE Osmonics) was performed according to manufacturer's instructions. Briefly, following gel electrophoresis and documentation, the gel was immersed in 0.25 N HCl for 15 minutes (5:1, HCl to gel volume ratio) with agitation, to fragment the DNA in order to achieve transfer of high molecular weight fragments. The gel was soaked in 1.0 M NaCl, 0.5 M NaOH for 30 minutes to denature the DNA to single strands to allow binding to the transfer membrane. The gel was neutralized by soaking in 0.5 M Tris-HCl, pH 8.0, 0.5 M NaCl for 30 minutes. The transfer solution employed was 20X SSC. Transfer was accomplished by upward capillary transfer of transfer solution through three pieces of 3MM filter paper (Whatman), gel, and then membrane, prewetted in 2X SSC. Following transfer, the compacted gel was re-stained with ethidium bromide (0.5-μg/ml) to test for transfer efficiency. The nucleic acid on the membrane was immobilized by baking in a vacuum oven at 80°C for 15 minutes. The DNA on the dry membrane was further immobilized by exposure to UV irradiation in a Model FB-UVXL-1000 UV crosslinker (FisherBiotech) set on the optimal crosslink function (120,000-μJ/cm^2).

4.2.6 DIG-labeled probe synthesis

DIG-labeled *lux* probes for Southern hybridization were generated using the PCR DIG probe synthesis kit (Roche). Briefly, *lux* genomic DNA (100-ng) from *V. cholerae* UM4057 was amplified in 1X PCR buffer containing 0.8 µM each primer (VCLUXA108F, VCLUXA757R for initial *luxA* probing), 200-µM dNTP, and 2.6 U of Expand enzyme mix, using the following cycling conditions: initial denaturation at 94°C for 3 minutes; 30 cycles of denaturation at 94°C for 1 minutes; annealing at T_m of primer set (i.e., lowest T_m of forward and reverse primer pair) - 5°C for 1 minute; extension at 72°C for 1 minute; and a final extension at 72°C for 10 minutes. A parallel PCR reaction (to assess size of the product accurately) was prepared using the same primers, Taq DNA polymerase, and standard 1X PCR reaction, as well as a negative control reaction, which contained MilliQ water instead of DNA. Gel electrophoresis, 2% NuSieve 3:1 agarose (FMC), in 1X TAE, 1 hr at 100V in model MPH horizontal gel apparatus (IBI), was carried out on the PCR product.

4.2.7 DIG Southern hybridization

Hybridization of digested genomic DNA of *V. cholerae* UM 4057 immobilized on nylon membrane, was performed using DIG-labeled lux probes, as prepared above, following the manufacturer's (Roche) instructions. Optimal hybridization temperature was determined by the following equation, $T_{opt} = T_m - 20°$ to 25° C, where $T_m = 49.82 + 0.41(\%G+C) - (600/l)$, and l = length of probe in base pairs. For the initial *luxA* probe, the optimal temperature for hybridization is 48.9° to 43.9°C, so 45°C was chosen. Blots containing digested DNA of *V. cholerae* UM4057 were prehybridized in (10 ml per 100 cm^2 membrane) DIG Easy Hyb solution, preheated to the hybridization temperature (45°C) in a hybridization oven. Blots were trimmed to approximately 100 cm^2, after transfer by removing the bottom

portion of the membrane, containing no DNA or DNA under 500-bp in length (as determined by the ladder). DIG-labeled probes were denatured by boiling for 5 min and then rapidly cooling in an ice bath. Denatured probe was added to pre-warmed DIG Easy Hyb solution at a rate of 2 µl probe per 1 ml hybridization solution. The prehybridization solution was removed (hybridization bottles were used as vessels) and the probe-hybridization solution mixture was added (3.5 ml per 100 cm^2 blot). The hybridization proceeded overnight (approximately 16 hours).

4.2.8 Hybridization washes and development

The following stringency washes and development are based on a 100 cm^2 blot. The hybridization solution containing probe was removed and the blot washed using two different stringency solutions: 2 x 5 min in 75-ml 2X SSC, 0.1% SDS at room temperature; and 2 x 15 min in 75-ml 0.5X SSC, 0.1% SDS at 65°C, both under constant agitation. The blot was washed in 75-ml of 0.1 M Maleic acid, 0.15 M NaCl, 0.3% (v/v) Tween 20, pH 7.5 for 5 min. The blot was incubated in 100-ml of 1X Blocking solution (Roche) for 30 minutes. The blot was then incubated with anti-digoxigenin alkaline phospatase (Roche, 75 mU/ml) in 20-ml 1X Blocking solution for 30 min. Blots were washed two times for 15 minutes in 100-ml of 0.1 M Maleic acid, 0.15 M NaCl, 0.3% (v/v) Tween 20, pH 7.5. The blot was allowed to equilibrate in 20-ml of 0.1M Tris-HCl, 0.1M NaCl, pH 9.5 for 5 minutes. The blot was transferred to a hybridization bag. One ml of CSPD (Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2´-(5'-chloro)tricyclo [3.3.1.$1^{3,7}$]decan}-4-yl)phenyl phosphate) was added to the membrane and the membrane was incubated for 5 minutes at room temperature. Excess CSPD was removed from the pouch, aftter which the pouch was sealed and incubated at 37°C to enhance the chemiluminescent reaction. The blot was exposed to BioMax X-ray

film (Kodak) for 15-20 minutes at room temperature, and then developed in a Mini-Medical series 90 film processor (AFP Imaging Corp., NY).

4.2.9 Stripping of Southern blot membrane

The alkali-labile DIG-11-dUTP probes used in this study are easily stripped from the membrane, so that the same membrane can be used for multiple probings. After detection, the membrane was rinsed thoroughly in deionized water. The membrane was washed twice, 15 minutes each, in 0.2 M NaOH, 0.1% SDS at 37°C. The membrane was rinsed in 2X SSC for 5 minutes and stored in the same buffer until used again.

4.2.10 *Lux* fragment cloning

Once *lux*-gene containing fragments were identified by Southern hybridization, large-scale *V. cholerae* UM4057 genomic DNA digestions with the appropriate restriction endonuclease was performed, as above. Gel electrophoresis was performed as above, and gel slices containing the *lux*-gene fragment were excised using a clean scalpel. The DNA was extracted using a QIAquick gel extraction kit (Qiagen) and concentrated using the method of Moore (1996). Briefly, 1/10 volume of 3M sodium acetate, pH 5.2, was added to the eluted DNA, and the mixture was vortexed. Two volumes of ice-cold 100% ethanol was added to the mixture, vortexed, and incubated at -20°C, overnight. The DNA was pelleted by centrifugation for 5 minutes in a microcentrifuge. The DNA was washed with 70% ethanol and then re-pelleted by centrifugation. The pellet was dried in a Speedvac evaporator for approximately 20 minutes. The DNA was resuspended in 25 to 50 µl sterile MilliQ water.

4.2.11 Vector preparation

A vector, pTOPOH/E, was used to accept *lux*-gene fragments extracted from digested *V. cholerae* UM4057 genomic DNA. The plasmid is a modified form of vector pCR-Blunt

II-TOPO (Invitrogen) in which the vector was digested with *Eco*RI, then ligated with a *Eco*RI-*Stu*I-*Eco*RI linker. The resulting plasmid contains a new and unique *Eco*RI and *Stu*I site, along with unique *Hin*dIII, *Kpn*I, *Sac*I, *Bam*HI, *Spe*I, *Pst*I, *Eco*RV, *Not*I, *Xho*I, *Xba*I, *Dra*II, and *Apa*I sites.

To accept restriction endonuclease generated *lux*-gene fragments, 5.0 µg of vector pTOPOH/E was digested with 3.0 µl of the same restriction enzyme for two hours at the appropriate temperature, in 110 µl 1X reaction buffer with 11.0 µg BSA. Following digestion, the vector was dephosphorylated by adding 5.0 µl of Antarctic phospatase and 11.0 µl of 10X AP buffer and incubating at 37°C, for 30 minutes. Both enzymes were heat inactivated at 65°C for 15 minutes.

4.2.12 Ligation and Transformation

Restriction endonuclease generated *lux* fragments were ligated into linear and dephosphorylated pTOPOH/E at a 3:1 insert:vector ratio (molar ends) in 20 µl 1X ligation buffer with 3.0 U (cohesive ends) or 6.0 U (blunt ends) T4 DNA ligase at room temperature for 1 hour (cohesive ends) or at 15°C overnight (blunt ends). Negative control ligations, no insert and no ligase, were performed in parallel. Two to four µl of the cloning reactions were added to 50 µl thawed, ice-cold, chemically competent One Shot TOP10 *E. coli* cells (Invitrogen). The genotype of these cells is F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G. The mixture was incubated on ice for 30 minutes. The cells were heat shocked at 42°C for 30 seconds, and immediately placed back on ice for 5 minutes. Two hundred fifty µl of S.O.C. medium was added to the cells and the cells were incubated in a shaking (225 rpm) 37°C incubator for

1 hour. Ten, 50 and 100 µl aliquots of the transformed cells were spread-plated onto LB amended with kanamycin and incubated at 37°C for 2 days.

4.2.13 Clone analysis

Clones were analyzed by PCR as follows. Clones were subcultured onto fresh LB plates, amended with kanamycin, and incubated overnight at 37°C. Single colonies were transferred into a PCR reaction tube containing 5 µl of MilliQ water. To each tube, 20 µl of Taq Master Mix containing 1.25 X PCR buffer, 250 µM dNTPs, 1 µM each primer and 0.625U Taq polymerase, was added. Primers used corresponded to those used to produce the DIG-labeled *lux* probe. For clones giving a positive PCR signal, plasmid DNA was extracted using the QIAprep Spin Miniprep kit (Qiagen). The presence of inserted *lux* fragment was confirmed by sequencing using flanking vector M13 primer sites. For fragments of 700-bp or smaller, this size ensures sense and anti-sense strand sequencing coverage to obtain accurate results, using the M13 sites. For fragments larger than 700-bp, the inserts were sequenced via primer walking, i.e., sequencing done by rounds, in which new primers were designed from sequence yielded from the previous round. In this case, the first round of sequencing was accomplished with the vector flanking M13 primer sites and original probe primers. The remaining sequence was filled in by primer walking until coverage of both strands was accomplished.

4.2.14 Sequence analysis and alignment

Open reading frames (ORFs) of the *lux* operon of *V. cholerae* were determined by comparing the sequence with published *lux* genes from other luminescent species and by using the NEBcutter V2.0 program (Vincze et al., 2003) which searches for start and stop codons using the bacterial genetic code. When two or more ORFs significantly overlapped,

the smaller one was dropped, keeping only the longest, non-overlapping ORF. Only ATG was accepted as an initiation codon. For each *lux* gene (6 total), multiple sequence alignments were performed using the ClustalX program (Thompson et al., 1997). Table 4.1 shows the sequences that were used for each alignment. From the alignments, trees were drawn using the neighbor-joining (NJ) method of Nei et al (1985).

Table 4.1. The *lux* gene sequences used for *lux* gene sequence similarity alignments and trees.

		***lux* gene sequence used**					
Species and strain	**Accession #**	***C***	***D***	***A***	***B***	***E***	***G***
V. cholerae UM4057	this study	+	+	+	+	+	+
V. cholerae TP	AY876056			+			
V. harveyi CTP5	X58791			+	+		
V. harveyi NBRC 15634	DQ436496			+	+		
V. harveyi B392	M10961			+	+		
V. harveyi B392	E12410			+			
V. harveyi B392	X07084	+					
V. harveyi B392	J03950		+				
V. harveyi B392	M27139						+
V. harveyi B392	M28815					+	
V. fischeri MJ-1	AF170104	+	+	+	+	+	+
V. fischeri MJ-1	X06758			+	+		
V. fischeri ES114	NC_006841		+	+	+	+	+
V. orientalis ATCC 33934	AB058948			+			
V. salmonicida NCMB 2262	AF452135	+	+	+	+	+	+
S. hanedai ATCC 33224	AB058949		+	+	+	+	+
P. leiognathi ATCC 25521	M63594	+	+	+	+	+	+
P. leiognathi 554	X08036			+	+		+
P. leiognathi Pl_1741	X65156	+					
P. leiognathi Pl_1741	X65612		+				
P. leiognathi Pl_1741	U66407					+	
P. phosphoreum NCMB844	X55458			+	+		
P. phosphoreum NCMB844	L21989	+					
P. phosphoreum	M65067				+		
P. phosphoreum	M64224		+				
P. phosphoreum NCMB844	X55459					+	
P. luminescens TT01	NC_005126	+	+	+	+	+	
P. luminescens Hm	X53783		+				
P. luminescens ZM1	AF403784			+	+		

4.2.15 Lateral gene transfer analysis

Several simple nucleotide descriptive statistics of the *lux* operon from *V. cholerae* UM4057 were compared with the *lux* operons of other luminescent species and with that of the whole genome of *V. cholerae* N16961, to include total G+C%, 1st nucleotide G+C%, 2nd nucleotide G+C%, and 3rd nucleotide G+C%. In addition, codon usage tables were prepared. The results from the codon usage tables were statistically analyzed using the Chi-Square frequency test.

4.2.16 Presence of mobile elements

Fifteen insertion sequences have been described in *V. cholerae*, 13 IS*5*s (ISVNR), 1 IS*605* (IS*1004*, *V. cholerae* O1), and 1 ISA*s1* (IS*1358*, *rfbQRS* of *V. cholerae* O139 Bengal) (Mahillon and Chandler, 1998). These insertion sequences can be detected by a conserved DDE motif, direct repeats, conserved terminal base pairs and terminal inverted repeats. The *lux* operon and flanking region of *V. cholerae* UM4057 was translated to protein residues and scanned for the DDE motif from IS903 (ISVNR), lai**D**-TG (71)-S-**D**GAYDTr-(67)K---gYh-RSls**E**TAMyRv**K**. One key feature that most mobile elements share, including insertion sequences, is the presence of short (10-40 bp) terminal inverted repeat sequences. The *lux* operon of *V. cholerae* UM4057 was scanned for various repeat sequences, using the FastPCR program.

4.3 Results

4.3.1 Sequencing by PCR

To evaluate the feasibility of the PCR-based approach, the PCR primer pair LUXA93F (5'-WGAAGRKTGTGGYTTCGA-3') and LUXA902R (5'-AYCTTCWGGYGTYCCTA-3'), were designed based on *luxA* sequences from *V. fischeri*

(GenBank Accession # AF170104, X06758), *V. harveyi* (E12410, M10961, X58791, AF147084, AF147086), and *V. cholerae* (partial, AF147090, M60437, M60438, M60439). An 827-bp fragment of *luxA* from *V. cholerae* was successfully amplified and sequenced. Subsequent PCR-based sequencing efforts aimed at *luxC*, *luxD*, *luxB*, and *luxE* failed, in that the designed degenerate primers amplified products of approximate size, but upon sequencing, were determined to be alternate products. This is mostly due to the fact that there is less sequence data deposited in GenBank for the other four *lux* genes. No further attempts were made using this approach.

4.3.2 Subgenomic cloning using Southern hybridization

Three enzymes, *Hin*dIII, *Eco*RV, and *Ssp*I, were chosen for quantitative restriction enzyme digestion, based on their ability to digest *V. cholerae* UM4057 DNA easily so that the median and mode fragments were approximately 1 to 4-kb in size, with a range of resolved fragments from 800-bp to approximately 12-kb in size, when the reaction was allowed to proceed for 4 hours. Other enzymes were either infrequent or frequent cutters, yielding large molecular weight or low molecular weight fragments. The *lux* operon and 5' and 3' flanking regions of *V. cholerae* UM4057 were sequenced by successive overlapping restriction digestions. Figure 4.1 provides a linear schematic representation of the 9.8 kb of genomic DNA that was sequenced, showing the pattern of the seven successive Southern hybridization and subgenomic cloning steps. The primer pairs listed in Table 4.2 were used to generate probes for each of the cloning steps; the number in column 3, Table 4.2 corresponds to the cloning step in Figure 4.1.

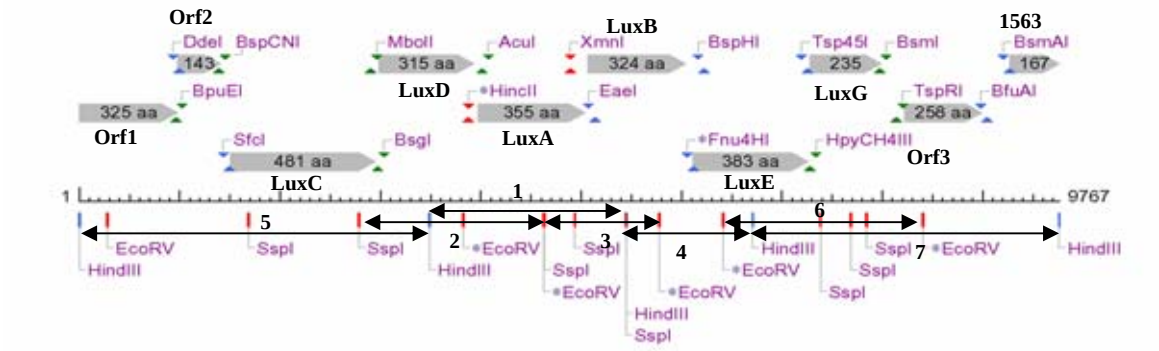


Figure 4.1. Schematic representation of the 9767 bp *V. cholerae* UM4057 *lux* operon and flanking region sequence determined by subgenomic cloning method. *Eco*RV, *Hin*dIII, and *Ssp*I sites are labeled as such, and the SPPS steps are numbered and shown with bold arrows below the ruler. ORF's are shown above the ruler.

To begin the subgenomic cloning approach, new *luxA* PCR primers (SPPS 1, Table 4.2) were designed (see Chapter 2 for details), in order to produce a smaller *luxA* product, better suited for DNA probe hybridization. Using *V. cholerae* UM4057 genomic DNA as the template, an expected 650-bp fragment of *luxA* from *V. cholerae* was successfully amplified, using *Taq* polymerase. A single band of approximately 800-bp was present, when the parallel DIG PCR reaction was performed. The incorporation of DIG-dUTP into the PCR product produces a shift in molecular weight that is dependent on the amount of nucleotide incorporated. For the 650-bp *luxA* product, this shift is approximately 150-bp. The DIG-labeled *luxA* probe was used to probe *V. cholerae* 4057 genomic DNA which had been digested with *Hin*dIII, *Eco*RV, or *Ssp*I, and transferred and immobilized onto a nylon membrane. The genomic DNA digestion with *Hin*dIII gave a sharp, single band of approximately 2.0 kb. This *luxA* fragment was cloned into vector pTOPOH/E, which had also been digested with *Hin*dIII. The resulting plasmid, pCG1, was sequenced using vector M13 primer sites flanking the insert and reverse primers of VCLUXA108F (-R) and VCLUXA757R (-F).

Table 4.2. PCR primers used for PCR cloning and sequencing and for producing Southern hybridization lux probes.

Name	SPPS #[a]	Sequence[b]	Target	Amp. size (bp)	Tm (°C)
LUXA93F		WGAAGRKTGTGGYTTCGA	*luxA*	827	48
LUXA902R		ATYCTTCWGGYGTYCCTA	*luxA*		
VCLUXA108F	1	CGAAGCGGTTTGGTTGCTA	*luxA*	650	59
VCLUXA757R		CGGGTAGCATTGACGTAGGA	*luxA*		
pluxD548F	2	GTGCGGAAGTCTTTGTTCGT	*luxD*	210	55
pluxD757R		GATCATGCGAGCTTCCAAGT	*luxD*		
luxA849F	3	AGGACATCGCGATACCAATC	*luxA-B*	532	58
luxB285R		TTGGTCGAGTAGCCCAGTTT	*luxA-B*		
pluxB418F	4	ACCACAGGTTATTGCCATCC	*luxB*	195	58
pluxB611R		TATTCTTGCCGAGTCGCATT	*luxB*		
luxCD83F	5	GCCGTTTCACAACCAAAGAT	*luxC-D*	153	60
luxCD235R		GGTCATCCACTTCCCAATGT	*luxC-D*		
pluxE359F	6	CTCGAGACCGCCTGAGTATT	*luxE*	201	58
pluxE559R		CGAGGTCATCTTTTGCGGTA	*luxE*		
luxE1135F	7	CCCCACATCACACCATAAGA	*luxE-G*	200	58
luxG163R		GTGAAGGGCAACTAGCAATA	*luxE-G*		

[a]Southern Probe Primer Set #
[b]W = A or T, Y = C or T, K = G or T, R = A or G

The sequence from the 1968-bp *Hin*dIII *luxA* fragment was digested with *Eco*RV and *Ssp*I using the NEBcutter V2.0 program (Vincze et al., 2003). The sequence contains 2 *Eco*RV sites at nt 335 and 1136 and 2 *Ssp*I sites at nt 1145 and 1448. Two new DIG-labeled *lux* probes flanking these two sites were generated using primer pairs pluxD548F/pluxD757R (SPPS 2) and luxA849F/luxB285R (SPPS 3), designed using the Primer3 program (Rozen and Skaletsky, 2000). The Southern blot used to probe for *luxA* fragments was stripped and re-probed with each of these two new probes separately. Probing with SPPS 2 revealed an *Ssp*I digested fragment of approximately 1.8 kb, while probing with SPPS 3 revealed an *Eco*RV fragment of 1.2 kb. Both fragments were cloned into vector pTOPOH/E, which had been digested with *Eco*RV. Subgenomic cloning step 2, using SPPS 2, yielded 1854 bp of sequence, which extended the known sequence 706 bp in the 5' direction. Subgenomic cloning step 3, using SPPS 3, yielded 1158 bp of sequence, which extended the known

sequence 323 bp in the 3' direction. At the end of subgenomic sequencing steps 1-3, the known sequence was nearly 3 kb.

Two new probes were generated, SPPS 4 and SPPS 5, by the Primer3 program (Rozen and Skaletsky, 2000) using the sequence between the 5' *Ssp*I and *Hin*dIII sites and the 3' *Hin*dIII and *Eco*RV sites, respectively (Table 4.2, Figure 4.1). Probing digested *V. cholerae* UM4057 genomic DNA with SPPS 4 revealed a *Hin*dIII fragment of approximately 1.2 kb, while probing with SPPS 5 revealed a *Hin*dIII fragment of 3.5 kb. Both fragments were cloned into vector pTOPOH/E, which had been digested with *Hin*dIII. Southern cloning step 4, using SPPS 4, yielded 1264 bp of sequence, which extended the known sequence 935 bp, in the 3' direction. Subgenomic cloning step 5, using SPPS 5, yielded 3495 bp of sequence, which extended the known sequence 2783 bp, in the 5' direction. At the end of sequencing steps 1-5, the known sequence was 6.7 kb (Figure 4.1).

The last two subgenomic sequencing steps were done successively, using probes generated by SPPS 6 and 7. A Dig-labeled probe generated by SPPS 6 identified a 2 kb *Eco*RV fragment from digested *V. cholerae* UM4057 genomic DNA. The fragment was cloned into *Eco*RV-digested pTOPOH/E and sequenced. The sequencing step yielded 1993 bp, which extended the known sequence 1691 bp in the 3' direction. A Dig-labeled probe generated by SPPS 7 identified a 3 kb *Hind*III fragment from digested *V. cholerae* UM4057 genomic DNA. The fragment was cloned in *Hin*dIII-digested pTOPOH/E and sequenced. The sequencing step yielded 3058 bp, which extended the known sequence 1361 bp in the 3' direction. No further sequencing was performed and the known *V. cholerae* UM4057 sequence was 9767 bp. The entire sequence is provided in Figure 4.2, with *lux* gene start and stop codons, restriction enzyme digestion sites used, and SPPS primers indicated.

AAGCTTCTGAGCAATACAAAGGGGTTCATAAAATCGGCTCCGTTGGTGTGGGAGTTGGGGCG
TGCTATGAAGACAAATCCAATCGTTTAGGTATTCGATTGCAAGATACGCTCAATGAGTCTGT
ATTACGTCATAAGCTCACTCTGCTTTGGGAAATTCGAGAAAAGCAAATTGAATCCGTCTTTG
AAGGGCGGCTTCACCTCAATTTTGAACAACAAGTGCAATACATACTCAGGCTCGGTAAACAA
CTAGAACCCTATTTTTGCTTCACTAATCAAGTGATATCACGCTTACTTCAACGTGGCGAAAG
CATGTTATTGGAATCCTCTCAGGCCACTTTTCTTGATAACAGTTTCGGAACTTACCCTTACA
CAGTGGCCTACTCCACCTTAATTCAGAGCTGCTTTGCTTCAATAGGCATTCCAGCTCAAACG
TTACAAGTATTAGGAGTGATGAAGGCGTACATGATCCGAGTTGGCAATGGTCCATTTCCGAC
AGAACTTGATGGTGACATTGCACAGTACATTCGAGTGAAGGGCAATGAGTTTGGTACGGTAT
CGCAAAGACCAAGGCGCTGTGGCTGGTTAGATCTTGCTTTGGTTGAACAAGCGATTCATCTC
AATGGCGTCAAAGAAGTGGCGATCACCAATCTTGATGTTCTCGGTGGCATGGATGAAGTTTG
GGTATGTAATGGATATTTATTACACGGAAAAAAAGTGTGCACTGATGAAGCTCTTCTTCATT
TTGATCAAGTCGAGCCTATTTACACAAAATTCCAAGGTTGGCCAAGACTCCCTAATGGCATT
CTAAAATTATCCGATTTACCCAAAGAACTACTTCATTATCTTGCTTATATTCAATCCTATAT
CAGTGCACCGATTCGATATATTTCCTATGGCGCAGAACGCGAATACACAATTCATATCGAAC
CTAACGGATTCAGCCAAATTATCCTTGCAGATTCTGAGGAATACGCATGATCAATTATGTTA
CTGGGTTTATGTTCTCTCAAGACCTATTAAACGTCGGATTAATCAAAAAATTAAAACCCAAA
TGGCAGAGTGGATTATATAACGGTATCGGAGGAAAGATTGAACATGATGAATCACCCTATGA
AGCCATCGCGAGGGAGTTTCAAGAAGAGACTGGCGTCGAGACAAAGCCAGATGAATGGAAGT
TGTATGTGACCTTAACTAGGCCAGATATTTATCGAGTTTATTTCCTTTGCATGATTAGTGAC
AAAGTCCATAACATTCGAACAACGGAAGAAGAAATTGTGAAATTATTACCTTGCGATGCATT
ACCTAAAAATGTCATTCATAATTTACGGTGGTTAATTCCTCTGGCCCTTGATAAATCATTAT
CGATGACGACACCCATTTGCATTGAAGAAATTAAAAACCCATAAATACCAATCCAAAAACCA
TCACACTGAGTCTATAAAGTTAATTCTTTATTTAAAAACACTGTAGCGCAGTAAAATATCAA
AAAAGAGCTTAAATAAT***ATG***AAAAAACACATGCCATTTATTATTAATGGAAGAGTGACTGAT
TGTTCAAAAAAATCAGAATCAGAACAAAAAAGCATTTGTTTTCGCGAAAACAGTCTCAACCT
AGATGTTTTAACCGATCACATCTCAGATGAAATAACAAGTTATAAAAACAGTCTGGAATTAA
CACTCAATAATATTGTTAATTTCCTTTATACCGTAGGTCAACGTTGGAAAAATGAAGAATAC
ACCAGAAGACGTAGCTATATTCGGGATTTGAAAAACTTCTTGGGTTATTCCGAAGAAATGGC
CAAATTGGAAGCCAATTGGATCGCCATGATTCTGTGCTCTAAAAGCGCGCTATATGACATTG
TACAAACTGAGCTAGGTTCACGTCATATATTGGATGAATGGGTAGCACAAGATGAGTGCTAT
GTTAAGGCCTTGCCTAAAGGCCGTACACTGCATTTATTAGCAGGCAATGTGCCTCTTTCAGG
CGTAACCTCTATTTTGCGCGCCATTCTGACCAAAAATCAGATAATCGTAAAAATGTCGTCGA
ATGATCCGTTTACCCCTCATGCATTAGCGATGAGTTTTATCGATGTCGATCCAAATCATCCC
ATTACCCAATCCATTTCCATTATTTACTGGCCTCATACGCAATGCACTCAGGTAGCCCAACG
TTTAATGCAAAAGATGGATGTGGTCGTCGCTTGGGGCGGTTCAGAAGCAATACGTTGGGCCG
TAGAGCATACCCCCCCGCATGCTGAGCTCATTAAGTTTGGCCCGAAAAAAAGCCTCACCATC
ATCAATGATCCTGAGAATTTGATCGAAGCGGCCGAAGGAGCCGCTCACGACATCTGTTTTTA
TGACCAACAAGCCTGCTTTTCAACGCAAAATCTTTTTTATATCGGTTCTCGTTTTCTTGAAT
TTAAGCAAGCGTTAAGAGAGCAATTACAGCGCTATGCTCGAATTTTGCCAAAGTCGCAATCC
AGTATTGATGAGCAAGCCGACTTCTCCTTAACTCTGCGTGAATGCCAATTTGCAGGGTTTAC
CGCCGAAATGGGTAACCAACAAAATTGGATGATGATTGAATCTCCAGCCGGCGTAGACCTGA
ATCATCCATTAGGGCGCTGTATTTATCTGCATCAAATGGCTTCATTTGAGGAAGTGCTGCCC
TTTGTCATTAAAGGACAAACTCAAACGGTTTCCCTCTTCCCTTGGTCCTGTTCATTGCAATA
TCGCGATCAACTCGCTGCTCACGGCGCTGAGCGTATCGTTGAGTCGGGGATGAAT***AATATT***T
TCCGCGTCGGTGGAGCCCATGATGCCATGCGTCCATTGCAGCGCTTAGTGCGTTTTATTTCC
CACGAACGCCCTA*GCCGTTTCACAACCAAAGAT*GTCGCCGTCGCTATTGAGCAGACACGCTA

TCTGGAAGAAGACAAATTCTTAGTCTTTGTACCT**TAG**TTACCCAAAAACAACATCATAAAGG
AGAAATGA***ATG***CCTCACTCATCACATTGGGAAGTGGATGACCCCTGCACCATTGATCATGTG
ATTTCTTTATCTGGAAATCAGCAAATTCATGTTTGGGAAACCCCTCCAACACTTAAATTTAA
TTCAATGGCAACGCGGCGGAATAGCATTTTAATCGCATCTGGCTTTGCTCGTCGGATGGATC
ATTTCGCAGGTCTAGCGCGCTATTTGTCCGCCAACGGATTTCATGTTTATCGCTATGATTCA
CTTCACCATGTTGGATTAAGCTCCGGCACGATCGATCAATACACGATGAGTATTGGCAAGCA
GAGTCTGGAAATCGTGATGGCTTGGCTTCATAACCGCGGAGTAACAGACATTGGGGTGATTG
CAGCGAGCTTATCCGCCCGAATTGCTTACGCTAGCGTAAATGAACTCGACCTATCCTTCTTG
ATCACCGCGGTTGGGGTG*GTGAATCTGCGCGATACCTT*AGAAAAAGCCCTTGGTTTTGATTA
CCTCAGCCTCCCTATTC***AAGCTT***TGCCAAACGATCTGGATTTTGAAGGGCATAAATTAG*GTG*
*CGGAAGTCTTTGTTCGT*GATTGTTTTGCAAATCACTGGGATAGCTTTTCATCCACGGTAGAA
CAAATTCGCCATCTCTCGATCCCTTTCATTGCGTTTACCGCCAATGGAGATGATTGGGTCAA
GCAAGACGAAGTTATTGAGATGCTTTCCCATATTCCTCGTGGTCAAAGCAAGCTGTATTCCC
TACTTGGAAGCTCGCATGATCTCGGTGAGAACTTAGTGGTACTCCGTAACTTCTACCAATCA
GTGACTAAAGCAGCAATCGCTCTCGATTCAAATAGTTTCGATATCAACATCCCTTTTGTGGA
GCCCACATTTGAGCAATTGACTATCGCAACCGTCAACGAGCGTCGAATGAAAAACCAAATTG
AAACAGAAACGATGATGCTGACA**TAA**ACCTACTCATACCTTACCTACCTTAATCAAGGACAT
ATT***ATG***AAATTTGGAAATTTCTTACTAACCTACCAACCACCTGAGTTATCACAAACAGAAGT
GATGCAACGCTTAGTGAATCTTGGACGAGCTTCAGAATCATGCGGTTT*CGAAGCGGTTTGGT*
*TGCTA*GAACATCATTTCACTGAGTTTGGTTTACTCGGTAATCCTTATGTTGCCGCCGCAAAC
CTCTTGGGAGCCACAAAACATCTTCATGTCGGTACGGCGGCGATCGTACTGCCGACCGCTCA
CCCCATTCGACAGCTTGAAGATGTAAACTTACTCGATCAATTGTCGAAAGGACGTTTTCGTT
TCGGCATTTGCCGTGGGCTCTATGACAAAGATTTTCGAGTGTTTGGCACTGACATGAACAAC
AGCCGCGCGTTGATGGATTGCTGGTATGACTTAATCACGACAGGCATGACACAAGGCTCTGT
CTCTGCGGACAACGAGCATATCCATTTCCCTGAAGTGAAAGTCCAACCTGCCCCCTATCATC
GTAGTGGTGCTCCTGTCTATGTGGTCGCTGAGTCTGCTTCAACCACTGAGTGGGCAGCAAAG
CGGGGTTTACCCATGATTCTGAGCTGGATTATCAACACTCATGAGAAAAAAGCGCAACTTGA
CCTTTATAACGAGATCGCGTTAGAGCATGGACAC***GATATC***CAG***AATATT***GATCACTGCCTCT
CCTACATCACCTCTGTGGATCATGATTCACAACGCGCAAAAGACATCTGTCGCCAATTTCTT
GCCCACTGGTATGACTCCTACGTCAATGCTACCCGCATTTTTGATGATTCAGACCAAACTAA
AGGCTATGACTTTAATAAAGGGCAATGGCGAGATTTTGTTCTAAA*AGGACATCGCGATACCA*
*ATC*GTCGTATTGATTACAGCTTCGAAATCAACCCAGTAGGAACACCAGAAGAATGTATTCGA
ATTATTCAGCAGGATATTGATGCAACGGGGATTACTAATATTTGCTGTGGATTTGAAGCCAA
CGGTTCAGAACAAGAAATTATCGCCTCAATGAAGCTCTTTCAAGCTGAAGTGATGCCTCACC
TCAAATCCACAAAC**TAA**CGCCAACTATTTTCAAAGGAAGAACCT***ATG***AAATTCGGATTATTT
TTCTTAAATTTCCTTCATTCTGGCCAATCATGCACAGAGGTTTTCGATGCCATGATCGATAG
TGTGAATTACGCGGAAAAAGGACACTTTGATCGACTGTTTATTTATGAGAACCATTTTAGTG
ATCACGGCATTGTAGGTGCCCCACTGACTGTAGCTAGCTTTTTATTGGGCATGACCGAGCGT
ATCAAGGTTGGTTCACTCAATCATATTTTGACCACTCACCATCCCGTCCGAACGGCTGAAGA
AACTGGGCTACTCGACCAAATGAGTCAAGGCCGTTTCATTTTAGGTTTCAGTGATTGTGAAA
ACCGCGACGAGATGAACTTTTTTAACCGCCCTTTAGATTCTCAGCAGCCAATATTCG***AAGCT***
TGTTATCAGATTATTAATGACGCTCTA*ACCACAGGTTATTGCCATCC*AAATAATGACTTTTA
TAGTTTCCCCAAAATTTCTGTTAATCCACATTGTTTTACCCAAGGGGGGCCGCAACAGTACG
TTTATGCCTCTAGTACACAGGTCGTGGAATGGGCAGCTAAACGGGCATTGCCGTTGACCTTT
AAATGGGATGATAGTAATGCGACTCGGCAAGAATATGCGCAACGTTATCAAGAGACAGCAAG
AAAACATGGGGTTGATGTTCAAGCCGTACGCCATCAACTCGCGTTATTGGTCAATCAAAATG
ATGATGGC***GATATC***GCGCGTGCAGAGGTTGGTCAATACCTATCCCAATACCTCACGGAACGC

TATCCCAGCGATGAAATTGAGGTCGTCTTAGAACGCATCATCAAAGAAAGTGCCATTGGTAC
ATACGAAGAAAGCACTCATGCCGCACGAGTTGCTATTGAAATGTGTGGTGCTTCTGATCTGC
TGCTCTCTGTTGAGTCGATAAAAGAGCCTGCTCACCGCCTGCATGTATTGGATGTGATTAAT
AGCAACATCGCTAAATACCATCAA**TAA**AAGATTTTTCTTTTTCCCTTGGGCCGCCGCATCCT
CCTTCGGTGGCGGCCCTTTTTTAAGGAATACACA***ATG***AATACCACGCTGTCCTTAATTGACA
CGCCTGCCGTTGAGAAATTTGATATTGAAGCAAGCTCCTACCTTGATGATTTAATCTTCATG
AGTGAGCCGAACTCATGGTCATACGAAGAGCAGAAAAACATTCGACATGATGTGATCATGAA
AGCCTTTCGTTGGCACTATCAGAACAATGCTGACTATCGTCGTTATTGCCAAACTATCGGTA
TTGGCCTTGAAATAGAGCATTTGGACCATATCCCCGTTTATCCAACGTCTGTATTTAAAACG
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GCTATGGAATGAAATTGGTGGGTACATGGTTTGACCATGAAATGGAGCTTGTTAATTTAGGT
CCAGATCGATTTAACGCTCACAACATCTGGTTTAAATATGTGATGAGTCTTGTTGAATTACT
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TGTACCGAATTCA***AAGCTT***GGGAAAAACCGCTTGCTTAATTGGCCCGCCCTATTTTATTTAT
CTTCTATGTCAGCACATGAAAGCAGGAAAAATCCACTTCCGAGCTGGGCATAAACTTTACAT
CATCACTGGAGGTGGGTGGAAAACTCACCAGAACTCCGCCTTAAATAGAGATCAATTCAATC
AATTACTAATGGAAACTTTTGGTCTCAACGATATAAGCCAAATACGCGATACATTCAACCAA
GTTGAACTCAATACTTGCTTTTTTGAAGATGATCAGCAACGCAAATGTGTTCCACCTTGGGT
GTATGCTCGGGCGTTAAATCCCAGAACTCTTCAACCTTTATCTGATGGAGAAATAGGTTTAA
TGAGTTATATGGATGCATCCGCGACAAGCTACCCTGCATTTTTAATTACGGATGATCTTGGT
TACGTTCATCAGCCCACTGAGGAAATACCTTATACAAGAGTACAAATTGTTCGCCGAATTAA
TACACGAGCCCAAAAAGGCTGTGCCCTAAAGATGTCACAACATTTTTCACTC*CCCCACATCA*
*CACCA***TAA***GA*AGAATAGGAACAAGAAA***ATG***AAATTTTTATGCCACGTTCACACAGTTCAGCG
CCTAGCCCCCGGGATCTACCAAGTCATTTTAGCGCCACACTACCCCTTGGATTTTAAAGCTG
GACAATATTTGAAGCTCACATTGGCGGGGAAAGATCGCTACTTTTCTATTGCTAGTTGCCCT
TCACAGCCAGGGCTTATCGAACTGCATATTGGAGCATCAAAGACTGATGAAGGCGTTCTTAG
TGCTATTGCAGCCTTACATGAATTTAAAGAAGCAGAATTATCACTAGAGATTGAAGGGCCAC
TGGGTGATGCATGGCTTCGAAAAGAGAGTACTAACCCTATATTATTGATTGCCGGAGGAACG
GGAATATCCTACATCATGAGCTTACTTAGAAATGCCCTCCACCATAAACTGAAGCAAAATAT
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CGACATCTATTTATGTGGTCCAATAAATATGATTAAAGAAGGGAAGACACGTTTACTTGAAA
AATGCAGTGCAACTTCAGGCAATATGTATGGTGATGGATTAACCTACGTT**TAA**TCGGCATTT
TTAATAGAGCATTCAATTTGTCCTCTGCTTAGTAATGTCTGATTACTTACCAGAGATCGTTG
AGCCGCCGCAGTACAATGAAAAAACGGCCTATACTATACCCAGACAAACTGAGGTCGTGTTA
CCACGCACCTCTCTGTAAGGCCCATTCTTCATGGATATGGGTATCAGTGCTGTAAGCAGCAA
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AATGATGCGCTATTTTAATAACACCCTACTGAGCTGCTTGGCGCTTCTCTTTACGCCGATAG
CGTTAGCACAAACGTTAACCGCTGACTTTAGACATCGGCCACCAGAAATGGTGTTAGATGAA
GCAACAAACTCAATGACTGGACCACTCAAA***GATATC***GTTGAGCAAGCGACACAAAAGCTGGG
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CAAGGAGTACCAAGATTTAACTAAACTGAATGTTGGCGTAAAGCGAGGTACGGCTTATTTTG
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CGAATGCTAGAAGCAAAGCGCATTGATGCAATCATTGTTCTTGATGAAGCGGCGATTGAACA
AGAGCTGAAAAACATCAGTTTTACCAATTACAAAAAGGCCGATTACTTCTTCCCCAATGTGA
TAGGTAACTACTACGGTATGCCTAAAAATCATCCGCTGGCAGACAAACTTGCCGAAGTACTG
CAACAAATGGTGCAAAACGGTGAGGTTAACGCCATTTACCAAAAATACGGATTAACTCCTAA
TTAACTCACATCAAAGGCTTTGAGGTTACAGCAGGTAATGATCCCTAAGCTCCTTTAGGGAT
CATTACCTCATTCATAGAGTCTATCTATGCTCATGGGGATAAACTGACTTACCGCCTATCTG
CAACATAAAGTCGTTTGGGTACACAAAGTCTCTGTTGAAGGTTGTGTTTAGCTCATTCAGCA
TGTTAAAAAACAACGTTTTTTTGAACTTTCCTTATCATCCTTAGTCTGAATCCCGTCTCTAA
TTGATTGATGAAATAAATGAGTTTTTT***ATG***ACAAAACGTTGGACACTTCTTGCAACCTTAGT
AGGTGTTGGCTTGGTAGGCGGTTATTACACTCTCGCTTATACCCCTGAGTCTTCATCCCCAT
TACCGACCCTCAACGTCAGCCGGGGAACAATAGAGAAACAAGCGGTCGCGGTTGGTCAAATC
ATGCCTTCACATTCGGTGGCAATTAAATCGCAAATCAACGGTATTGTCGGTGAAATTTATGT
GCGTGAAGGGCAACATGTTGGGCAAGGACAACCCTTGATTAAGGTTCGTCCGAATCCAACAC
CACAGGCGTTGACCGATGCCTCCACAGAACTGATGCAAAGTGAAGCAAATCTTGAATCAGAC
TTACAACGCTTAGCCAACCTTGAGCGCTTAGTGAAACAGCAAATCATTCCTGCCAATTATGA
TGATTACGTCCGCGCGAAAGCCGACGTCAAAGCTAAGCAAGCCGAAGTGCAGCAAAAACGCC
AAAACCTAGAGTTGATCCGCAGCGGTG***AAGCTT***

Figure 4.2. The *lux* operon sequence of *V. cholerae* UM4057 with 1.5 kb of 5' flanking region and 1.8 kb of 3' flanking region. Start (italics) and stop (underlined) codons of *lux* genes (*luxCDABEG*) are shaded in gray and in bold. *Hin*dIII, *Eco*RV, and *Ssp*I restriction endonuclease recognition sites (AAGCTT, GATATC, and AATAAT, respectively) used in Southern hybridization cloning and sequencing are italicized, underlined, and in bold. SPPS primers used to make *lux* probes are shown shaded, with the forward or left primer italicized and the right or reverse primer underlined.

4.3.3 Organization of the *V. cholerae lux* operon

The organization of the *lux* operon of *V. cholerae* UM4057 is *luxCDABEG* and is illustrated in Figure 4.3. The *luxCDABEG* operon of *V. cholerae* is most similar in arrangement to that of *Shewanella hanedai* and non-*luxF Photobacterium leiognathi* (Figure 4.4). The *V. cholerae lux* operon lacks the oppositely transcribed *luxR-luxI* regulatory pair found in *V. fischeri*, *luxH* found in *V. harveyi*, *luxF* found in some strains of *Photobacterium leiognathi* (or *P. mandapamensis*, Ast and Dunlap, 2004) and *phosphoreum*, *luxR1, R2*, and *I* found in *V. salmonicida*, and it contains the *luxG* gene not found in *Photorhabdus luminescens*.

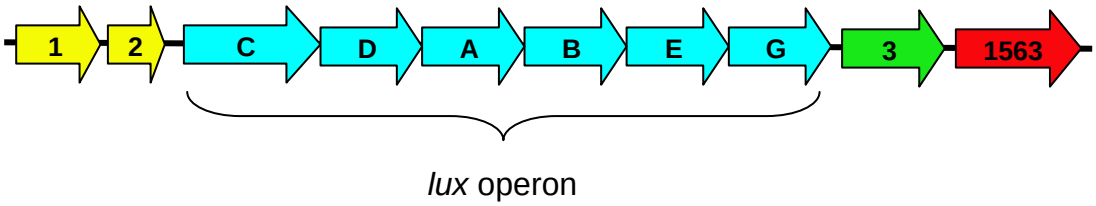


Figure 4.3. The *lux* operon of *V. cholerae* UM4057, with flanking regions. *luxCDABEG* genes are labeled with corresponding letters. Numbered ORFs indicate putative proteins flanking the *lux* operon. 1563 indicates gene VC1563, from *V. cholerae* N16961 (Heidelberg et al., 2000).

4.3.4 Upstream and downstream flanking regions

The DNA located immediately upstream of *luxC* (~100 bp) is an A+T rich region (Figure 4.2). There is no putative Shine-Dalgarno (SD) translation initiation sequence immediately upstream (9-11 bp) of *luxC*, although only 55% of gamma proteobacteria genes are SD-led (Chang et al., 2006). A search for SD sequences (GGAGG, GGAA, GGAG, GAGG, AAGG, and AGGA) within the upstream 500-bp of sequence yielded 4 sites (-426, -381, -334, and -243, from the *luxC* start codon). These are presumably SD "background noise"; i.e., appearance of the SD sequence without any as yet known biological function. In the 1.5 kb of 5' flanking sequence (upstream of *luxC*), two non-*lux* ORF's were identified (Figure 4.3). The first, ORF1, encodes an adenylosuccinate synthase, which catalyzes the first step in the *de novo* biosynthesis of AMP. The amino acid residues are 99% (314/315) identity match with protein COG0104 from *V. cholerae* RC385, a luminescent strain isolated from the Chesapeake Bay (accession number ZP 00752785), and 58% identity match, the next closest, to an adenylosuccinate synthase from *Photorhabdus luminescens* subsp.

Laumondii. ORF2 encodes a *MutT*/NUDIX phosphohydrolase, the closest match of wihich was with *Photorhabdus luminescens* subsp. Laumondii (56%).

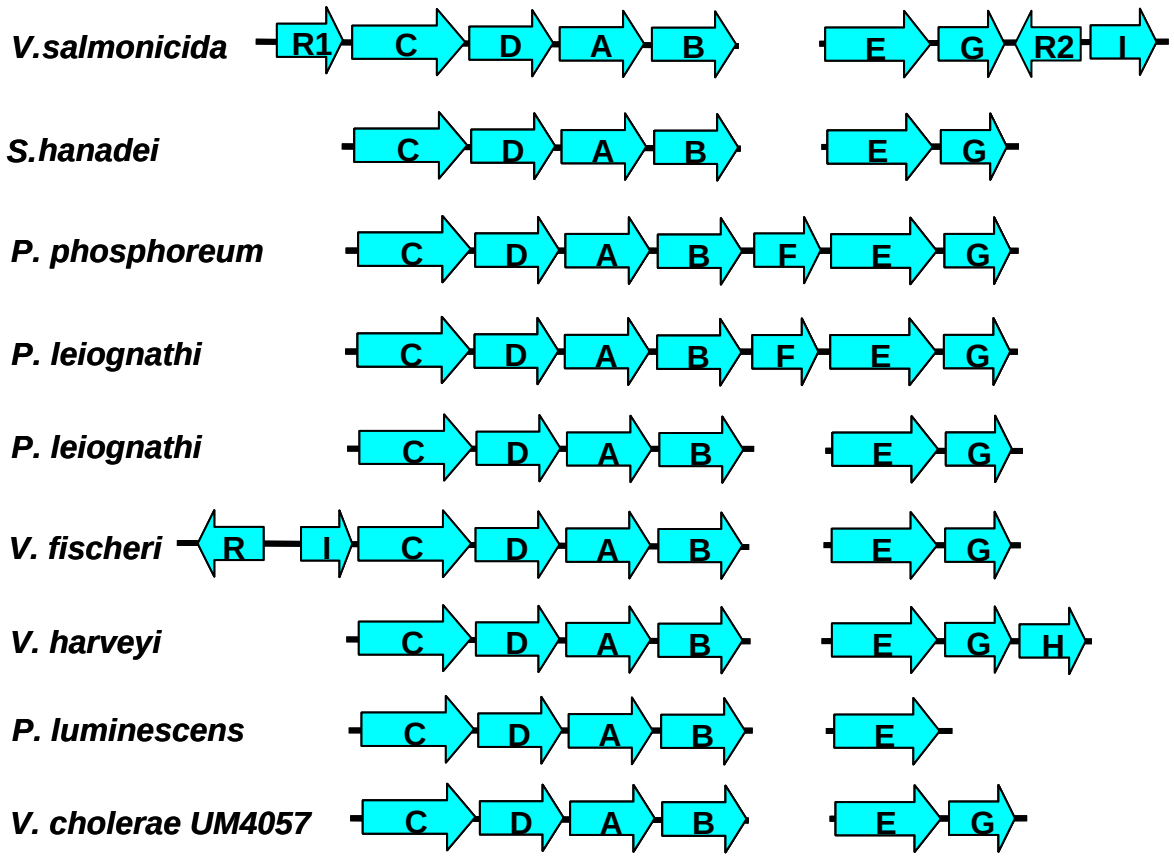


Figure 4.4. *lux* operon arrangement comparison, including the *lux* operon of *V. cholerae* UM4057, among luminescent bacterial species. *lux* genes are indicated by corresponding letters. Two different operon arrangements are found within the *P. leiognathi* species, and both are shown.

The downstream 1778 bp of sequence (3' from *luxG*) contained 2 non-*lux* ORF's, ORF3 and ORF4, or VC1563 (Figure 4.3). ORF3 is a 258 residue extracellular solute binding protein (family 3), of which the closest match is that of *Shewanella* sp. (50%) and *Magnetococcus* (44%). ORF4 corresponds to VC1563, a membrane fusion protein, from *V.*

cholerae N16961 O1 El Tor. This provided a clue as to the chromosomal location, on the larger chromosome I, of the *lux* operon relative to a clinical strain of *V. cholerae*. This location was confirmed by further 5' upstream sequencing, which reveals VC1560 from *V. cholerae* N16961, followed by two additional ORFs, the closest matches being *Photorhabdus luminescens* (C. Wimpee, personal communication; Figure 4.5). Figure 4.5 illustrates this chromosomal divergence between luminescent *V. cholerae* UM4057 and pathogenic O1 El Tor N16961.

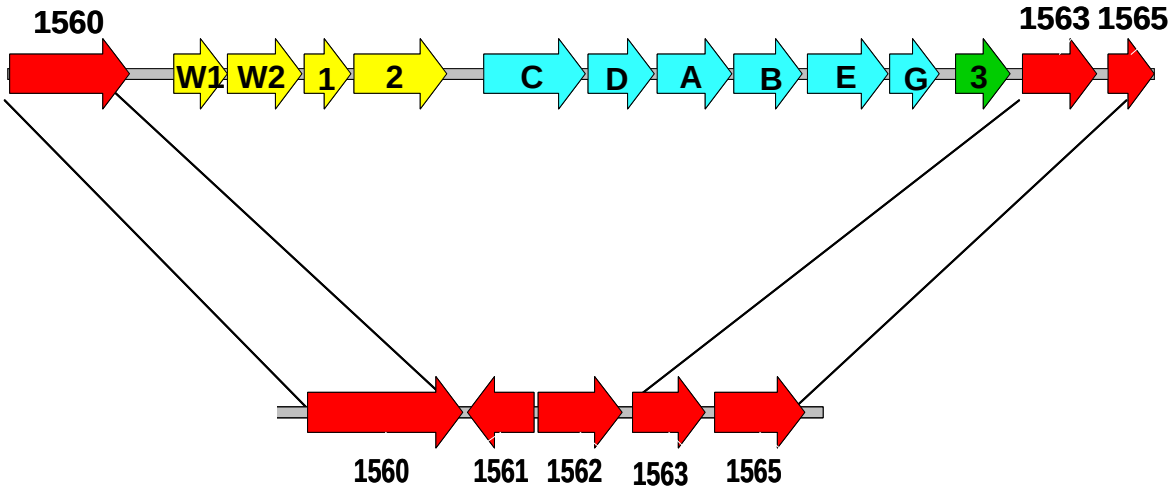


Figure 4.5. Schematic representation showing the chromosomal location of the *V. cholerae* UM4057 *lux* operon relative to the genome of *V. cholerae* N16961. Genes 1560-1565 were sequenced and identified previously (Heidelberg et al., 2000). ORF's W1 and W2 were identified by C. Wimpee (personal communication).

4.3.5 Sequence similarity

The sequence similarity or identity of *V. cholerae* UM4057 *lux* operon genes to those of other luminescent bacteria was analyzed. The NJ trees of all six *lux* genes are shown in Figure 4.6 and the sequences used for alignment are summarized in Table 4.1. In all cases,

A

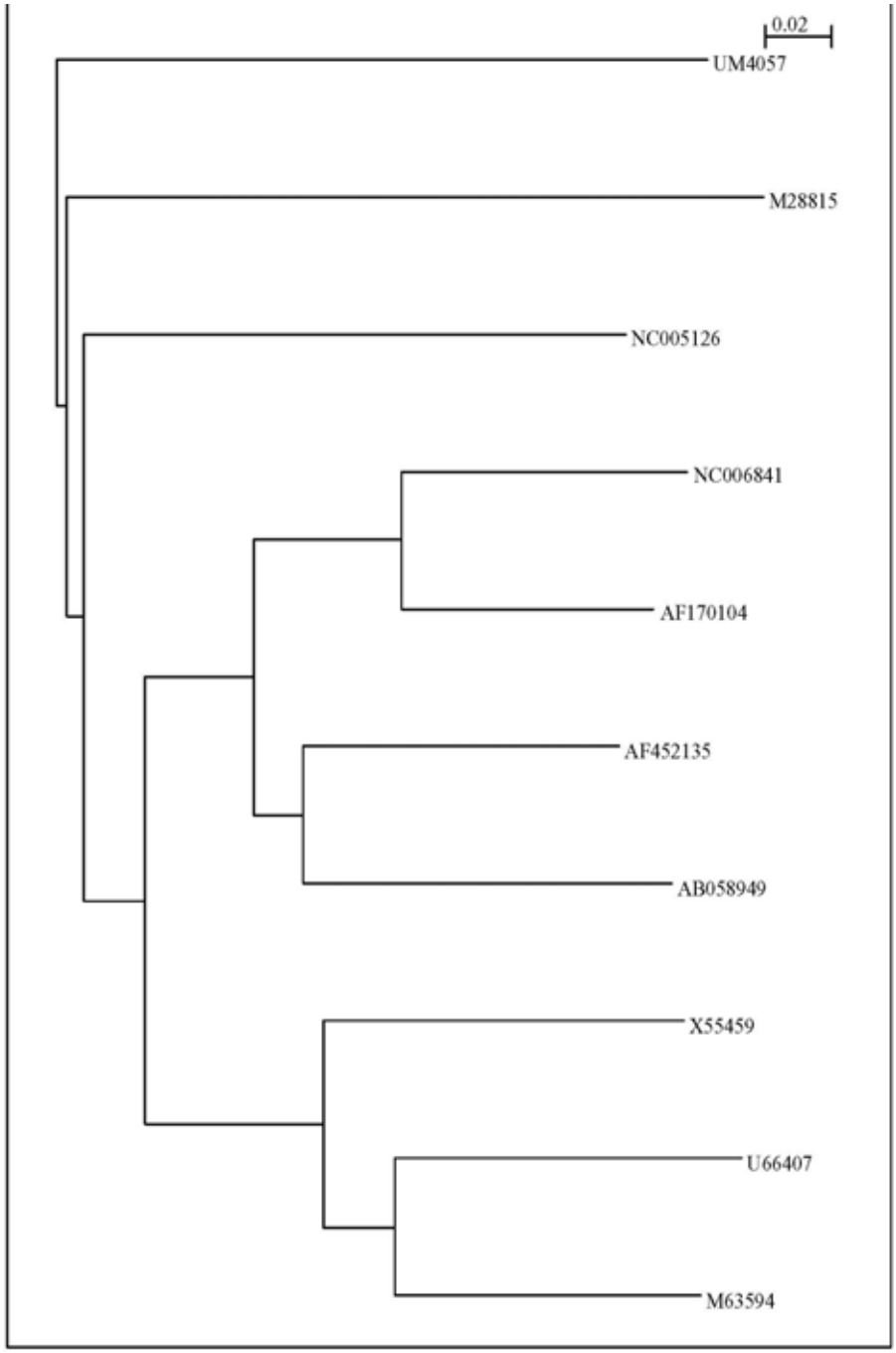


Figure 4.6. NJ Trees of nucleotide sequence similarities following multiple sequence alignment using ClustalX for the 6 *lux* genes found in *V. cholerae* UM4057 compared to other published sequences. Species are indicated by NCBI accession number of deposited sequence. See Table 4.1 for species – accession number conversion. A., *luxC*; B., *luxD*; C., *luxA*; D., *luxB*; E. *luxE*; F., *luxG*.

B

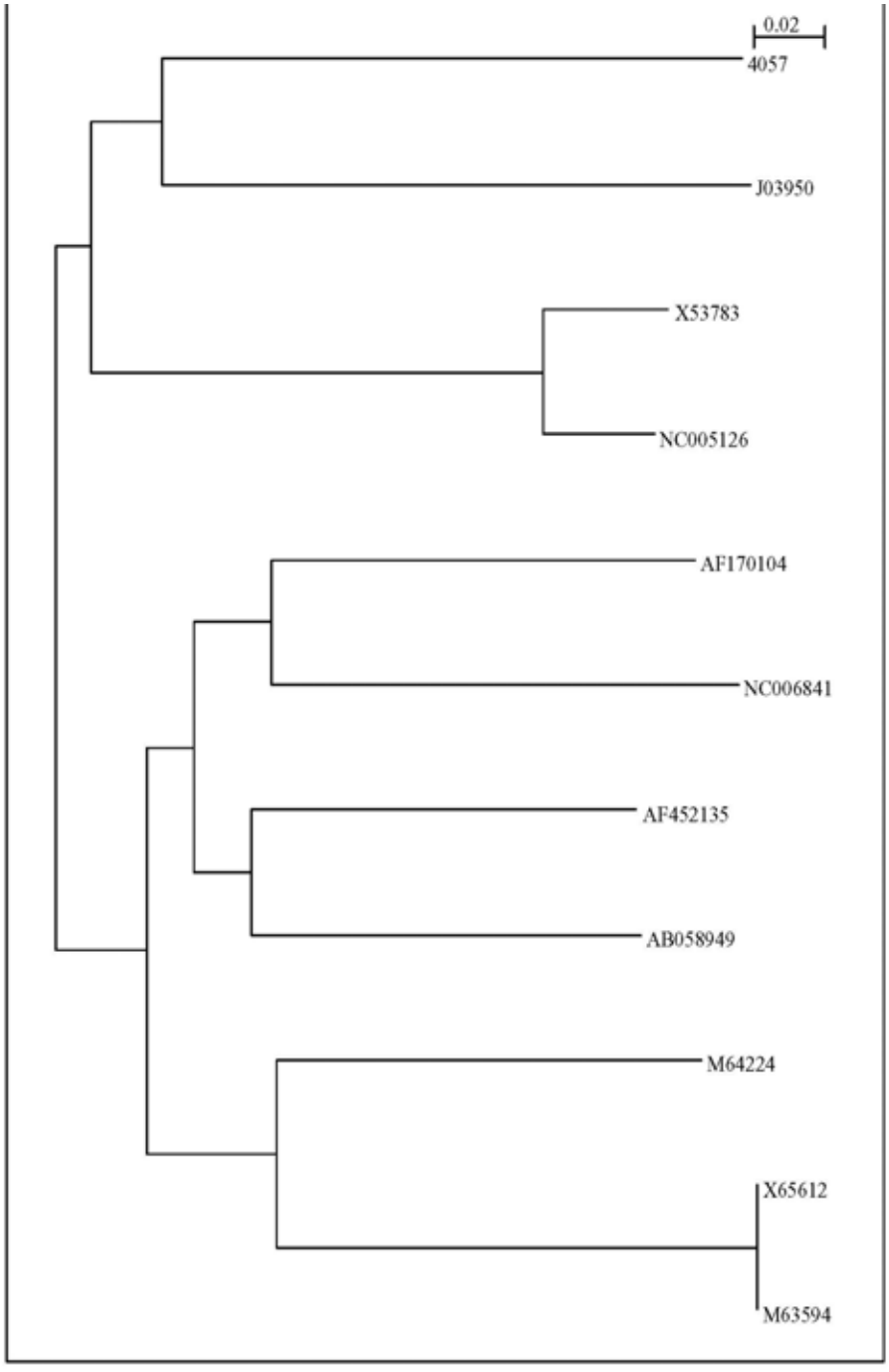


Figure 4.6. NJ Trees of nucleotide sequence similarities following multiple sequence alignment using ClustalX for the 6 *lux* genes found in *V. cholerae* UM4057 (continued).

C

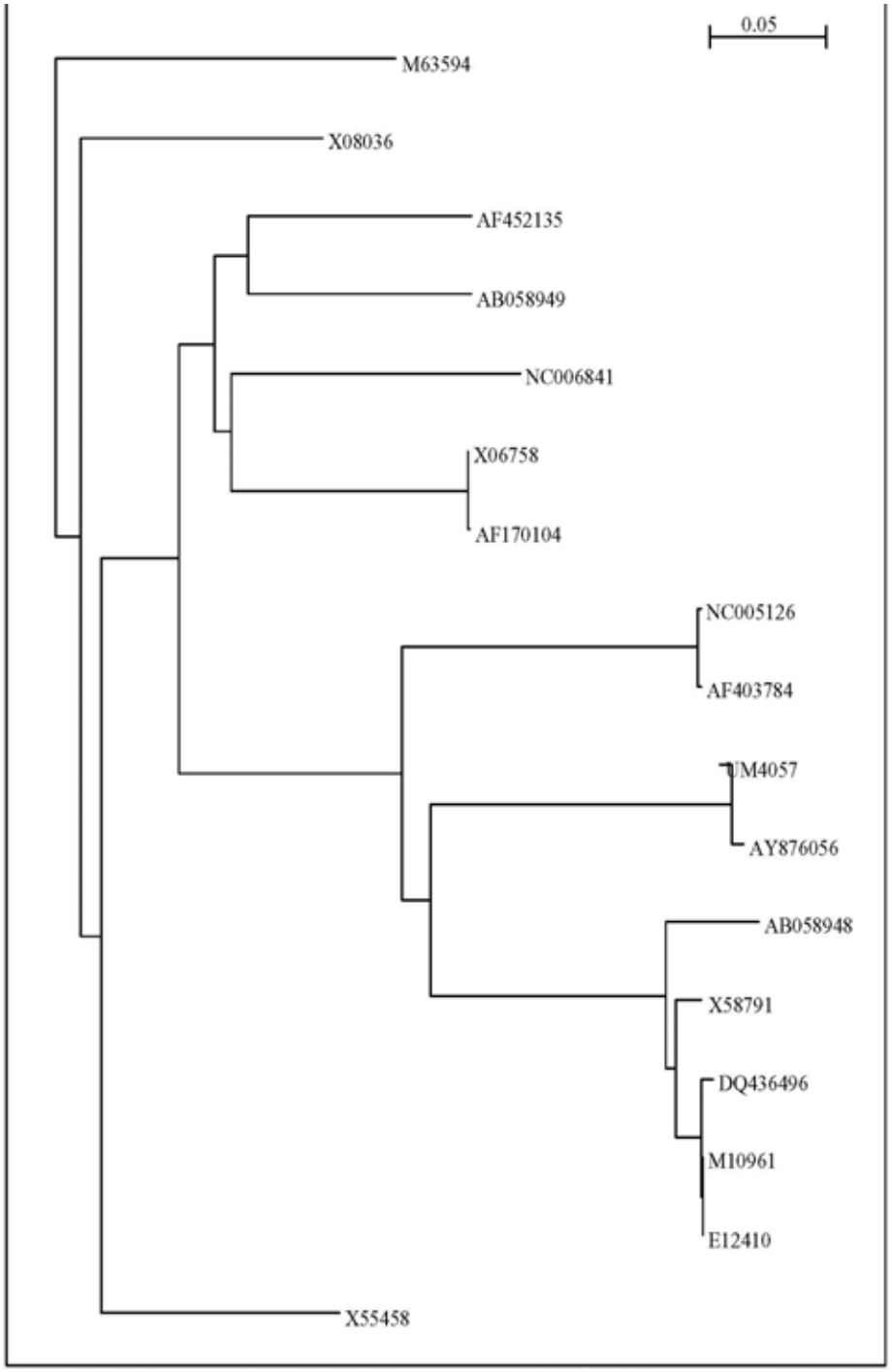


Figure 4.6. NJ Trees of nucleotide sequence similarities following multiple sequence alignment using ClustalX for the 6 *lux* genes found in *V. cholerae* UM4057 (continued).

D

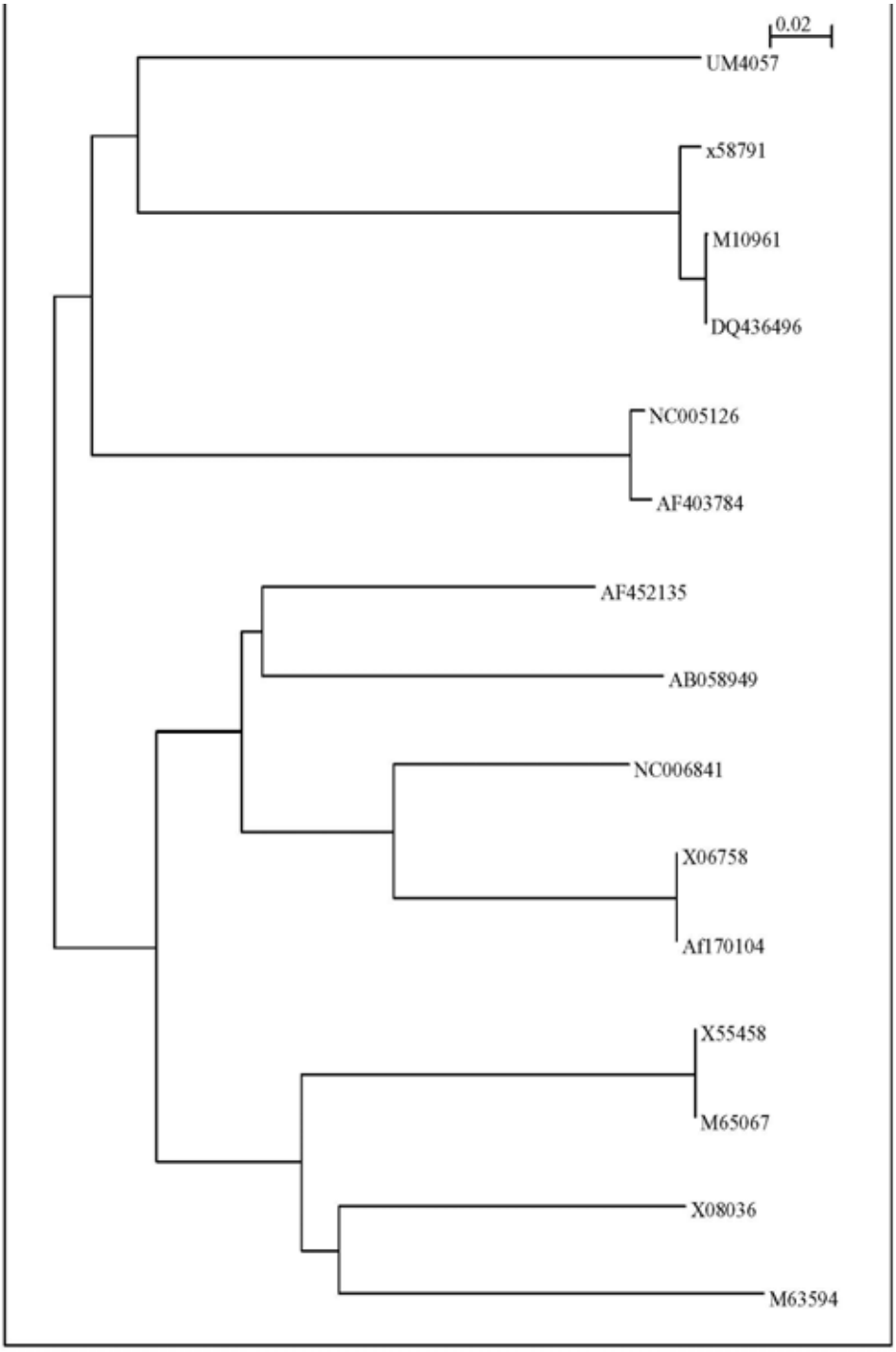


Figure 4.6. NJ Trees of nucleotide sequence similarities following multiple sequence alignment using ClustalX for the 6 *lux* genes found in *V. cholerae* UM4057 (continued).

E

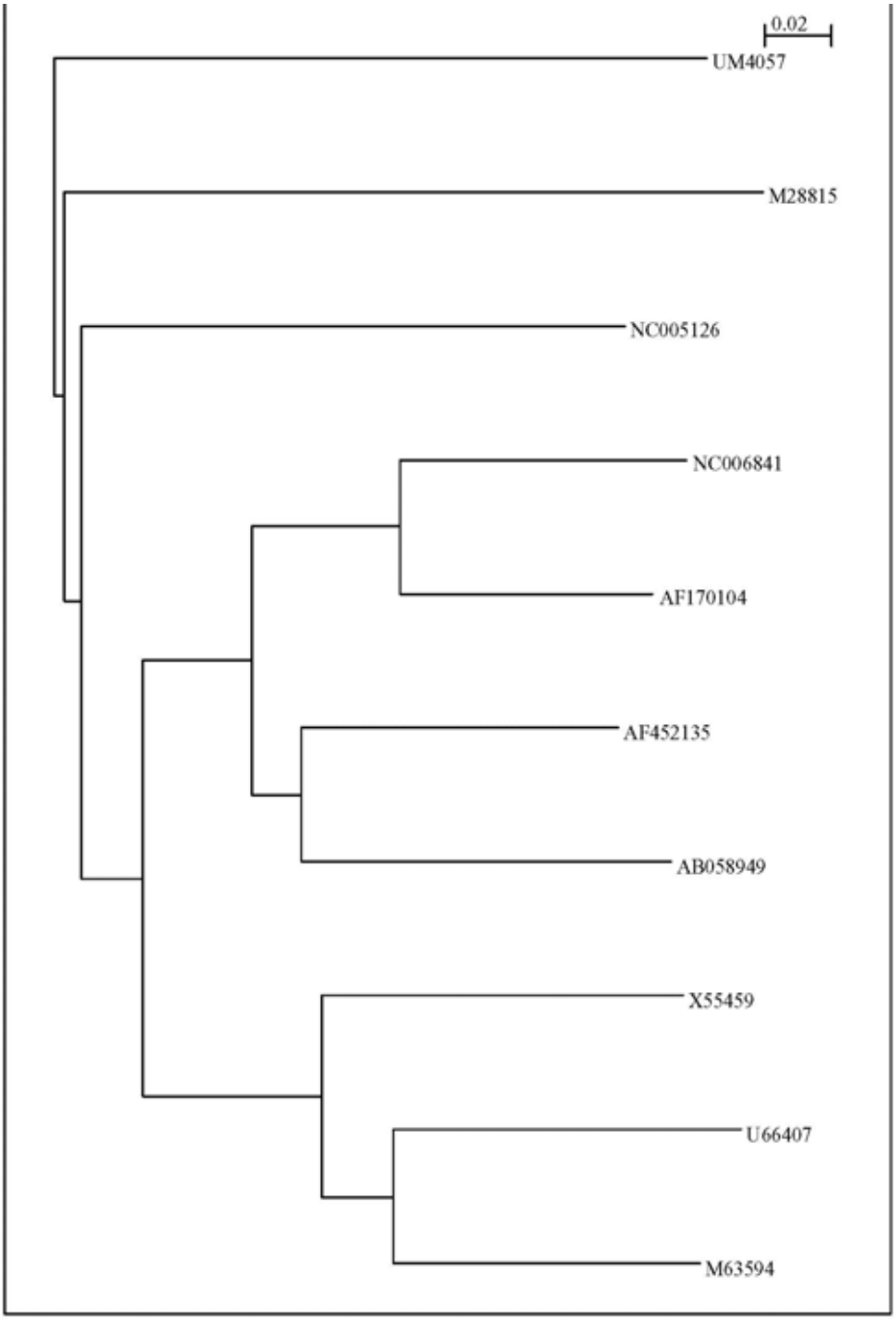


Figure 4.6. NJ Trees of nucleotide sequence similarities following multiple sequence alignment using ClustalX for the 6 *lux* genes found in *V. cholerae* UM4057 (continued).

F

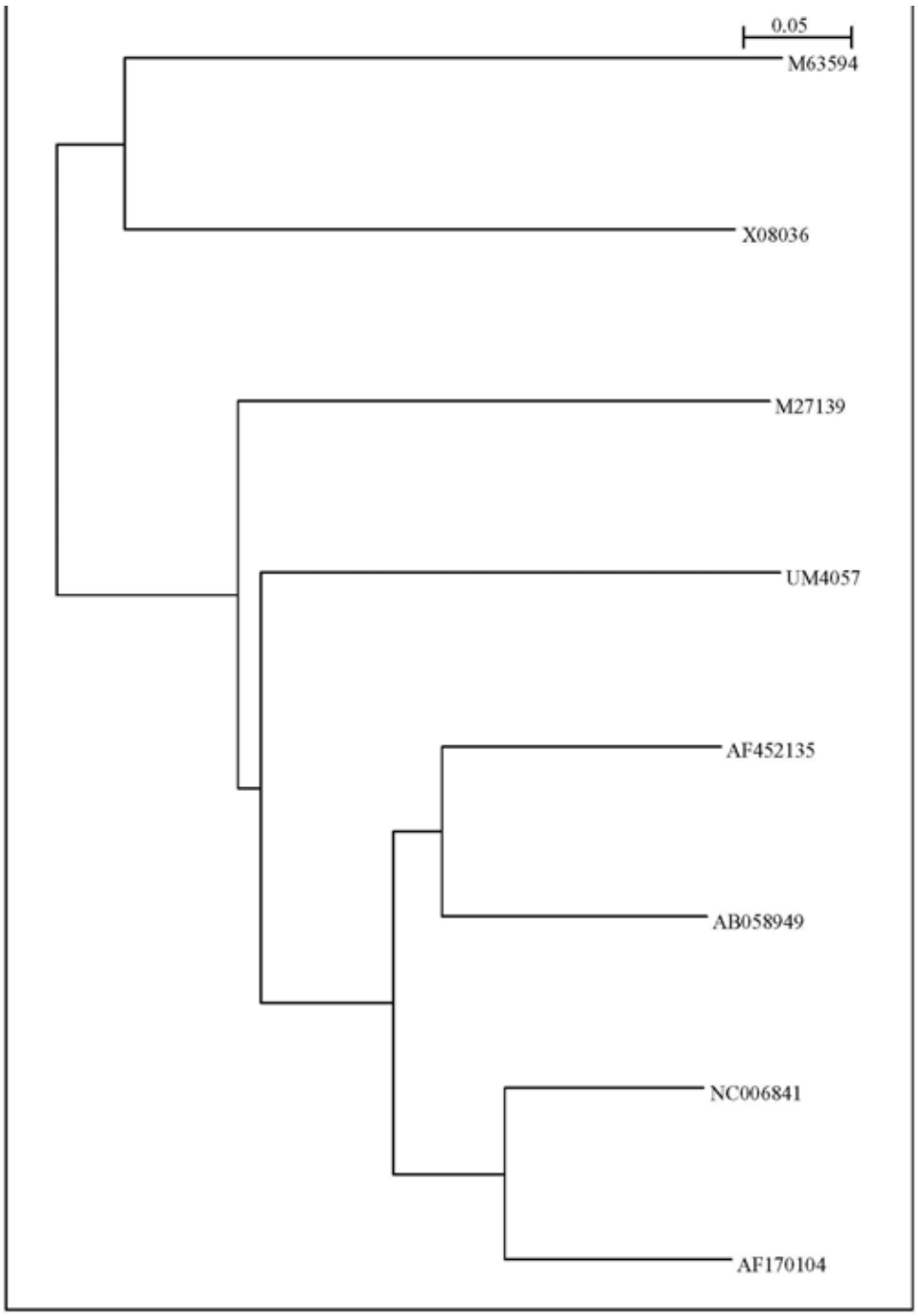


Figure 4.6. NJ Trees of nucleotide sequence similarities following multiple sequence alignment using ClustalX for the 6 *lux* genes found in *V. cholerae* UM4057 (continued).

the sequences of the *lux* genes were most similar to those of *V. harveyi*, except in the case of *luxA*, in which the sequence from *V. orientalis* was also very similar to that of *V. cholerae* UM4057. Unfortunately, this is the only *lux* gene from this species in the NCBI database. Not surprisingly, the two species of *Photobacterium* were closely related to each other and these two species were the least similar to *V. cholerae*. The two species, *S. hanedai* and *V. salmonicida,* were closely related and grouped closely with *V. fischeri*. Surprisingly, the sequences from *Photorhabdus luminescens* always appeared as more similar to *V. cholerae* and *V. harveyi* than any other species, except in the case of *luxG*, which is absent in *P. luminescens*.

4.3.6 G+C % content

The G+C% content was determined for *V. cholerae lux* operon and the *lux* operon of several other luminescent species (Table 4.3). As with the sequence similarity analysis, the G+C% content showed that the *lux* operon of *V. cholerae* is most similar to that of *V. harveyi*, regardless of whether total operon or 1st, 2nd, and 3rd letter G+C% content is used. In

Table 4.3. G+C% content of seven luminescent bacterial species.

Organism	Chromosomal %G+C	*lux* operon %G+C	1st letter GC%	2nd letter GC%	3rd letter GC%
V. cholerae	47/47[a,b]	43	52.2	37.1	41.3
V. salmonicida	38-40	35	35.4	35.4	36.1
V. fischeri	39/37[a]	35	34.4	33.7	34.2
V. harveyi	45-47	44	52.2	35.8	42.7
P. luminescens	43	37	37.9	36.4	37.9
P. leiognathi	38	37	37.5	35.4	38.6
P. phosphoreum	40-44	34	ND	ND	ND

[a]Numbers are for both chromosomes, larger/smaller.
[b]*V. cholerae* N1961, 1st letter, 56.6; 2nd letter, 38.7, 3rd letter, 49.0

addition to examining the G+C% content differences between luminescent species, the G+C% content of the *lux* operon was compared with the whole genome for the various luminescent species (Table 4.3). In all cases, the *lux* operon G+C% content was lower than that of the whole genome.

4.3.7 Codon usage analysis

Codon usage tables were prepared using the five essential genes of the *lux* operon, *luxC* to *luxE*, of the species used, except *P. phosphoreum*, which contains *luxF* between *luxB*

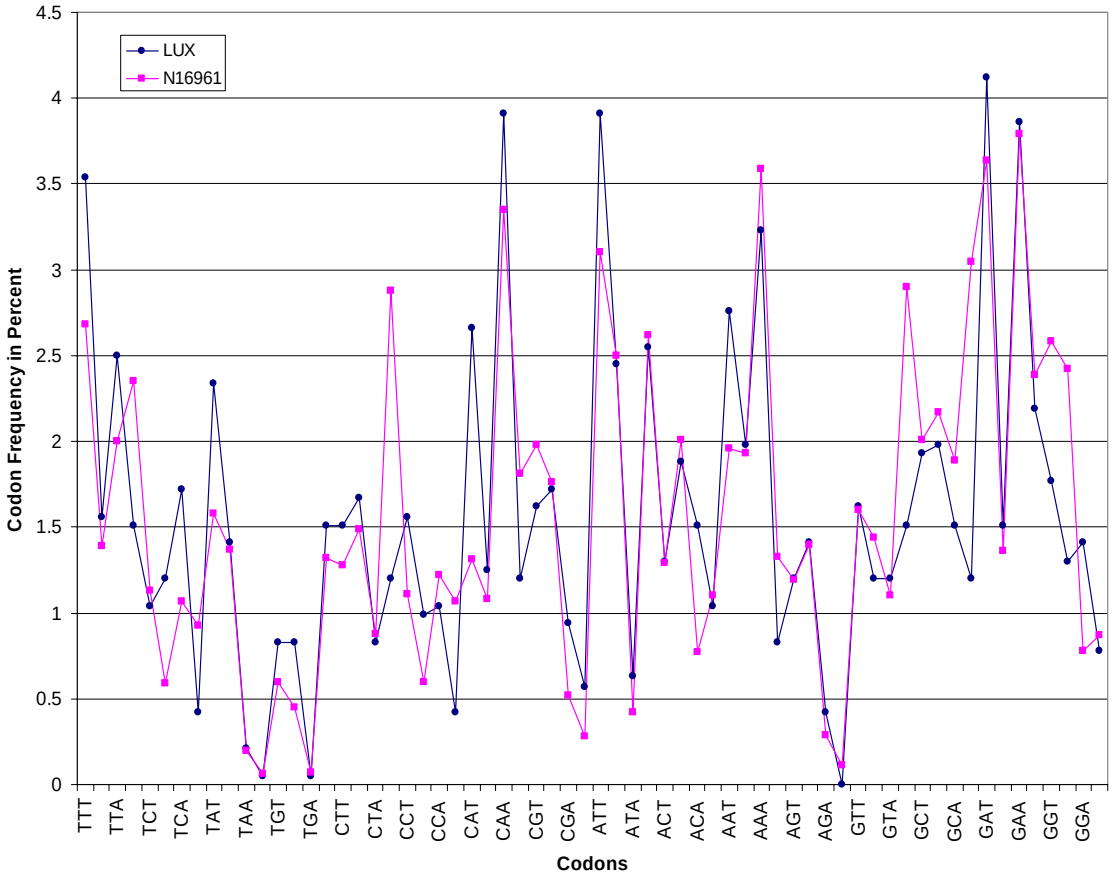


Figure 4.7. Codon usage patterns for *lux* operon of *V. cholerae* UM4057 and whole genome of *V. cholerae* N16961. Codons are in the order: NNT, NNC, NNA, NNG.

and *luxE*. The tables were adjusted so that the total number of codons analyzed was 1900 for all species, the mean of the *lux* codon number of the different luminescent species operons. Results of the chi-square analysis showed that the *lux* operon of *V. cholerae* was significantly different from all other *lux* operons analyzed; however, it was also significantly different from the whole genome of *V. cholerae* strain N16961 (p = 0.05). The codon usage patterns of *V. cholerae* UM4057 *lux* operon and the whole genome of *V. cholerae* N16961 are

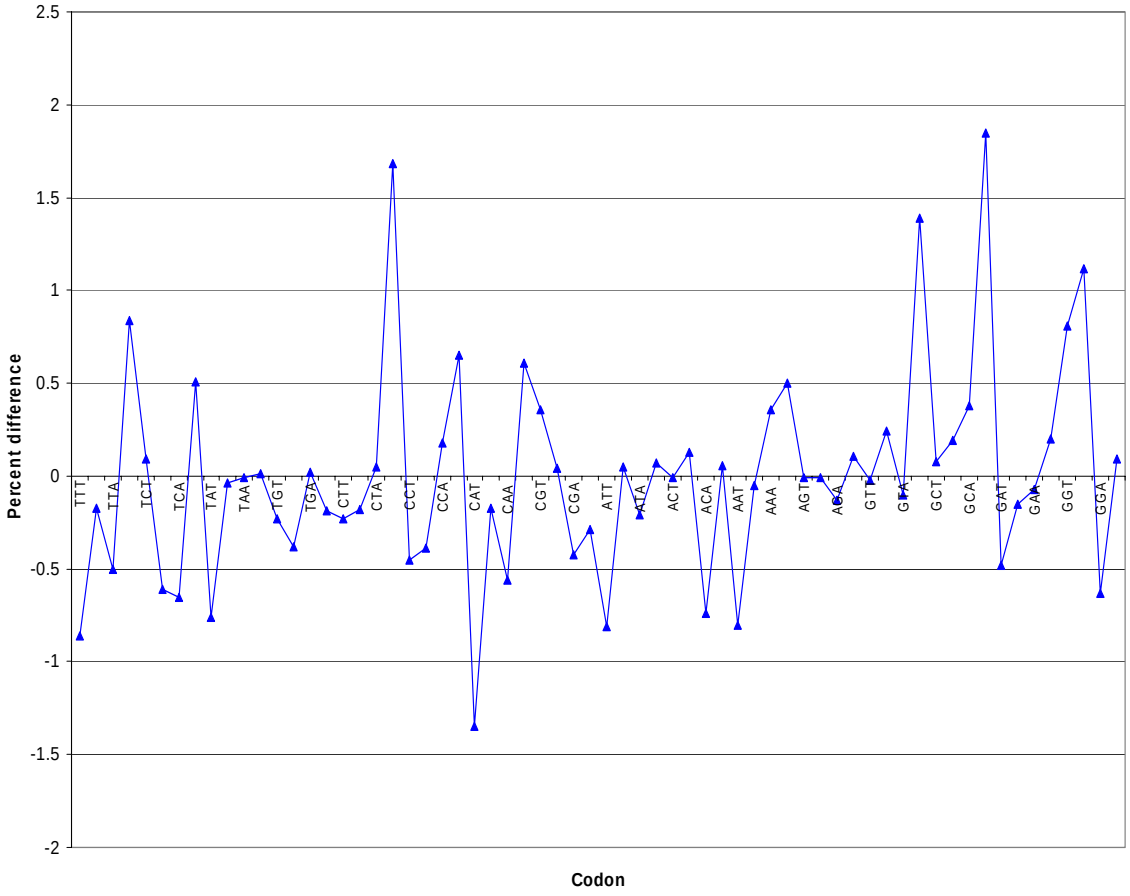


Figure 4.8. Difference in codon usage pattern, given in percent, between the *lux* operon of *V. cholerae* UM4057 and whole genome of *V. cholerae* N16961. Codons are in the order: NNT, NNC, NNA, NNG.

presented in Figure 4.7, and the difference comparing *lux* to the whole genome is presented in Figure 4.8. From data in Figure 4.7, the two codon usage patterns are concluded to be similar, with a few codons used at the exact same frequency. However, Figure 4.8 presents the difference more clearly demonstrating significant differences in codon usage pattern

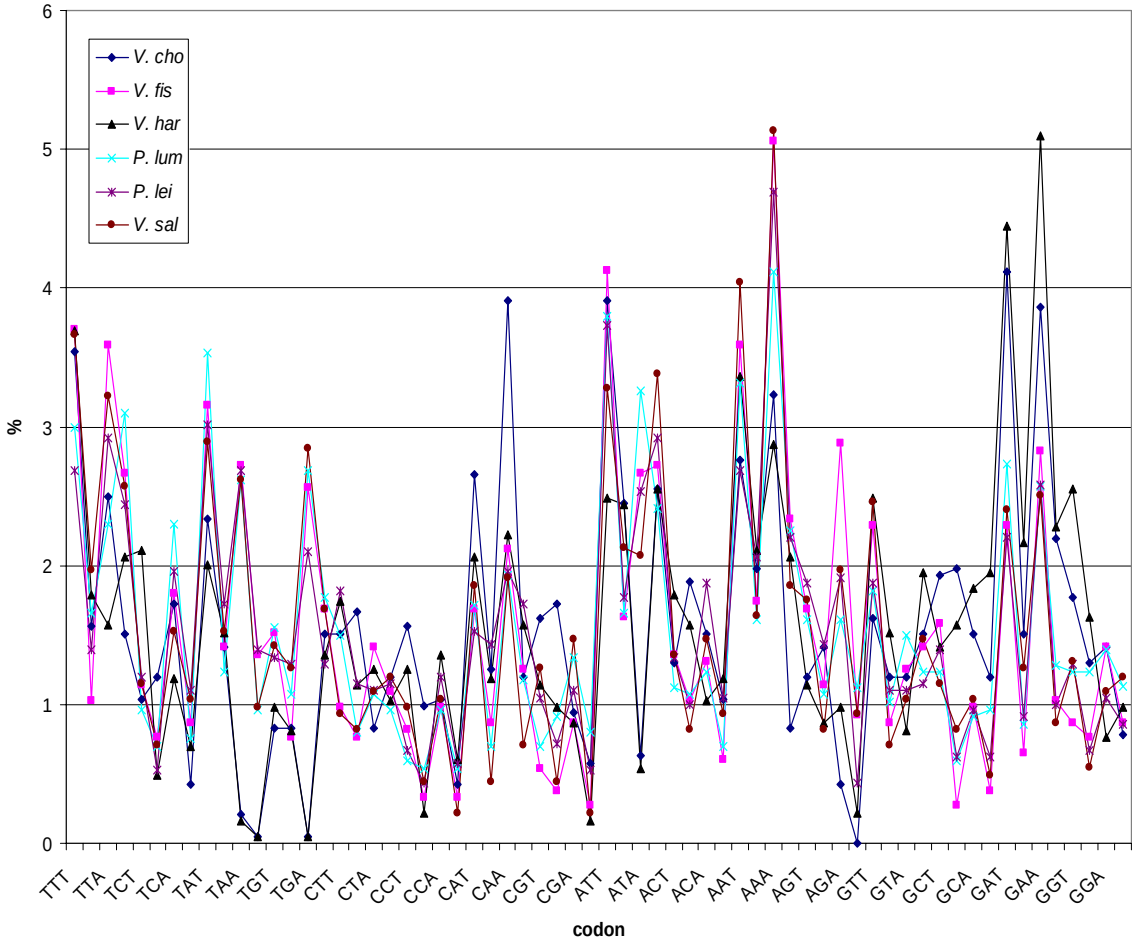


Figure 4.9. Codon usage patterns for 6 species of luminescent bacteria, *V. cholerae* UM4057, *V. fischeri* MJ-1 (AF170104), *V. harveyi* B392 (X07084, J03950, M10961, M28815), *P. luminescens* TT01 (NC_005126), *P. leiognathi* ATCC 25521 (M63594), and *V. salmonicida* NCMB 2262 (AF452135). Codons are in the order: NNT, NNC, NNA, NNG.

between the two DNA sequences. Figure 4.9 shows codon usage patterns for six different luminescent species, including *V. cholerae*. Results presented in the graph support the results obtained from the G+C% analysis, in that *V. cholerae* and *V. harveyi* are shown to be closely related. Eighteen of the 64 possible codons showed a less than 1% difference in codon usage between *V. cholerae* and *V. harveyi*. Indeed, although all species were found to be significantly different by chi-square analysis, the chi-square value for *V. cholerae*-*V. harveyi* codon usage was the lowest, while that for *V. fischeri*-*V. cholerae* comparison was highest.

```
>Repeat 1
Similarity=69%
 6990         taaacgcaacgactagtagaagtttttt---cgttcataactcaagttga       6944
              :|||||: ||:| || |||:|||  |     ||| |||:|::|:|||||:
 429          caaacgttacaagtattaggagtgatgaaggcgtacatgatccgagttgg       478

>Repeat 2
Similarity=68%
 2408         tgctcttggctatatttttctaaaacgcaacttttcgtccgaacaaccag      2358
              | | ||:::::|||||||  |:   || || | |::||||||||::|:::
 5282         ttcactcaatcatattttgacc---actcaccatcccgtccgaacggctga      5329

>Repeat 3
Similarity=58%
 187          agtttctgcctaagttaaacgaaagagcttaaagggtttcgtctcactcg       138
              || |::|||:| :|||:|||:|:            | |||:||||| | |
 587          agatcttgctttggttgaacaag-----------cgattcatctcaatgg       625
```

Figure 4.10. Three direct-reverse repeats contained in the *lux* operon and flanking DNA of *V. cholerae* UM4057 as determined by the FastPCR program.

4.3.8 Presence of mobile elements

Four possible repeat elements were found in the *lux* operon and flanking region sequence from *V. cholerae* UM4057, using the FastPCR program. One simple repeat was found at nt 1556 to 1582, aaaaa**tcagaatcagaa**caaaaaaag. Three direct-reverse repeats were found and are

presented in Figure 4.10. One of the direct-reverse repeats, Repeat 1, occurs 5' of *luxC* and again in *luxE*, to include 79% of this gene. The nucleotide sequence of the *V. cholerae* UM4057 *lux* operon and flanking regions was translated to protein residues. The sequence was searched in the three reading frames for the DDEK consensus motif of IS*903*, but no evidence of an insertion sequence was found.

4.4 Discussion

Within the *Vibrionaceae*, bioluminescence shows no obvious phylogenetic pattern, with respect to gene distribution or sequence similarity. For example, based on the *lux* gene sequence, G+C% content, and codon usage patterns obtained in this study, it is concluded that *Vibrio cholerae* and *V. harveyi* are closely related. However, there are many non-luminous species of *Vibrio* that are more closely related to *V. harveyi* than *V. cholerae,* such as *V. campbelli, V. alginolyticus, V. parahaemolyticus*, and *V. natriegens*. Furthermore, many *V. cholerae* strains are nonluminescent, including human pathogenic strains of serogroup O1 and O139 (see Chapter 2).

The bioluminescent species, *Photorhabdus luminescens,* further complicates the picture. From results of the analyses of nucleotide sequence similarity, G+C% content, and codon usage patterns, *P. luminescens* was concluded to be more closely related to *V. cholerae* and *V. harveyi* than *V. salmonicida, V. fischeri*, and the two luminescent *Photobacterium* species. The strongest conclusion, based on its distant phylogenetic placement, is that the species, *P. luminescens*, acquired the *lux* operon via lateral gene transfer.

Evidence of *lux* operon mobility has been obtained for several strains of the terrestrial luminescent species, *P. luminescens*. Meighen and Szittner (1992) reported four ERIC

sequences in *P. luminescens* strain Hw, upstream of *luxC*, between *luxD* and *luxA*, and two between *luxB* and *luxE*; and one in *P. luminescens* strain Hb, located between *luxB* and *luxE*. Furthermore, there is an ERIC repeat element upstream of *luxC*, between *luxD* and *luxA*, and immediately downstream of *luxE* in *P. luminescens* stain ZM1 (Zavil'gel'skii et al., 2002). An 18-bp repeat element located upstream of *luxC* has been reported in the *lux* operon of *V. fischeri* (Knight and Papadakis, 1999). An analysis of the 9.8 kb *lux* region from *V. cholerae* UM4057 revealed four repetitive elements, with one (Repeat 1) bracketing *luxC* to *luxE*. Furthermore, there are four genes flanking the 5' end of the *lux* operon in *V. cholerae,* including W1 and W2 (Figure 4.5), that have close sequence similarity to a cluster of open reading frames in *Photorhabdus luminescens*. Based on *luxA* sequence similarity, it has long been assumed that *P. luminescens* obtained *lux* genes via lateral transfer from *Vibrio*. The results obtained in this study establish that the donor of the *P. luminescens lux* genes was a close ancestor to luminescent *V. cholerae.*

Nucleotide analysis, including G+C% content and codon usage patterns, yielded inconclusive results with respect to the origin of *lux* genes in *V. cholerae*. The %G+C content was lower in the *lux* operon, compared to the whole genome of *V. cholerae*, but this was true for all of the luminescent species analyzed, including *P. luminescens*, a result unaffected if only one letter, such as 1st or 3rd, of the codon or the total G+C% was used. Similar results were obtained from the analysis of the codon usage pattern. *V. cholerae* was found to be statistically different in codon usage than all other luminescent species analyzed ($p=0.05$), but was also statistically different from its whole genome ($p=0.05$). Both analyses are confounded, given that the whole genome of another strain of *V. cholerae*, N16961, was used for this comparison, an O1 El Tor clinical strain which is non-luminescent.

Unfortunately, the whole genome of strain UM4507 could not be sequenced. Also confounding the analyses is the fact that all of the whole genomes used contained laterally transferred genes, which affected the G+C% analysis and codon usage pattern. The results of this study emphasize the importance of establishing a standardized method for determining gene origin, that is, occurrence of laterally transferred genes, other than by sequence homology.

The sequence of the *lux* operon of *V. cholerae* UM4057 revealed that it has an operon organization identical to *S. hanedai* and non-*luxF* containing *P. leiognathi*. An examination of results presented in Figure 4.4, and assuming that *P. luminescens* acquired the *lux* operon by horizontal gene transfer, allows an evolutionary history of the *lux* operon to be postulated. Mutational analysis determined that only the region from *luxC* to *luxE* is essential for luminescence expression (Engebrecht and Silverman, 1983; Martin et al., 1989). *LuxG* appears to be a supplemental gene common to the *Vibrionaceae* and added to the operon. The *lux* operon was laterally transferred to *P. luminescens* in the form of *luxCDABE* or *luxCDABEG*, with subsequent loss of *luxG*. It is difficult to determine exactly when and how this event occurred since the presence of numerous ERIC elements within the *lux* operon of *P. luminescens* could have facilitated the horizontal transfer in multiple steps. From an early *luxCDABEG* ancestor, specialization of the *lux* operon occurred within the different phylogenetic lineages. Addition of *luxH* by *V. harveyi*, *luxR-luxI* by *V. fischeri,* and *luxF* by *Photobacterium* sp. most likely represents adaptation of *lux* expression by each organism in response to their ecological niche. The same can be concluded for *V. salmonicida* (*luxR1, R2*, and *I*) but the latter is less clear.

Among the conclusions drawn in this study is that species such as *V. cholerae* and *S. hanedai* possess an ancient and unmodified form of the *lux* operon. As for other, non-luminescent members of the *Vibrionaceae*, it is assumed that these species lost the *lux* operon in the absence of environmental selective pressure. Another important factor; however, is that under-representation of several of the species with respect to number of strains isolated and sequenced and for which sequence data are available, places a bias on the interpretation of distribution of luminescence. For example, only a single luminescent strain of *V. vulnificus* has been isolated to date. This anomaly may be explained by the results of this study (See Chapter 2) that luminescence is not found in pathogenic O1 or O139 strains of *V. cholerae*, attributable to the observation that pathogenic organisms appear to reduce their genome content, discarding genes coding for metabolic pathways important for existence only in the aquatic environment as opposed to the host, whether invertebrate or vertebrate. In this study, several weakly-luminescent or dark strains of *lux*$^{+}$ *V. cholerae* were found, contributing to underestimation of the incidence of this trait. Indeed, it is very likely that more luminescent members of the *Vibrionaceae* await discovery and characterization.

Chapter 5 EXPRESSION OF LUMINESCENCE IN *V. CHOLERAE*

5.1 Introduction

5.1.1 Spontaneous/natural dark (K) variants

The occurrence of dark variants of luminous bacteria was first described over 100 years ago (Beijerinck, 1889; Beijerinck, 1916). These K variants spontaneously arise from bioluminescent *V. fischeri* and to some extent *V. harveyi* maintained axenically in laboratory culture (Hastings and Mitchell, 1971). When the selective pressure to maintain a high level of luminescence expression, namely light organ symbiosis, is removed, the bacteria apparently repress light production for reasons yet to be explained. This trend toward dark variants has made research on *V. fischeri* quite challenging. Nevertheless, K variants of luminescent *V. harveyi* have been characterized rather well.

The occurrence of luminescence expression of K variants ranges from 1% to 0.001% in the wild type population (Hastings and Nealson, 1977). These variant cells contain low levels of intracellular luciferase and other luminescent reactants (Hastings and Nealson, 1977). Furthermore, they contain normal levels of inducer and are not induced by exogenous cAMP, which normally neutralizes catabolite repression. *V. harveyi* K variants are relatively stable and the phenotype is inherited, although apparent "wild type" revertants can be isolated easily (Keynan and Hastings, 1961). There is some evidence that K variants are pleiotropic in their defect, with colony morphology and flagellation also altered in *V. harveyi* K variants (Hastings and Nealson, 1977).

Conditions that contribute to formation of K variants are not completely understood. K variants grown under conditions of rapid growth, with aeration, do not revert to the luminescent wild type (Hastings and Nealson, 1977). However, growth conditions without

aeration and elevated temperature leading to dense cultures give rise to K variants (Hastings and Nealson, 1977).

Among the marine symbiotic luminescent species, *V. fischeri, V. harveyi*, and *Photobacterium* species, the occurence of natural K variants is infrequent, although symbiotic and environmental isolates do demonstrate a range of luminescence expression. Of the documented cases, a dark defect of *V. fischeri* B-61 was attributed to low to no autoinducer production (Nealson, 1977). Other "dim" strains were shown to be stimulated by exogenous riboflavin (Doudoroff, 1938). Interestingly, "super bright" strains have also been isolated, an example of which is *V. fischeri* MJ-1 (Nealson, 1977).

5.1.2 Catabolite repression of bioluminescence

Many inducible operons, such as the lactose (*lac*) operon, are not only controlled by their respective inducers and regulatory genes, but they are also controlled by the concentration of glucose in the environment or growth medium. The ability of glucose to control expression of a number of different inducible operons is called catabolite repression. Catabolite repression generally acts on operons involved in the degradation of compounds used as energy sources. Since glucose is apparently the preferred energy source for many bacteria, the ability of glucose to regulate expression of other operons ensures that the bacteria will utilize glucose first, i.e., before any other carbon source, as a source of energy.

There is an inverse relationship between glucose concentration and cAMP concentration in bacteria. That is, when glucose levels are high, cAMP levels are low and when glucose levels are low, cAMP levels are high. Transport of glucose into the bacterial cell inhibits the adenyl cyclase, the enzyme which produces cAMP. In the bacterial cell, cAMP binds to a cAMP binding protein called CRP. The cAMP-CRP complex (but not free

CRP protein) binds to a site in the promoter of catabolite repression sensitive operons and acts as a positive regulator of the operon, when glucose is absent.

The luminescent system of *V. harveyi* is subject to catabolite repression (Nealson et al., 1972). That is, growth in glucose represses light production and the addition of exogenous cAMP neutralizes this repression. At least one strain of *P. leiognathi* (Henry and Michelson, 1970) and *P. phosphoreum* WSU (Makemson, 1973) show permanent glucose repression that is not rescued by addition of cAMP. In the case of *V. fischeri*, catabolite repression is not reversed by addition of cAMP. However, the extent of repression is both reduced and transient, as the cells become glucose-adapted (Ruby and Nealson, 1976).

5.1.3 Aldehyde limitation

Hastings and Nealson (1977) state that any "growth situation or genetic lesion that results in repression of luciferase synthesis also leads to aldehyde limitation, as demonstrated by stimulability of luminescence of cells by exogenous aldehyde." This phenomenon was first described when researchers recognized that eclipse phase cells from a batch culture exhibited a drop in luminescence even though the cells contained a normal level of luciferase (Nealson et al., 1970). The defect was corrected by addition of exogenous aldhehyde. Aldehyde limited strains are very dim, producing light at 1% to 0.001% of wild type (Cline and Hastings, 1971; Cline and Hastings, 1974), similar to K variants. The natural aldehyde of the luminescence reaction has been proposed to be tetradecanal (Ulitzur and Hastings, 1979), although other long-chain endogenous aldehydes have been isolated from luminescent *P. phosphoreum* and *V. fischeri* (Shimomura et al., 1974).

5.1.4 Luminescence expression in *V. cholerae*

Luminescence and expression levels of luminescence have not been well studied in *V. cholerae*. This is largely because of an earlier report that the luminescence phenotype was found in approximately 10% of *V. cholerae* strains (West et al., 1986). Later evidence revealed that "dark" (defined here as having all or some elements of the *lux* operon, but emitting no visible light) and "dim" or "weakly luminescent" strains of *V. cholerae* can be isolated from the environment (Palmer and Colwell, 1991). Results presented in Chapter 2, show that this phenotype is common among *V. cholerae* isolates from the environment. Characterization of luminescence expression in *V. cholerae* will provide insight into the ecological implication of bioluminescence, by determining how this phenotype is expressed and repressed.

In this chapter, luminescence expression of *V. choloerae* Groups 0, I, II, and V is investigated in detail. Isolates are chararcterized according to luminescence expression, with some effort made to determine normal expression levels and establish a classification system for expression based on relative light units. Luminescence expression defective *V. cholerae* are further investigated to determine if the same characteristics hold true for this species compared to symbiotic species of this and related species, which have been better characterized and, more importantly, to explain the nature of the defect(s). Defective strains were tested for aldehyde limitation and autoinducer production, as well as examined to determine differences, if any, in the *lux* operon sequence, content, and topography.

5.2 Materials and Methods

5.2.1 Bacterial strains

Gourps I, II, and V (defined in Chapter 2) were used to define "normal" luminescence expression and characterize expression variants. Twelve environmental *V. cholerae*

Table 5.1. Luminescence defective strains of *V. cholerae* employed in the luminescence expression assays.

Strain UM-#	Source	Plate lum[a]	Hybridizatio with *V. fischeri luxA* probe[b]	Low-level lum[b]	*luxA* PCR[c]	Lum[c]
4056	Mud Lake, LA	-	+	6.9	+	15
4057	water, LA	-	+++	10.1	+	2101
4071	Apalachicola Bay, FL	-	-	-	+	0
4072	Apalachicola Bay, FL	-	+++	-	+	0
4075	Apalachicola Bay, FL	-	+++	-	+	0
4082	Chesapeake Bay, MD	-	+++	-	+	0
4086	oyster, FL	-	+++	9.5	+	1167
4089	Chesapeake Bay, MD	-	+++	11.8	+	209630
4091	Tillamook Bay, OR	-	-	7.0	+	18
4102	Tillamook Bay, OR	-	+++	11.1	+	18471
4103	Tillamook Bay, OR	-	+++	7.1	+	19
4157	England	-	+++	10.0	+	412

[a]West et al., 1986
[b]Palmer and Colwell, 1991; arbitrary light units = (mean cpm – cpm of blank)/10-ml (OD_{660})
[c]This study; relative light units = # of events per second per 100-µl culture

strains from Group I were used in the following luminescence expression assays (Table 1). These strains were previously characterized as non-luminescent (West et al., 1986) or as dimly luminescent (Palmer and Colwell, 1991). Indeed, as shown in Chapter 2, these strains were found to express luminescence at various levels that were independent of cell growth. However one of the strains, UM4089, was found to expresses luminescence at a relatively high level. *V. harveyi* BB120 (wild type), *V. fischeri* MJ100 (wild type), and *V. cholerae* biotype *albensis* ATCC14547 served as positive controls for luminescence and *E. coli* DH5α and uninoculated medium were used as negative controls for the expression experiments.

5.2.2 Luminescence assay and expression classes

The luminescence assay employed in this study is a three-step culturing procedure that selects for optimal luminescence expression, as previously defined in Chapter 2. *V. cholerae* strains were subcultured from –80°C frozen stock onto Marine agar 2216 (Difco) plates and incubated overnight at 30°C. Single colonies were inoculated into fresh Marine broth 2216 (Difco) and incubated overnight at 30°C with aeration (200rpm). The overnight culture was diluted 1:500 or 1:1000 and transferred to 125 ml flasks or 16 x 125 mm culture tubes containing fresh medium, and incubated at 30°C, with aeration (200rpm). Luminescence was measured at successive time points using a model LB96P luminometer (EG&G Berthold, TN). Relative light units, RLUs, is defined as the number of events observed per second per 100 μl culture. Simultaneously, measurements of cell concentrations were taken by measuring absorbance at 600nm (OD_{600}), using a Beckman DU640 UV/VIS spectrophotometer.

As an expansion of the rate of incidence of luminescent *V. cholerae* cells (Chapter 2), the expression levels of strains of the three groups were analyzed to determine normal expression levels and more accurately define luminescence expression classes.

5.2.3 Growth-curve luminescence kinetics

Growth and luminescence curves were generated for three strains of *V. cholerae* to determine how the kinetics of luminescent *V. cholerae* compare with that of *V. harveyi* and *V. fischeri.* To assess luminescence levels along a prolonged growth curve, overnight broth cultures were diluted using six 10-fold serial dilutions. One ml of the -6 dilution was used to inoculate the assay test vessels, i.e., 16 x 125 mm test tubes. Luminescence, culturable plate

counts on Marine agar, and absorbance at 600 nm, OD_{600}, were measured hourly for a minimum of 24 hr.

5.2.4 Luminescence repression

To determine if dark (K) variants can be generated experimentally, three luminescent Chesapeake Bay *V. cholerae* strains, RC457, RC512, and RC538, were grown in LB broth at 37°C, under static conditions, for 6 months. The cultures were subcultured into fresh LB broth every 2-5 days and periodically (approximately once per month) subcultured into marine broth for luminescence expression assay at 30°C, with shaking. In separate experiments, the effect of elevated temperature was analyzed by conducting each of three luminescence assays at 37°C, instead of 30°C.

To compare results of the repression experiments, "normal" (30°C, Marine broth, aeration) growth and luminescence curves for each of the three test strains were determined, using 36 replicates and six timepoints. A logarithmic regression line was estimated from the data for comparison with repressed samples.

5.2.5 Autoinducer production

Autoinducer from luminescent test strains was harvested at the time of maximal luminescence expression, determined by luminescence bioassay. Autoinducer from non-luminescent species was harvested at several timepoints along the growth curve. Cells were removed from culture by centrifugation at 5000 x *g* for 10 minutes, followed by 0.22 μm filtration using a syringe top filter.

5.2.6 Autoinducer induction assays

To test whether luminescence defective *V. cholerae* strains produce expected levels of autoinducer, autoinducer induction bioassays were performed, following the procedure of

Bassler et al. (1997). *V. harveyi* BB170 (*luxS* autoinducer sensor, Bassler et al., 1997) was used as the autoinducer induction assay reporter. *V. harveyi* BB120 or BB170 was used as positive control (autoinducer donor), while *V. fischeri* strains MJ100 or MJ203 or uninoculated, sterile medium was used as a negative control in the autoinducer induction assays. The *V. harveyi* BB170 reporter strain was grown overnight in AB medium at 30°C. The culture was diluted 1:5,000 in fresh medium and cell free culture supernatants from test strains were added to final concentration of 10%. Induction of luminescence was measured at multiple time points using a model LB96P luminometer (EG&G Berthold, TN) or a TD20/20 luminometer (Turner Designs, CA).

5.2.7 Aldehyde limitation assay

To test whether luminescence-defective strains are aldehyde limited, decanal (Sigma Aldrich) was added at a final concentration of 0.01% to cultures of seven luminescent defective *V. cholerae* strains, UM4056, 4075, 4082, 4086, 4091, 4103, and 4157, grown according to the basic luminescence assay. Experimentally, decanal is used as it is readily available from commercial sources and less expensive than tetradecanal, but luminescence was measured immediately after addition since this aldehyde is toxic to bacterial cells.

Luminescence was assayed prior to aldehyde induction. After addition of the aldehyde, the culture was vortexed, and assayed immediately for luminescence. This assay was performed in the laboratory of Dr. E. Stabb, University of Georgia, with modification of the luminescence assay. That is seawater tryptone (SWT) medium (Boettcher and Ruby, 1990) was used as the culture medium and the assay temperature was 28°C. A TD20/20 luminometer (Turner Designs, CA) was used to measure luminescence.

5.2.8 Genotypic characterization of luminescence defective strains of *V. cholerae*

Three different genotypic characterizations were carried out employing luminescent *V. cholerae* strains from Group I, a survey of the 6 *lux* operon genes, *luxA* nucleotide analysis, and a survey of the 5' and 3' regions of the *lux* operon, to determine if the defects were caused by mutation or deletion in the *lux* operon and/or surrounding DNA.

Genotypic survey of the lux operon. A PCR survey of the 6-gene *lux* operon of the 12 luminescent *V. cholerae* strains of Group I was conducted to determine if absence or alteration of one of the structural genes was responsible for the observed luminescence defects. PCR primers used were either Southern hyrbridization probe primers determined from sequencing the *lux* operon of *V. cholerae* UM4057 (Chapter 4) or newly designed

Table 5.2. PCR primers used for genotypic characterization of luminescence defective strains of *V. cholerae*.

ID	Sequence	Target	Amp. size (bp)	T_m (°C)
VCLUXA108F	CGAAGCGGTTTGGTTGCTA	*luxA*	650	59
VCLUXA757R	CGGGTAGCATTGACGTAGGA	*luxA*		
pluxD445F	GTGAATCTGCGCGATACCTT	*luxD*	179	59
pluxD624R	ATGGCGATTTGTTCTACCG	*luxD*		
pluxB418F	ACCACAGGTTATTGCCATCC	*luxB*	195	58
pluxB611R	TATTCTTGCCGAGTCGCATT	*luxB*		
luxC466F	GTGCCTCTTTCAGGCGTAAC	*luxC*	198	59
luxC664R	GTTGGGCTACCTGAGTGCAT	*luxC*		
pluxE359F	CTCGAGACCGCCTGAGTATT	*luxE*	201	58
pluxE559R	CGAGGTCATCTTTTGCGGTA	*luxE*		
luxE1135F	CCCCACATCACACCATAAGA	*luxE-G*	200	58
luxG163R	GTGAAGGGCAACTAGCAATA	*luxE-G*		
VcluxAI-F	ACGAGCGTCGAATGAAAAAC	*luxA*	674	50
VcluxAI-R	CCAGCTCAGAATCATGGGTAA	*luxA*		
VcluxAII-F	CCTGTCTATGTGGTCGCTGA	*luxA*	638	53
VcluxAII-R	GATTGGCCAGAATGAAGGAA	*luxA*		

primers generated using the Primer3 program (Rozen and Skaletsky, 2000). These are listed in Table 5.2. Genomic DNA (100-ng) prepared from *V. cholerae* luminescence-defective strains was amplified in 1X PCR buffer containing 0.8 μM of each primer, 200 μM dNTP, and 0.625 U of *Taq* DNA polymerase, using the following cycling conditions: initial denaturation at 94°C for 3 minutes, 30 cycles of denaturation at 94°C for 1 minutes, annealing at T_m of primer set (i.e., lowest T_m of forward and reverse primer pair) - 5°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. Negative control reactions, which contained MilliQ water instead of DNA, were performed for each of the six *lux* gene PCR reactions. Gel electrophoresis, 2% NuSieve 3:1 agarose (FMC), in 1X TAE, 1 hr at 100V in a model MPH horizontal gel apparatus (IBI), was carried out on the PCR reactions.

luxA nucleotide analysis. To test whether changes in the nucleotide sequence of one of the *lux* structural genes could account for the luminescence expression defects, PCR primers were designed to clone and sequence the *luxA* gene, in two halves, of nine of 12 luminescence *V. cholerae* strains from Group I. The primers used, VcluxAI (F/R) and VcluxAII (F/R), are presented in Table 5.2. PCR reactions were carried out according to the protocol described above, using *Pfu* polymerase. Blunt-ended PCR products were cloned into vector pCR-Blunt II-TOPO using the Zero Blunt TOPO PCR cloning kit (Invitrogen) and transformed into One Shot TOP10 chemically competent *E. coli* cells (Invitrogen) according to manufacturer's instructions. The resulting plasmids were extracted from the host cells, using QIAprep spin miniprep kit (Qiagen). Proper insertion of PCR amplicons was tested by digestion of 1.0 μg of plasmid DNA with *Eco*RI, followed by gel electrophoresis. The inserted fragment was sequenced using flanking vector M13 primer

sites (forward and reverse), and an ABI 3100 automated capillary DNA sequencer. Nucleotide and corresponding protein sequences for the nine strains were compared using the ClustalX program (Thompson et al., 1997).

Commonality of 5' and 3' gene arrangement. To determine if changes in the flanking regions of the *lux* operon upstream of *luxC* (5') and downstream of *luxG* (3') may have contributed to the luminescence expression defects, PCR primers for regions at each end of the *lux* operon of luminescent *V. cholerae* UM4057, including part of the flanking genes, were designed. PCR was carried out on 98 strains of luminescent *V. cholerae*, 86 strains from Group II and 12 from Group I, and the products were analyzed by gel electrophoresis. Those strains that did not amplify with the PCR primers and suspected of having a different gene arrangement were further analyzed by Southern blot hybridization. For analysis of the 5' end of the *lux* operon, total genomic DNA was digested with *Cla*I. For analysis of the 3' end, genomic DNA was digested with *Nsi*I and *Eco*RV. The digests were separated on 0.75% agarose and blotted onto nylon membranes. PCR products from each end of the *lux* operon of strain UM4057 were labeled with ^{32}P by random-priming and hybridized to the membranes.

5.3 Results

5.3.1 Distribution of luminescence expression among *V. cholerae*

As stated in Chapter 2, the incidence of luminescence among strains of *V. cholerae* from the Chesapeake Bay, MD, and Bangladesh observed in this study was 52% and 5%, respectively. The two collections of strains were re-analyzed according to expression level. Among 116 luminescent strains from Chesapeake Bay, 85.3% expressed luminescence at a

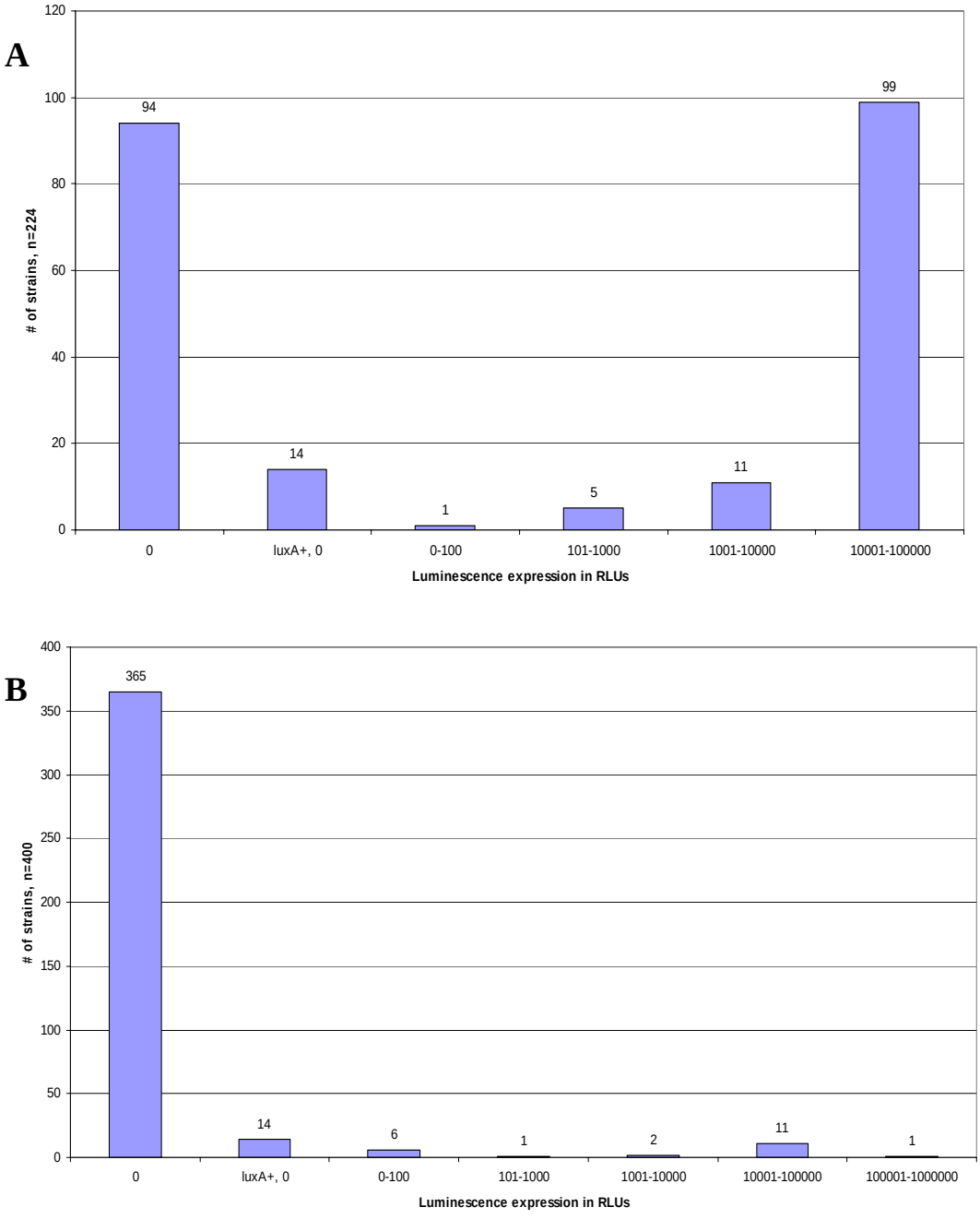


Figure 5.1. Distribution of luminescent *V. cholerae* from A. Chesapeake Bay (Group II), and B. Bangladesh (Group V), by luminescence expression level. Relative light units (RLUs) are defined in Materials and Methods. Expression level of 10 RLUs is the lowest value considered luminescent.

level of 10,000-100,000 RLUs. These values are approximately 10% of the level of expression of *V. fischeri* and *V. harveyi*, measured under the same conditions (this study). This expression range is characterized as "normal" expression, since this log range represents the highest percentage of luminescent isolates from either group and includes the mean expression value of all luminescent isolates from both groups. Nearly 15% (17) of the Chesapeake Bay luminescent isolates had some defect in luminescence expression (Figure 5.1A), with one strain demonstrating an expression level less than 0.1% of the "normal". These defects are illustrated more clearly in Figure 5.2A, which shows expression level (log scale) compared with cell growth or cell density. From Figure 5.2A, it is clear that two of the 16 defective strains are a result of low cell density at the time luminescence was measured. The two strains grew much slower than the other 222 isolates and never reached an OD_{600} above 0.5. Eleven isolates (9.5%) from Chesapeake Bay, MD expressed luminescence at 10% of normal and 5 (4.3%) expressed luminescence at 1% of normal.

Of the luminescent strains from Bangladesh, 52% expressed light at levels considered "normal" (Figure 5.1B, 10,000-100,000). Interestingly, 29% were "dim", that is, expressing light within the 0-100 RLU range. An RLU value of 10 (25 less the background level of 15) was the lowest value accepted as luminescent. The other two expression classes included three isolates. Also, one strain expressed light above normal levels for *V. cholerae*, approaching expression levels of *V. fischeri* and *V. harveyi*. With respect to *V. fischeri* and *V. harveyi*, these strains are termed "super brights". By examining expression of luminescence versus cell density, two of the six "dim" strains were related to slow culture growth (Figure 5.2B).

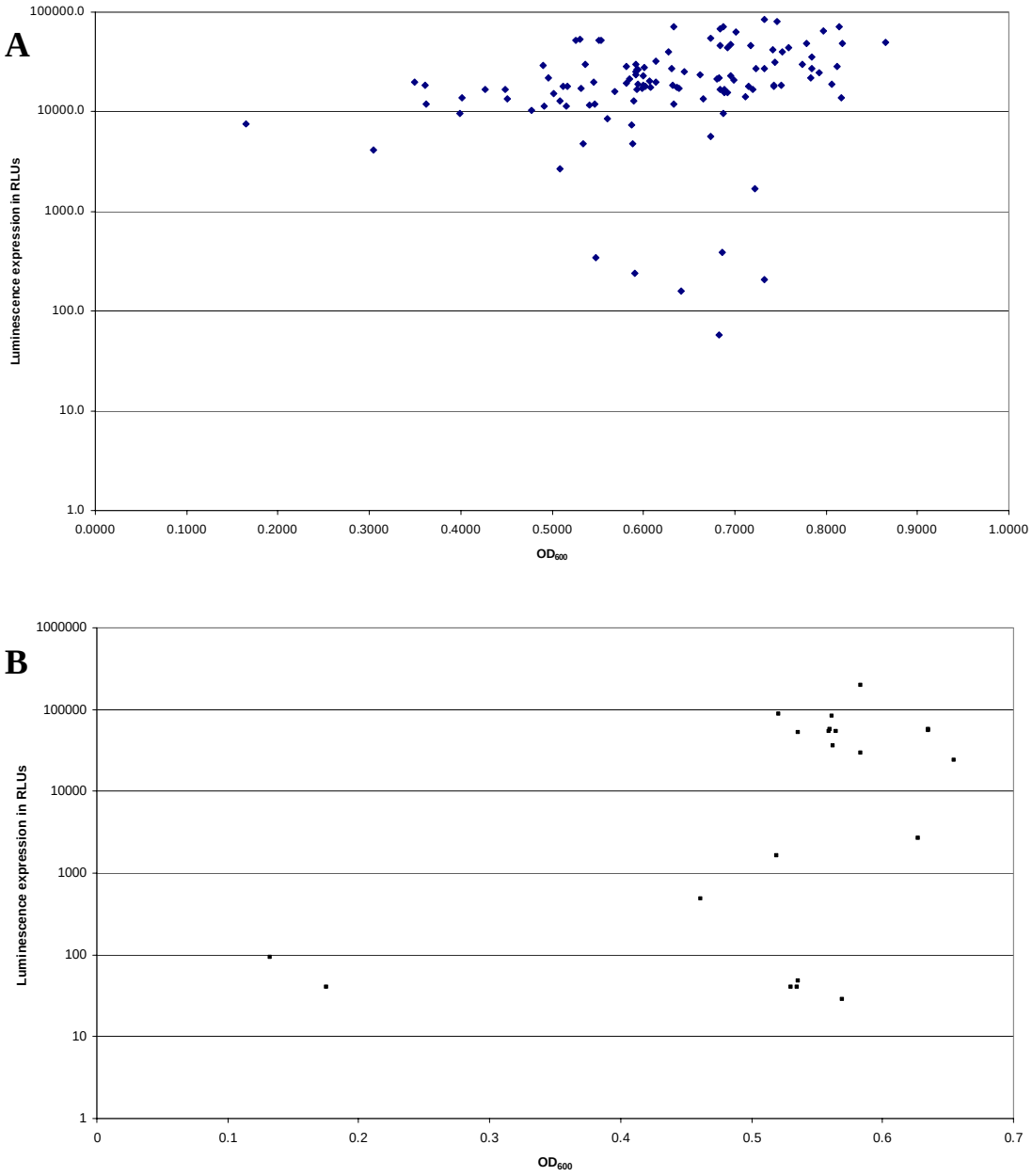


Figure 5.2. Distribution of luminescent *V. cholerae*. A. Chesapeake Bay (Group II), and B. Bangladesh (Group V), by luminescence expression level compared with cell density at the time of measurement (absorbance, or OD600). Relative light unit (RLU) is defined in Materials and Methods.

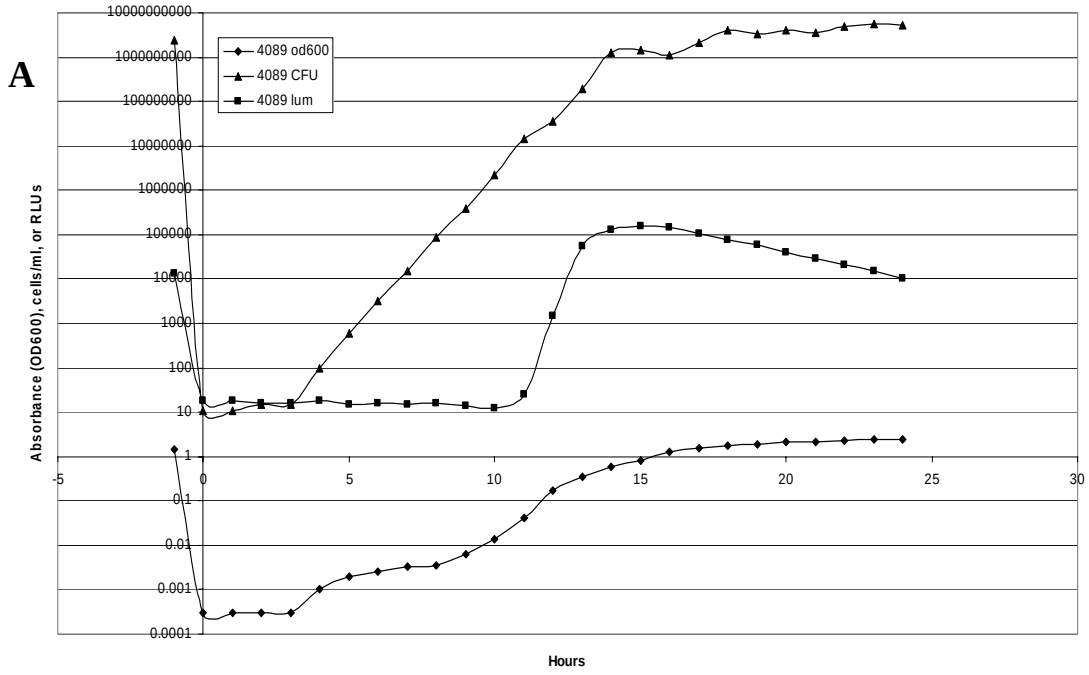
In addition to the visibly luminescent strains, both groups contained "dark" strains; i.e., strains that contain the *lux* operon but do not emit visible light. There were 14 such

isolates (6.3%) in Group II, and 14 isolates in Group V (4.2%) (Figure 5.1). As there is no definition for K variants, with regard to expression level, for the purposes of this study "dark" (0 RLUs) and "dim" (0-100 RLUs) variants are considered to be K variants. Thus, a classification system based on expression range is adopted in this study, with expression from 0-100 RLUs ("dim") comprising Class I, 101-1,000 RLUs comprising Class II, 1,001-10,000 RLUs comprising Class III, and 10,001-100,000 RLUs comprising Class IV (normal expression), and "super bright" (>10,0001 RLUs) comprising Class V. The "dark" strains comprise Class 0.

The twelve luminescent *V. cholerae* UM strains from Group I were also classified by luminescence expression. Strains UM-4071, 4072, 4075, and 4086 are "dark" strains (Class 0). That is, they did not express luminescence. Strains UM-4056, 4091, and 4103 are "dim" strains (Class I), with barely detectable light expression (~20 RLUs). Strain UM4102 expresses luminescence at a normal level (Class IV) and UM4089 expresses luminescence at a high level (Class V). Strain UM4157 is a Class II luminescent strain, while UM-4057 and 4086 are Class III emitters.

5.3.2 Luminescence kinetics related to growth

The growth and luminescence of three strains of luminescent *V. cholerae* from Group I, UM4086 (Class III), UM4089 (Class V), and UM4103 (Class I), as well as *V. fischeri* MJ-100 and *V. harveyi* BB-120, serving as positive controls, were assayed over a 24 hour period (Figure 5.3A-E); *V. fischeri* and *V. harveyi* were assayed longer since their growth was slower (Figure 5.3D and E). For the three strains of *V. cholerae*, luminescence expression began during mid-to-late exponential growth, consistent with an autoinduction controlled



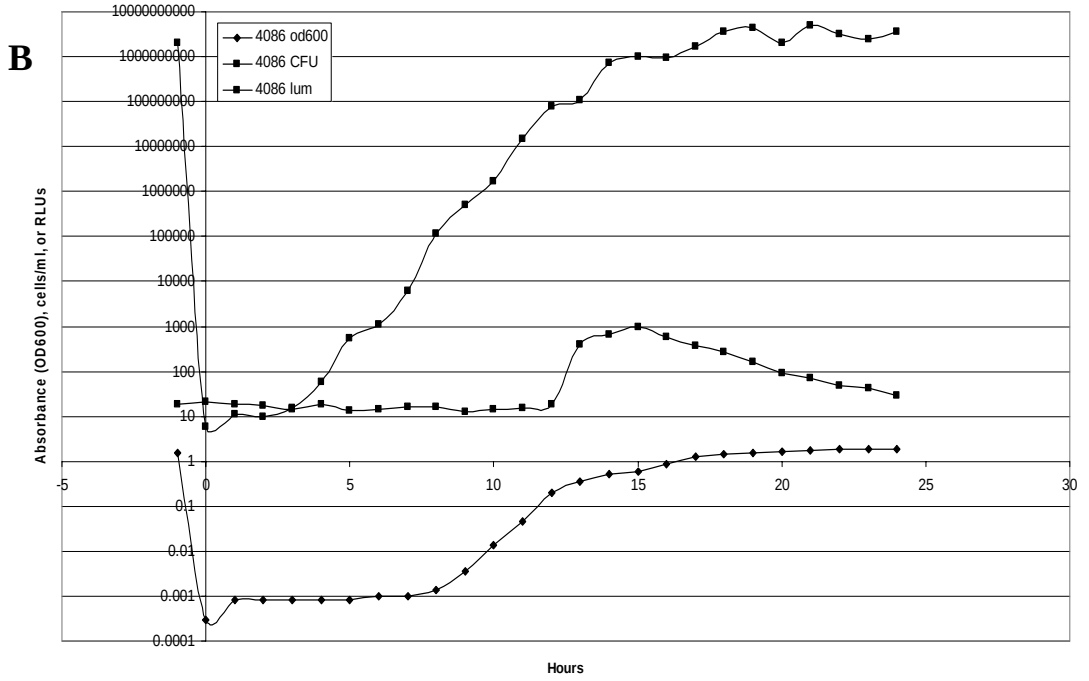
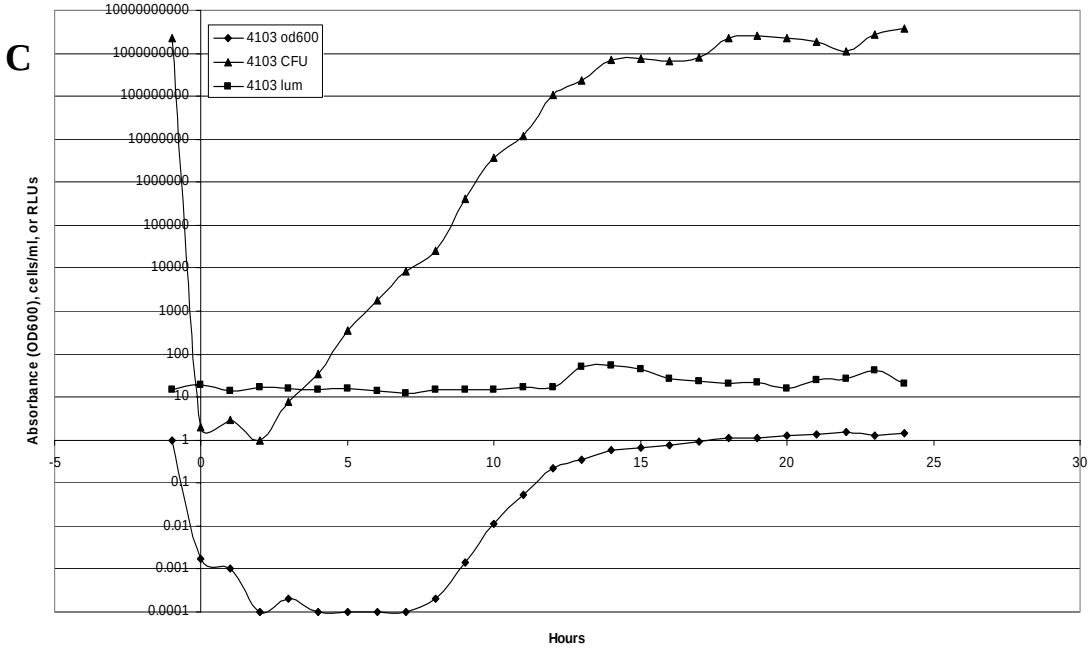


Figure 5.3. Growth and luminescence kinetic curves for A. *V. cholerae* UM4089, B. *V. cholerae* UM4086, C. *V. cholerae* UM4103, D. *V. fischeri* MJ100, and E. *V. harveyi* BB120. Cell density = culturable cell plate counts (cells/ml) and absorbance of culture at 600 nm (OD600) and luminescence = RLUs, n =9.



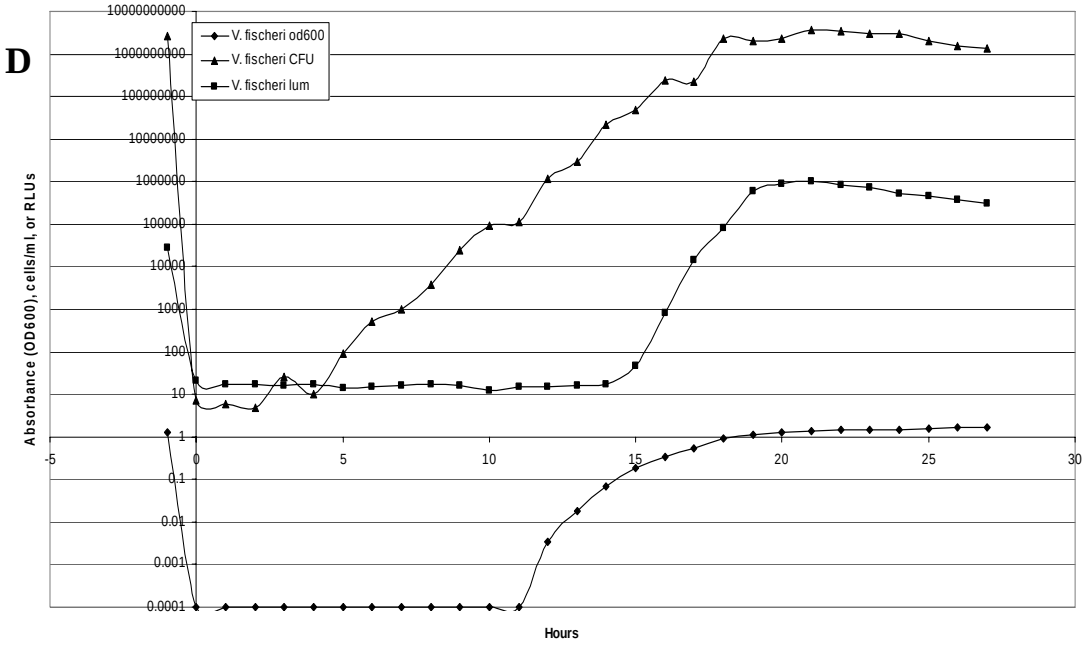


Figure 5.3. Growth and luminescence kinetic curves for *V. cholerae*, *V. fischeri*, and *V. harveyi* (continued).

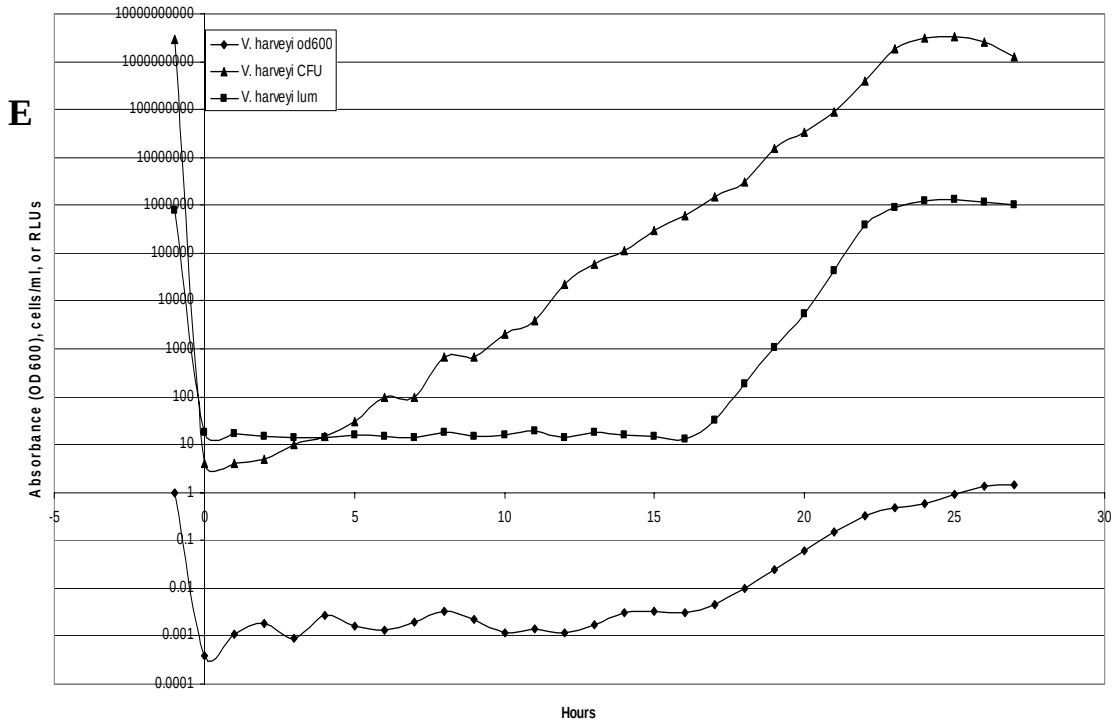


Figure 5.3. Growth and luminescence kinetic curves for *V. cholerae*, *V. fischeri*, and *V. harveyi* (continued).

phenotype (Figure 5.3A-C). The two control strains, MJ-100 and BB-120, were significantly brighter in luminescence expression than the three strains of *V. cholerae*. The brightest strain of luminescent *V. cholerae*, UM4089, had a maximal light expression value of 10^5 RLUs (Figure 5.3A), while the control strains were one log higher (Figures 5.3D and E). In addition, decay of luminescence expression was more rapid among the luminescent *V. cholerae* strains, compared to *V. fischeri* and *V. harveyi*, the control strains.

The maximum values of luminescence expression for the three strains of *V. cholerae* were consistent with those determined previously (Table 5.1, and Chapter 2). Indeed,

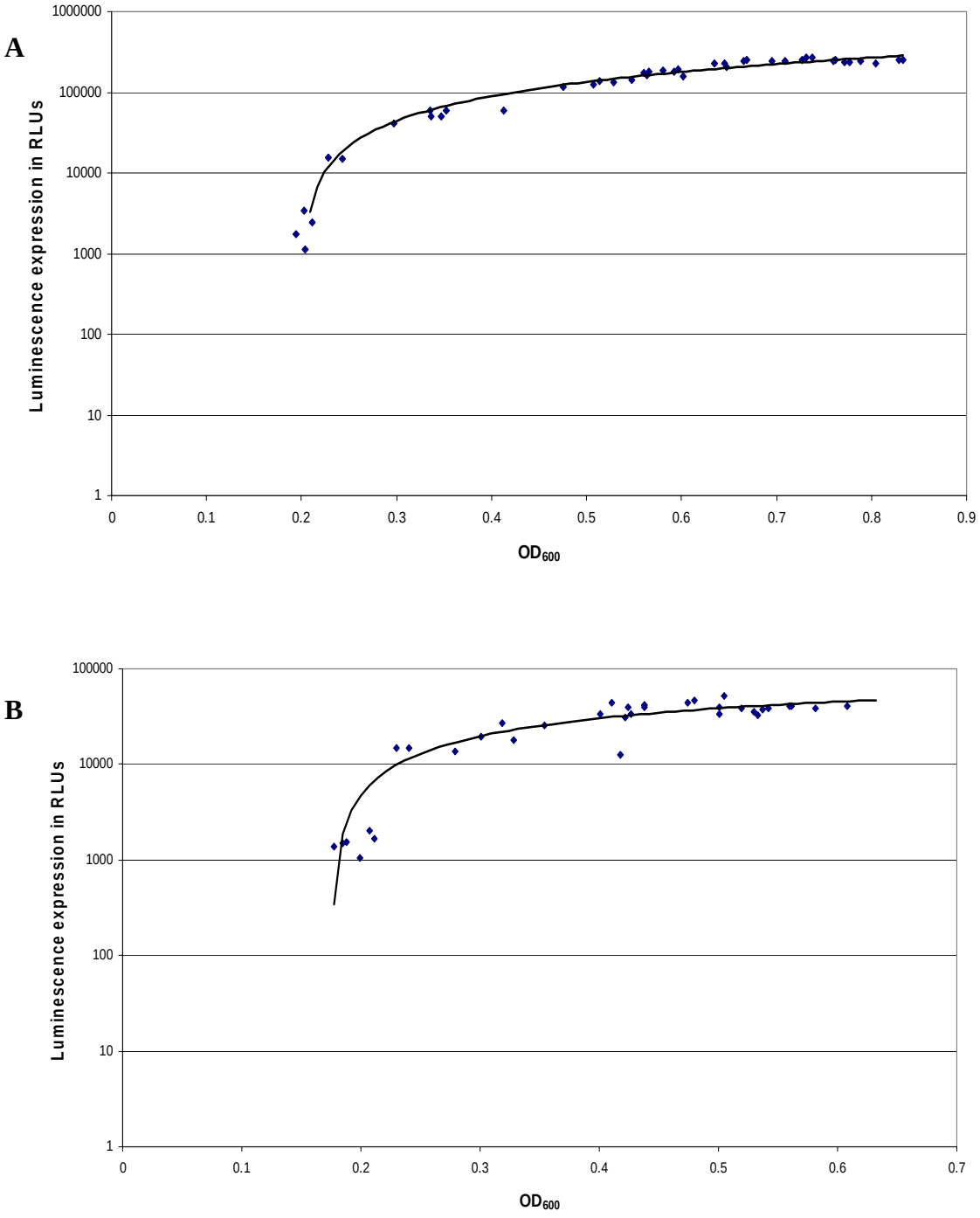


Figure 5.4. Luminescence expression of A. *V. cholerae* RC457, B. *V. cholerae* RC512, C. *V. cholerae* 538, compared with cell density at time of measurement. Results are shown using logarithmic scale, since growth during the time measurements were made was exponential. Regression line of best fit is shown and was used to define "normal" luminescence expression of the isolates over the growth period shown.

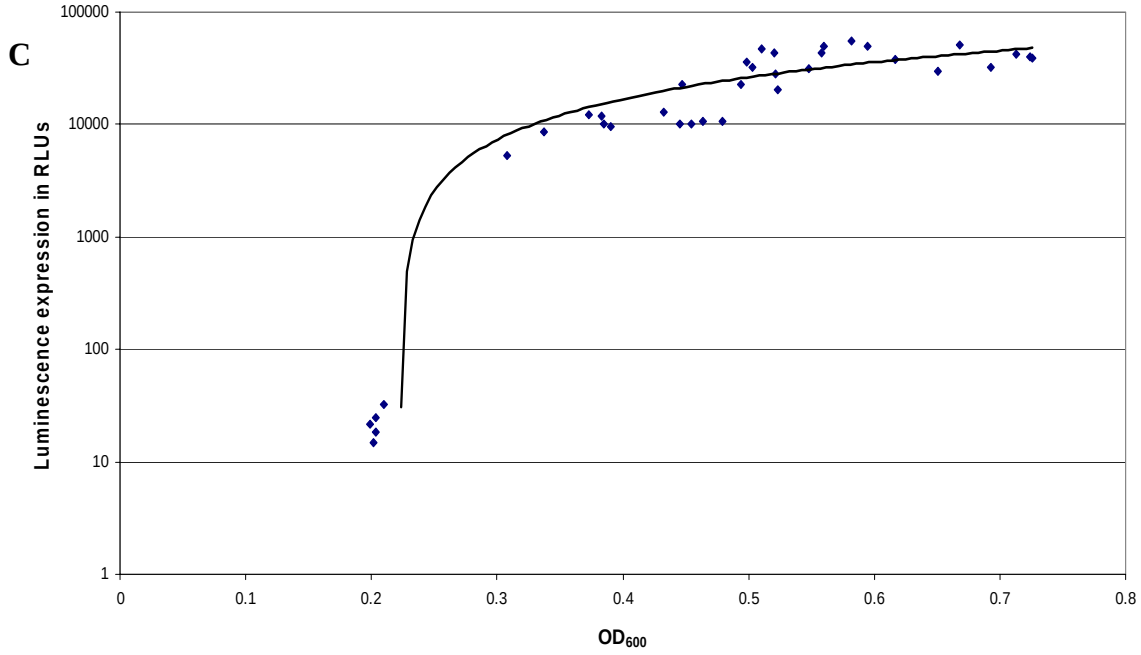


Figure 5.4. Luminescence expression of *V. cholerae* compared to cell density (continued).

expression was stable and relatively unaffected by slight variations in experimental procedure. One trend that was apparent for the three strains was onset of expression correlated with maximum expression level. *V. cholerae* UM4089 expresses luminescence at a normal level (Class IV). For this strain, onset of luminescence expression occurred at a lower cell density, 1.5 x 10^7 cells/ml or OD_{600} of 0.0421, compared to the other *V. cholerae* strains assayed. Onset of luminescence expression is early in *V. fischeri* and *V. harveyi*, as well. For *V. cholerae* UM4086, a Class III strain, onset of luminescence expression occurred later in growth, at a higher cell density of 7.6 x 10^7 cells/ml or OD_{600} of 0.1937. For UM4103, a "dim" strain, or Class I, onset was even later, at a cell concentration of 1.1 x 10^8

cells/ml or OD_{600} of 0.2177. The growth versus luminescnece profile of *V. cholerae* biovar *albensis* was similar to that of *V. cholerae* UM4089 (not shown).

5.3.3 Production of K variants

As stated above, Classes 0 and I represent K variants. K variants of *V. cholerae* could be produced artificially. The three strains were inoculated into LB medium and grown in conditions which were hypothesized to select for K variants, elevated temperature (37°C) and

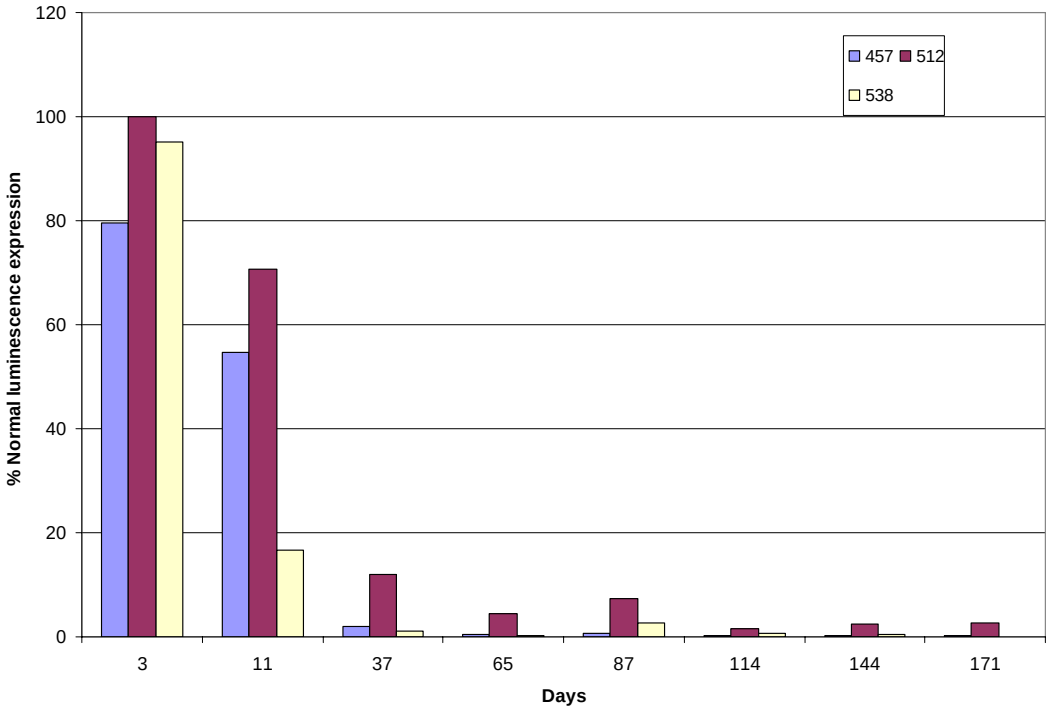


Figure 5.5. Repression of luminescence of luminescent *V. cholerae* RC457, RC512, and RC538 over a six month period measured during growth in LB medium at 37°C under static conditions.

static growth. Aliquots of three luminescent *V. cholerae* strains were assayed for luminescence expression approximately once per month for 6 months. Using luminescence bioassay conditions (Marine Broth, 30°C, aeration), which are considered optimal for

luminescence selection, growth-luminescence assays were performed on three strains, 42 replicates each, to determine "normal" expression profiles. These profiles are shown in Figure 5.4A-C.

Expression values of repressed cultures were compared to normal expression levels at the given culture density, as determined by absorbance at 600 nm. Results are shown in Figure 5.5. All three strains expressed luminescence at a level 10% of normal after the first month. Luminescence of strain RC538 decreased to 28 RLUs (0.02%), and that of

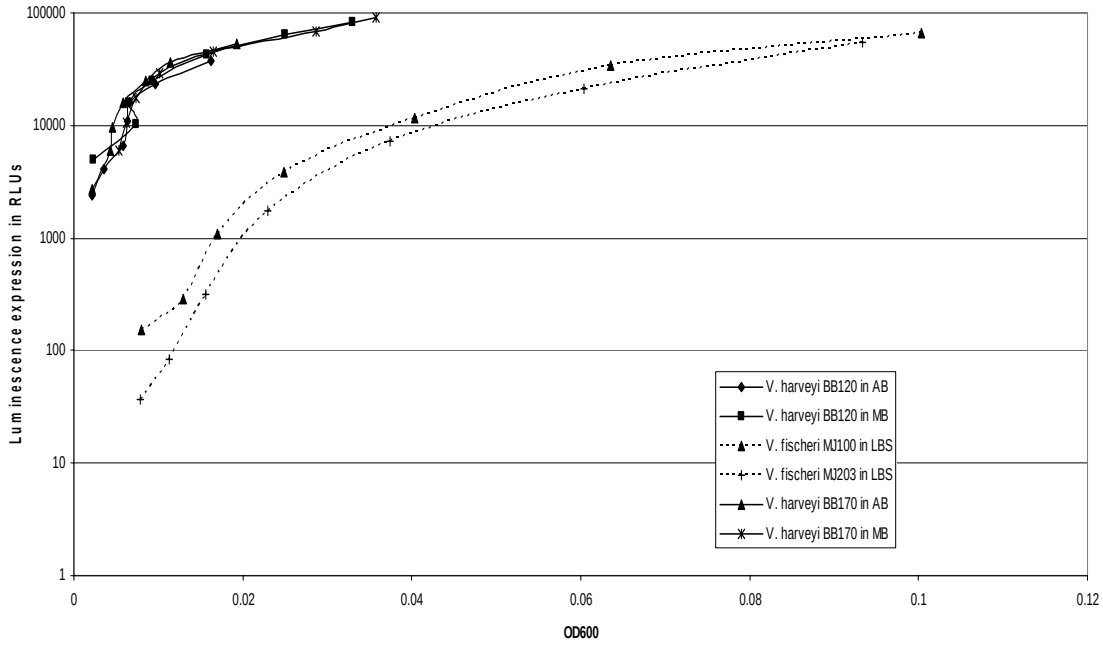


Figure 5.6. Response of luminescence autoinducer-2 (LuxS) reporter strain *V. harveyi* BB170 to exogenous autoinducer prepared from two strains of *V. harveyi*, BB120 (wild type) and BB170 (system-2 sensor) harvested after growth in Marine Broth 2216 or autoinducer bioassay medium (Greenberg, et al., 1979), and two strains of *V. fischeri*, MJ100 (wild type) and MJ203 (Vf autoinducer donor, lux-) after growth in LBS medium (Dunlap, 1989). Dotted lines = negative control; i.e., luminescence expression without exogenous autoinducer.

RC457 decreased to 168 RLUs (0.13%) after 6 months. Interestingly, RC512 continued to emit light that was 2.7% of normal, and appeared to stabilize at that level. Therefore, for some strains, it appears that completely dark mutants (Class 0) can be produced (RC538), while other strains are resistant to becoming completely "dark" (RC512).

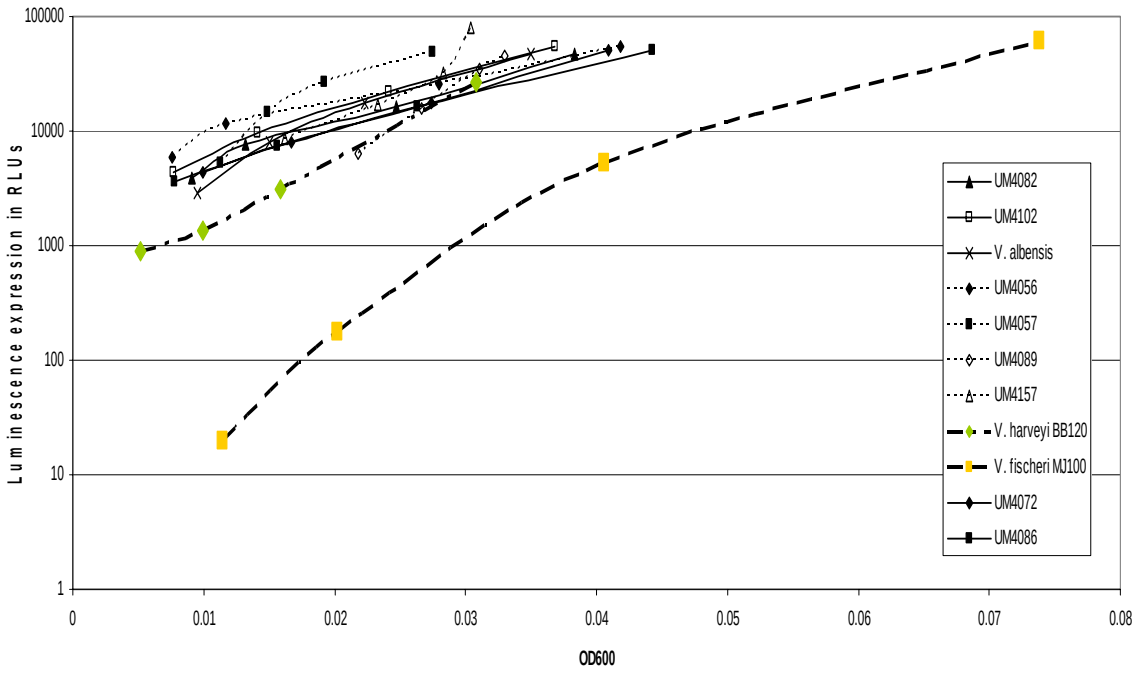


Figure 5.7. Response of luminescence autoinducer-2 (LuxS) reporter strain *V. harveyi* BB170 to exogenous autoinducer prepared from nine strains of *V. cholerae*, UM4056, UM4057, UM4072, UM4082, UM4086, UM4089, UM4102, UM4157, and biovar *albensis*, after growth in Marine Broth. *V. harveyi* BB120 (wild type) served as positive control and *V. fischeri* MJ100 (wild type) as negative control. Dotted lines = positive and negative controls.

5.3.4 Autoinduction

Control experiments were performed with two autoinducer-2 donors, *V. harveyi* BB120 and BB170, and two strains of *V. fischeri,* serving as negative controls (Figure 5.6).

Exogenous autoinducer-2 from *V. harveyi* significantly induced luminescence in the reporter strain, *V. harveyi* BB170, while *V. fischeri* conditioned media did not, regardless of culture medium employed. Conditioned media from *V. cholerae* UM strains that contained autoinducer-2, induced luminescence as effectively as conditioned media from *V. harveyi* and *V. cholerae* biovar *albensis* (Figure 5.7), indicating normal autoinducer production in luminescent defective strains. For clarity, only results of seven of the 12 strains from Group I are shown in Figure 5.7, but results for the other five strains were similar. Conditioned

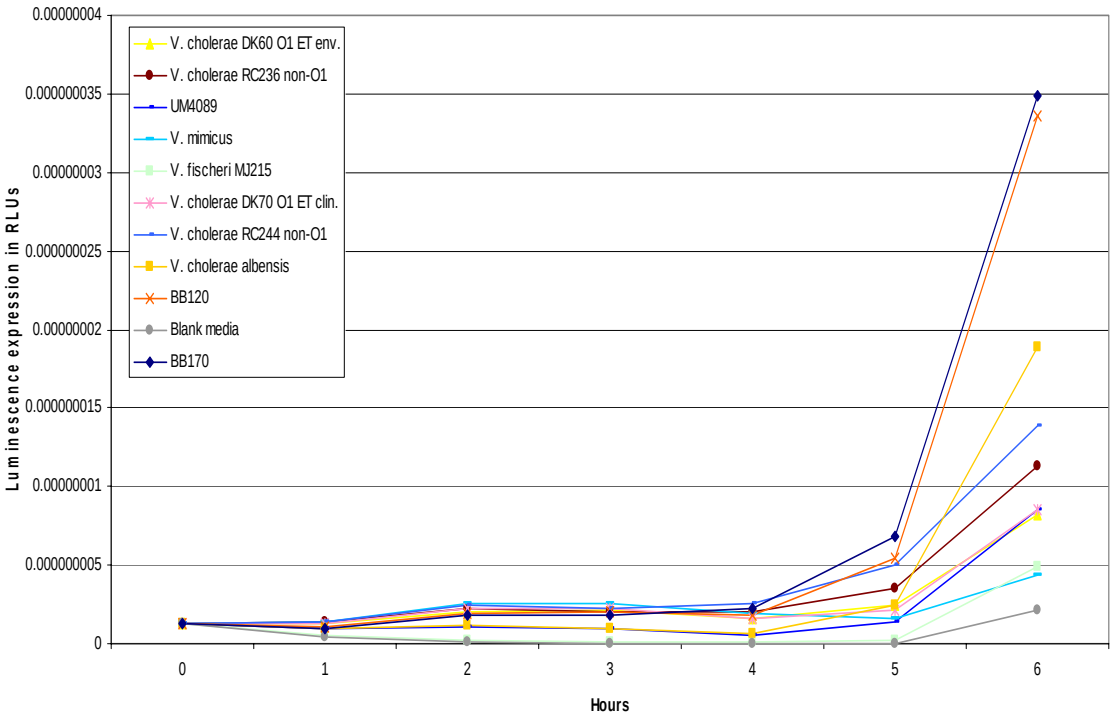


Figure 5.8. Response of luminescence autoinducer-2 (LuxS) reporter strain *V. harveyi* BB170 to exogenous autoinducer prepared from six strains of *V. cholerae*, UM4089, RC236, RC244, DK60, DK70, and biovar *albensis*, after growth in Marine Broth or Brain Heart Infusion Broth. *V. mimicus*, *V. harveyi* BB120 (wild type), and BB170 (reporter) = positive controls; and *V. fischeri* MJ215 ($VfAI^-$) and uninoculated medium = negative controls.

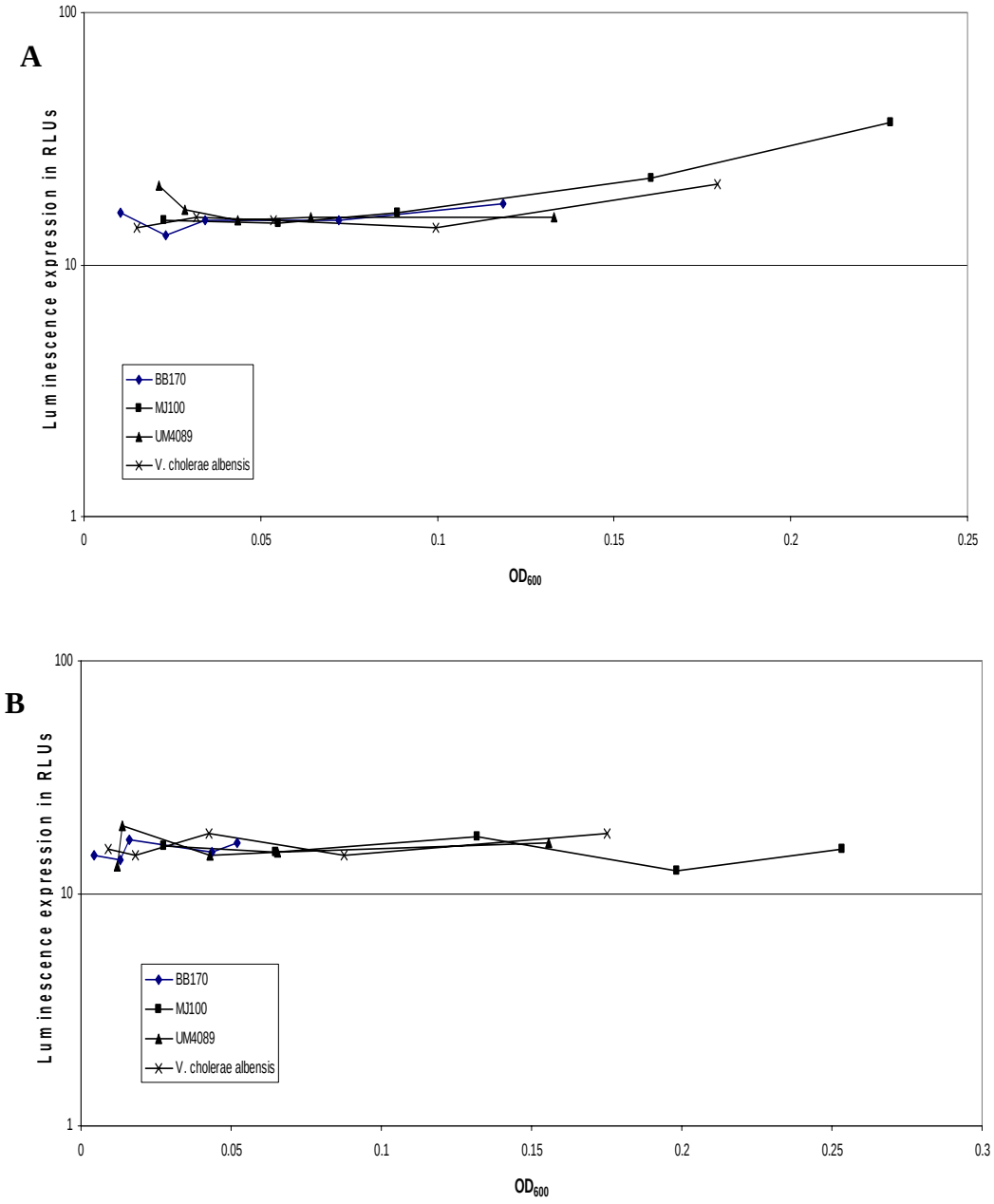
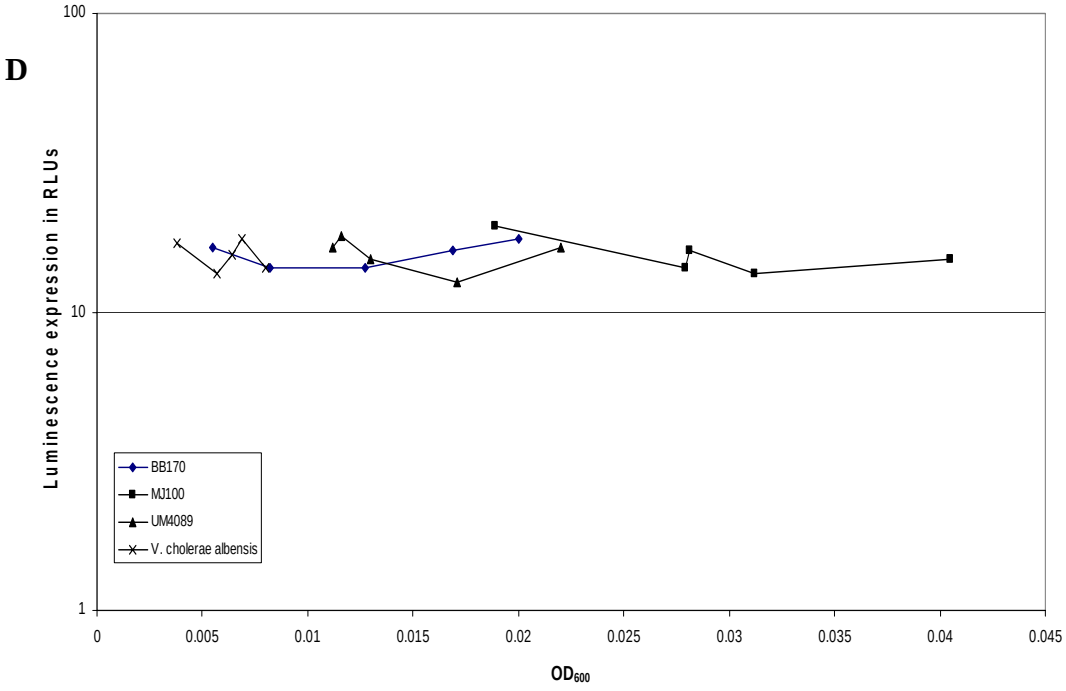


Figure 5.9. Response of luminescence defective strains A. *V. cholerae* UM4056, B. *V. cholerae* UM4075, C. *V. cholerae* UM4082, D. *V. cholerae* UM4086, and E. *V. cholerae* UM4091, to exogenous autoinducer from *V. harveyi* BB170, *V. cholerae* UM4089, *V. cholerae albensis*, and *V. fischeri* MJ100 (negative control).



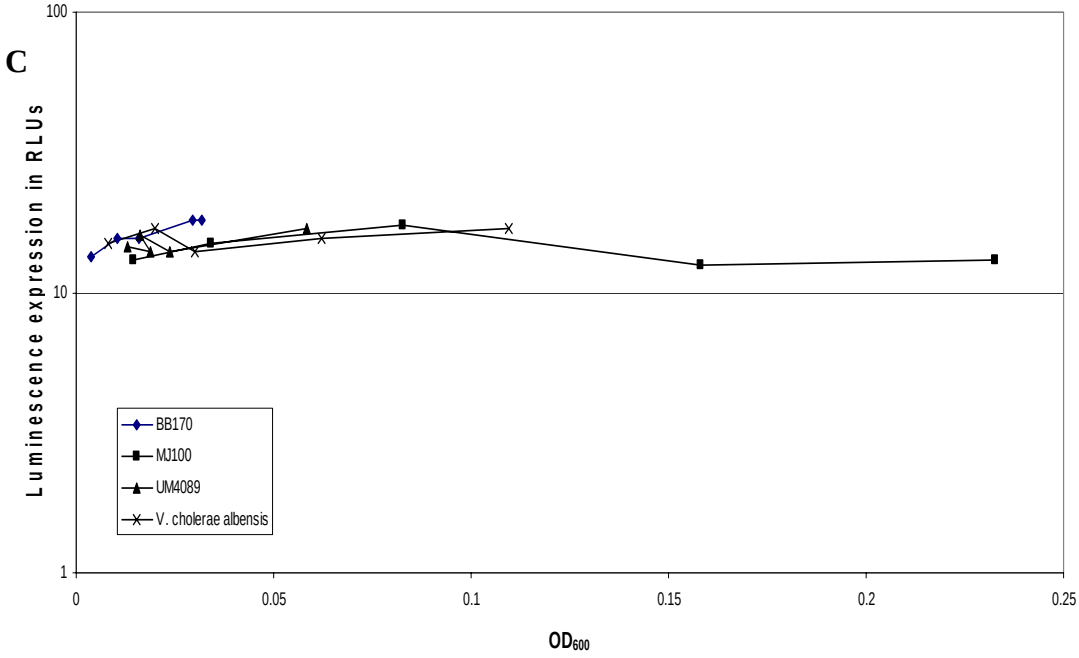


Figure 5.9. Response of luminescence defective strains of *V. cholerae* to exogenous autoinducer (continued).

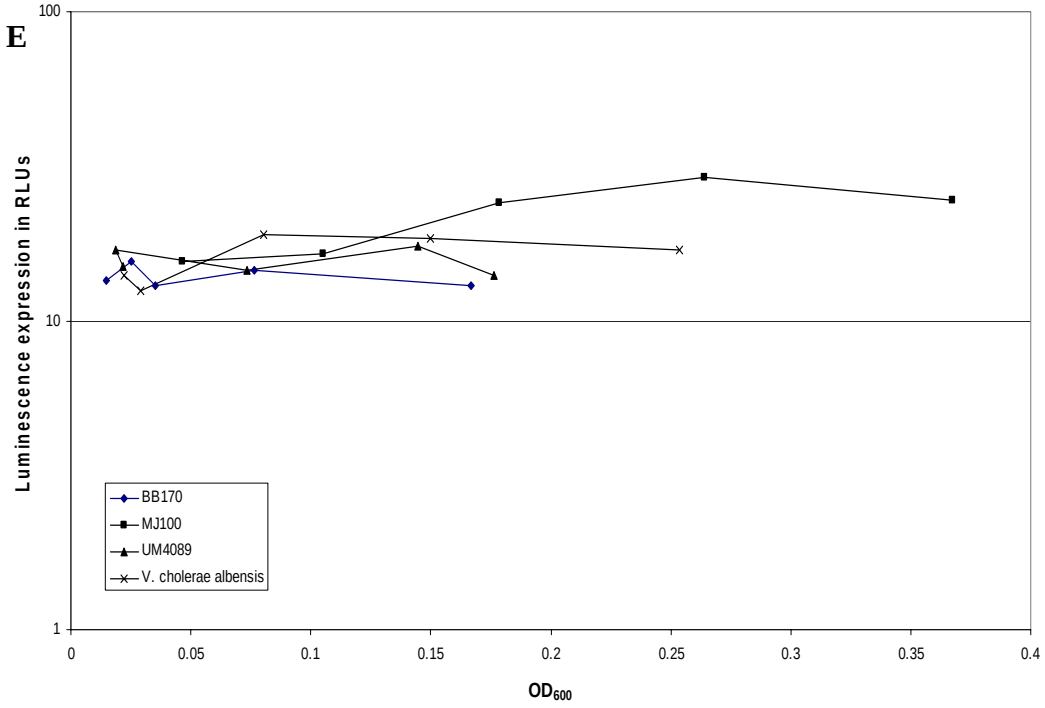


Figure 5.9. Response of luminescence defective strains of *V. cholerae* to exogenous autoinducer (continued).

media from non-luminescent *V. cholerae* was also found to be effective in inducing luminescence in *V. harveyi* BB170 (Figure 5.8), but *V. mimicus* conditioned medium did not stimulate luminescence above negative control levels (Figure 5.8, uninoculated medium, or *V. fischeri* MJ215). Conversely, exogenously added autoinducer, in the form of conditioned medium, from *V. harveyi* BB170, *V. cholerae* UM4089, and *V. cholerae* biovar *albensis* was unable to induce luminescence above defective levels in luminescent defective *V. cholerae* (Figure 5.9A-E).

5.3.5 Aldehyde Induction

Five of seven of luminescent defective *V. cholerae* strains from Group I, UM4056, UM4075, UM4091, UM4103, and UM4157, were luminescent after addition of exogenous aldehyde (Table 5.3). The extent of induction caused by addition of decanal varied

Table 5.3. Aldehyde (decanal) induction of luminescent defective *V. cholerae* with decanal, n=3.

		Luminescence (RLUs)		
Strain ID	**Class**	**pre-aldehyde**	**Post-aldehyde**	**Fold change**
UM4056	I	0.216	6.906	32.0
UM4075	0	0.018	2.397	133.2
UM4082	0	0	0	N/A
UM4086	III	631	566	0.9
UM4091	I	0.801	23.69	29.6
UM4103	I	0.326	6.002	18.4
UM4157	II	14.08	67.4	4.8

dramatically from strain to strain. Two of the strains, UM4082 and UM4086, were essentially unaffected by addition of decanal (Table 5.3).

5.3.6 *lux* operon gene survey

All twelve of the *V. cholerae* strains of Group I contained all six *lux* operon genes as determined by PCR (results not shown), using primers designed employing *V. cholerae* UM4057 sequence (Table 5.1), indicating high sequence homology of the *lux* operons of the luminescent defective strains.

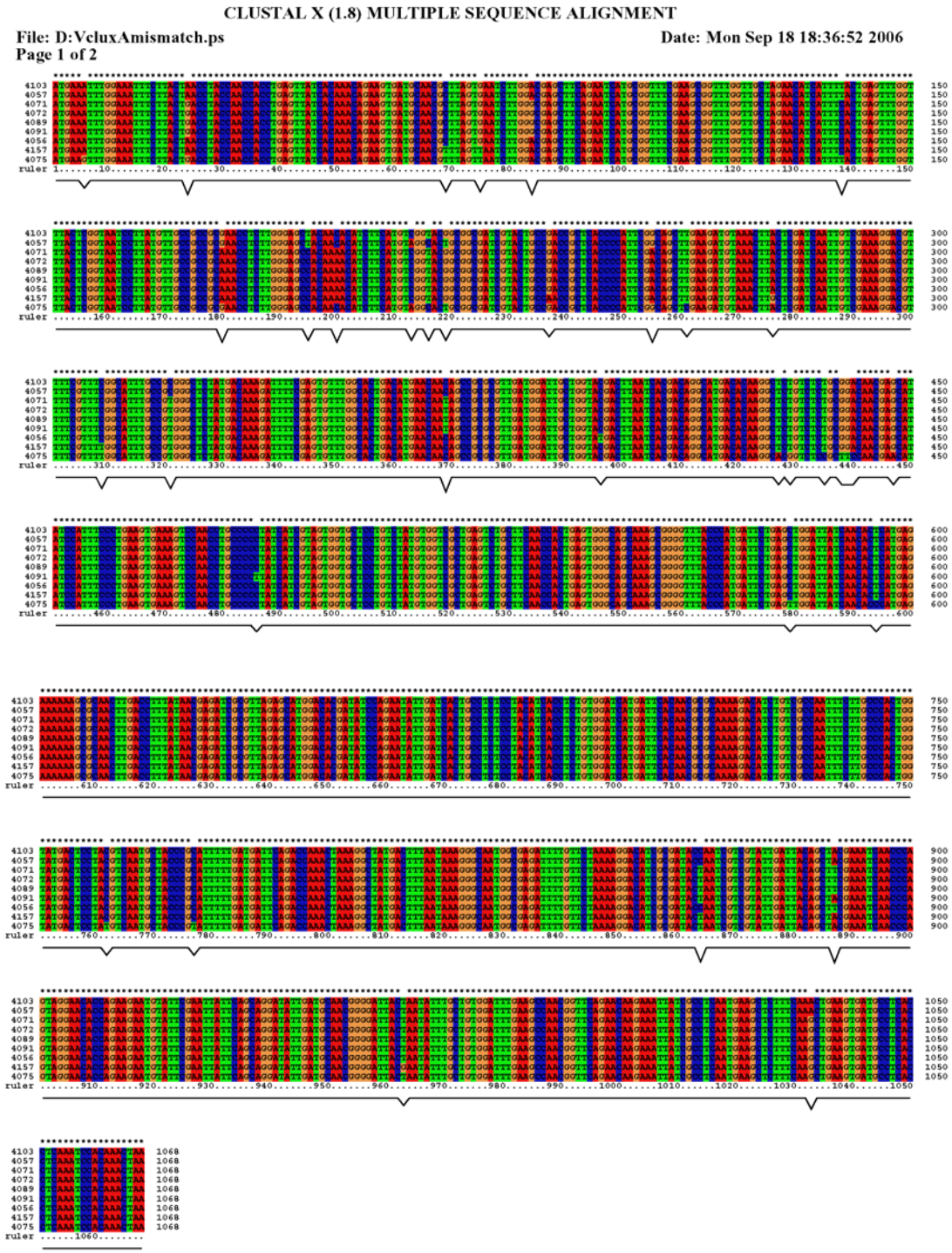


Figure 5.10. Multiple sequence alignment generated by Clustal X (Thompson et al., 1997) of *luxA* nucleotide sequence of nine *V. cholerae* strains from Group I, UM-4056, 4057, 4071, 4072, 4075, 4089, 4091, 4103, and 4157. Absence of asterisks above or a notch in the baseline beneath represent a nucleotide polymorphism.

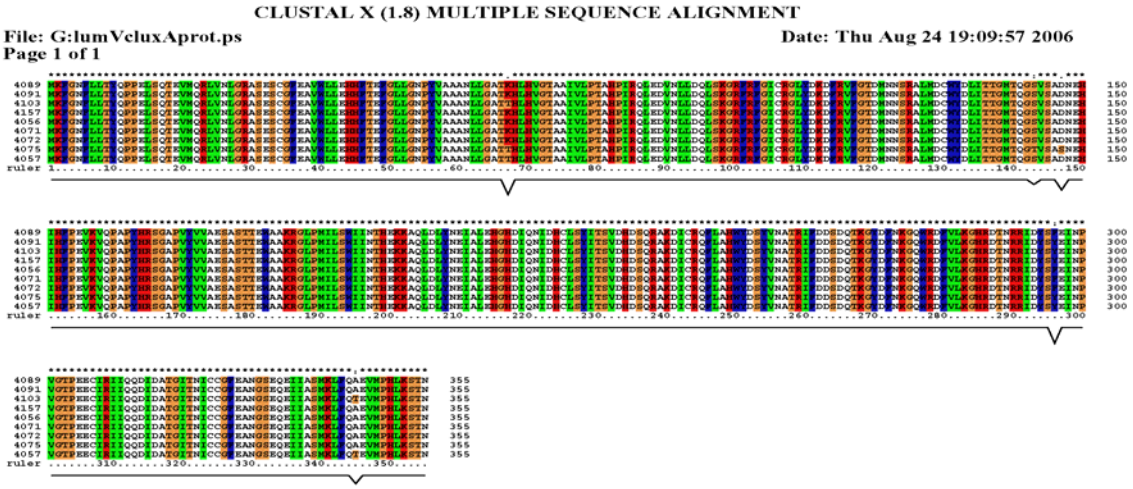


Figure 5.11. Multiple sequence alignment, generated by Clustal X (Thompson et al., 1997), of *luxA* amino acid residues of nine *V. cholerae* strains from Group I, UM-4056, 4057, 4071, 4072, 4075, 4089, 4091, 4103, and 4157. Absence of astericks above or a notch in the baseline beneath represent an alternate allele.

5.3.7 *luxA* sequence analysis

Sequencing of the *luxA* gene from 9 of the 12 luminescent defective *V. cholerae* strains from Group I, UM4056, 4057, 4071, 4072, 4075, 4089, 4091, 4103, and 4157, revealed 36 nucleotide positions in which a polymorphism occurred (Figure 5.10). The majority of the nucleotide substitutions are silent, occurring in the third letter of the codon, as there are only 5 amino acid differences in the translated sequence (Figure 5.11). There was no detectable nucleotide or amino acid mutation pattern, observable with respect to luminescence expression.

5.3.8 Commonality of 5' and 3' *lux* operon arrangement

The overall strategy to determine difference in the upstream and downstream flanking regions among luminescent *V. cholerae* strains is shown in Figure 5.12. Ten strains (10%) did not hybridize with either the 5' or 3' PCR primer pairs, RC379, RC380, RC433, RC435, RC438, RC430, RC431, UM4071, UM4072, and UM4075 (Figure 5.13). These ten strains were further tested using Southern hybridization with radiolabeled probes specific to the 5' and 3' regions of the *lux* operon (Figure 5.14). On the 5' end of the *lux* operon, the different

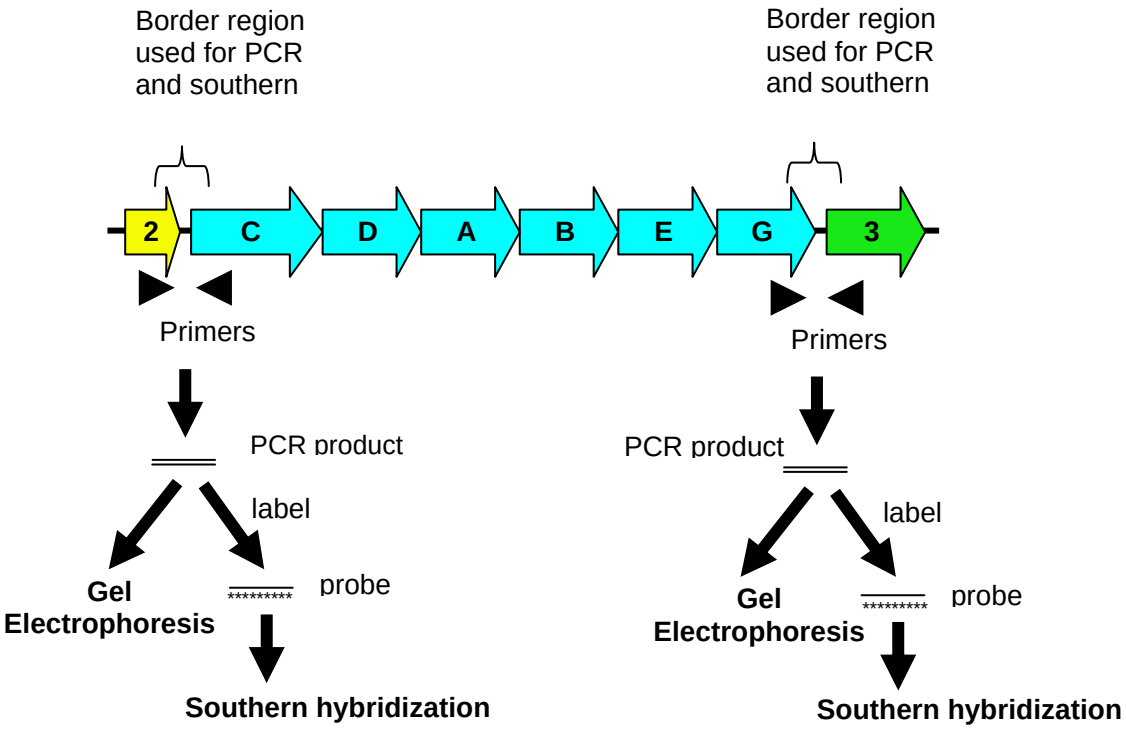


Figure 5.12. Experimental design to determine commonality of gene arrangement in the flanking regions of the lux operon. PCR to detect differences in the 5' and 3' regions was performed first. *V. cholerae* luminescent isolates that failed to generate an amplicon were probed to determine if an alternate gene arrangement existed.

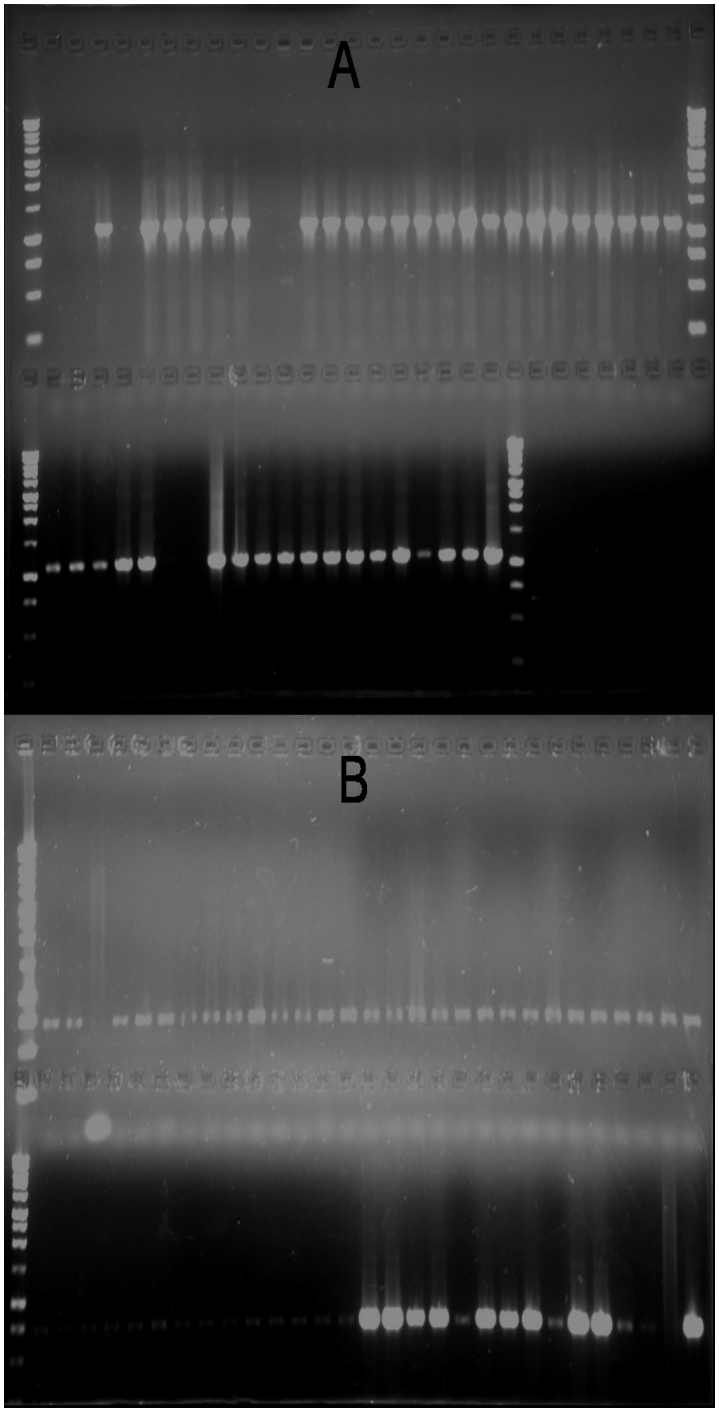


Figure 5.13. Examples of PCR amplification using primers for the 5' end (A) and 3' end (B) of the *lux* operon. Each strain was scored as all or none. In total, 98 strains were tested using these primer sets.

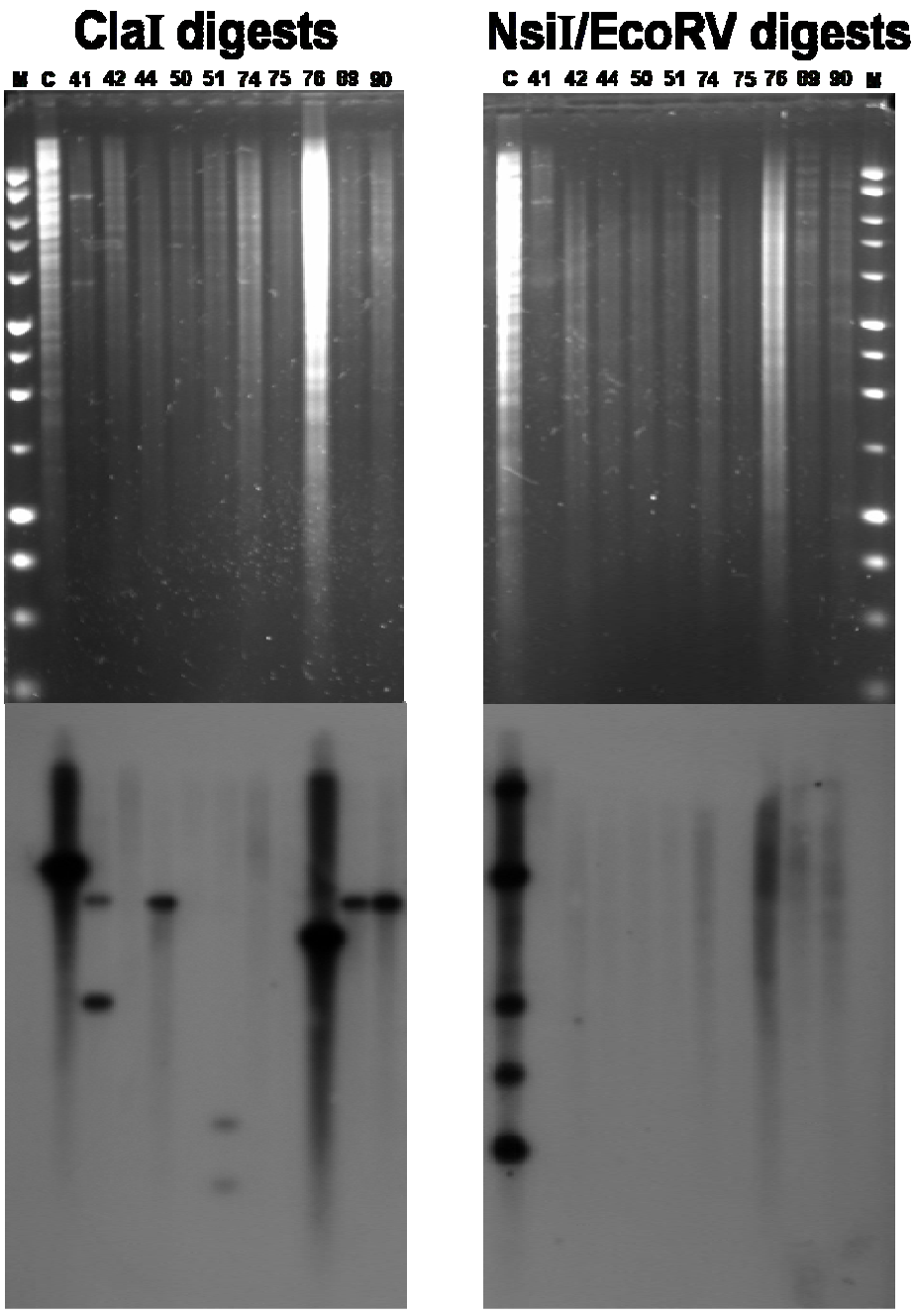


Figure 5.14. Results of Southern blot probe hybridization with radiolabeled probes specific for flanking regions of the *lux* operon of *V. cholerae* UM4057 for the 5' upstream region (*Cla*I digests, orf2-luxC probe) and 3' downstream region (*Nsi*I/*Eco*RV digests, luxG-orf3 probe). M=DNA marker; C=control, UM4057; 41=RC433; 42=RC435; 44=RC438; 50=RC430; 51=RC431; 74=UM4071; 75=UM4072; 76=UM4075; 89=RC379; and 90=RC380.

banding patterns among various strains were consistent with either sequence variability or a difference in genomic arrangement. On the 3' end of the operon, the lack of distinct bands implies that the region from *V. cholerae* UM4057 that was used as a probe might not be present in these strains. Since the blots were hybridized and washed at moderate stringency, the weak smears seen on the autoradiogram most likely represent non-specific background.

5.4 Discussion

5.4.1 Luminescence of *V. cholerae*

From Figure 5.1, it is apparent that the Chesapeake Bay, MD estuary, as a habitat, is selective for a luminescent phenotype of *V. cholerae,* compared to the rural tidal ponds of Bangladesh. A major difference in the two regions, besides geographical location, is overall climate, with Bangladesh having a subtropical climate and the Chesapeake Bay a temperate climate. Based on the results from this study, another difference is the incidence of pathogenic *V. cholerae* (serogroups O1 and O139) not containing the *lux* operon. The two habitats have similar salinity and pH. A more detailed analysis of additional environmental parameters no doubt will identify other factors important in distribution of the luminescent phenotype, e.g., plankton or fish species comprising their systems ecology.

A majority of luminescent strains from Chesapeake Bay, MD express light at the 10^4-10^5 level (Class IV), defined as normal for *V. cholerae*, although stronger expression can occur. Only one of the 116 Chesapeake Bay isolates examined was found to be "dim" (Class I), while 14 "dark" (Class 0) isolates were detected. There were only 16 Chesapeake Bay isolates with luminescence expression below normal but brighter than the "dim" isolates (Classes II and III). For luminescent strains from Bangladesh, the distribution was bi-modal, with 52% expressing luminescence at normal levels and 29% being "dim", but it is

recognized that this pattern may not hold true when a larger sample of luminescent strains is analyzed. The majority of the defective luminescent strains in the two groups were independent of cell growth (Figure 5.2).

These "dark" and "dim" isolates constitute natural K variants of luminescent *V. cholerae*, apparently occuring frequently. Natural K variants have not been reported for *V. fischeri* or *V. harveyi*, although K variants can arise in laboratory maintenance of luminescent strains of both species. K variants of luminescent *V. cholerae* were also readily produced by varying culture conditions in the laboratory (Figure 5.5). Luminescence bioassay conditions were defined as optimal for luminescence expression (Chapter 2) and can, therefore, be considered selective for luminescence expression since the results are consistent and stable over time. Temperature of incubation is set at 30°C, the highest temperature for maximal luciferase activity, which also allows relatively fast growth rate. Adequate aeration is an important factor since elemental oxygen is a reactant in the luminescence reaction. Marine broth 2216 was found to allow maximal luminescence expression experimentally (Chapter 2), but this was expected since the medium is a seawater based medium (mimicing conditions of habitat) with low glucose content (less catabolite repression). These three parameters were altered to determine if K variants could be induced. LB medium was used, which has a higher yeast extract (and glucose) content and promotes rapid growth, as were static conditions to minimize oxygen content. Finally, the incubation temperature was elevated to 37°C, a temperature at which luciferase is expected to be inactive. From results of earlier experiments, it is clear that the first two modifications encourage sub-optimal luminescence expression (see Chapter 2, medium and oxygen optimization). The effect of elevated incubation temperature was investigated by conducting parallel luminescence bioassays in

which each of the three steps involved (from frozen stock to agar plate, from agar plate to overnight broth, and from overnight broth to assay vessel and incubation for 6-7 hours) were performed at 30° and 37°C. Elevation of the incubation temperature to 37°C of any of the three steps yielded significant reduction in luminescence, ranging from 54% to 14% of the normal expression levels. However, it appears that luciferase from *V. cholerae* is active at elevated temperatures.

5.4.2 Luminescence in *V. cholerae* defective strains

The nature of the luminescence defects of *V. cholerae* UM strains from Group I was investigated extensively to determine the mechanism and the extent the phenotype is repressed in *V. cholerae*. Time from onset of luminescence to maximal expression is short for luminescent *V. cholerae,* compared to *V. harveyi* and *V. fischeri* (Figure 5.3). For *V. harveyi* and *V. fischeri*, onset of luminescence, also known as the critical quorum number, is exactly in the middle of the exponential growth stage. For *V. cholerae* UM4089, a strain with high luminescence expression, this is also the time of onset of luminescence. For the two luminescent defective strains, time of onset of luminescence is much later and is correlated with the extent of the defect. Apparently, *V. cholerae* has a stronger feedback repression of luminescence (Figure 5.3A-C) that is expressed beginning at approximately mid-exponential growth. Interaction of the repressor with the *lux* operon can account for the growth versus luminescence profiles shown in Figure 5.3B and 3C.

5.4.3 Exogenous autoinducer luminescence induction

Autoinducer-2, the product of *luxS* gene, is essential for positive regulation of the *lux* system 2 quorum sensing systems. In *V. cholerae*, luminescence is controlled by the *lux* system 2 regulatory cascade. Defective luminescence expression could be explained by

production of low levels of autoinducer by the bacteria. This is true for one "dark" strain of *V. fischeri,* B-61, and is attributed to low to no autoinducer production (Nealson, 1977). To investigate this possibility for *V. cholerae* luminescent strains, exogenous autoinducer from *V. cholerae* luminescence defective strains was tested for ability to induce luminescence in a *V. harveyi* reporter strain (Figures 5.7 and 5.8) and exogenous autoinducer from a luminescent *Vibrio* sp. was tested for ability to induce stronger expression of luminescence in *V. cholerae* luminescence defective strains (Figure 5.9). According to results of these experiments, the defective *V. cholerae* strains produced autoinducer at levels similar to normal luminescent strains (Figures 5.7 and 5.8) and addition of extra autoinducer did not cause the defective *V. cholerae* strains to luminesce above levels without added autoinducer (Figure 5.9A-E). Thus, this type of dark variant appears not to occur frequently.

5.4.4 Aldehyde limitation

To determine if *V. cholerae* luminescent defective strains are stimulated by exogenous aldehyde, decanal was added to cultures and luminescence was assayed. In five of the seven such experiments, aldehyde increased luminescence, but to differing degrees. Interestingly, the aldhehyde did not induce two of the strains, UM4082 (Class 0) and UM4086 (Class III) (Table 5.3). Interpretation of these results is not simple, since aldehyde limitation can be caused by an abundance of growth, regulatory, or genetic explanations. The results obtained in this study suggest that, for some of the luminescent defective strains, the defect is caused by multiple factors, some perhaps occurring at a higher level than that of *lux* regulation, since the defect was not overcome.

5.4.5 Genetic causes of luminescence defects

Alterations in the *lux* operon sequence may explain the luminescence defect observed among "dark" and "dim" strains of *V. cholerae*. Such changes include loss of an entire structural gene, mutation of a *lux* structural gene as to reduce activity, and changes to the flanking regions, especially the 5' upstream sequence which presumably contains an mRNA transcription promoter site. Each of these possibilities was investigated.

None of the *lux* genes were missing in the luminescent defective *V. cholerae* UM strains. This result is altogether not surprising since altered expression is more commonly achieved by changes in regulation. However, the possibility for the opposite outcome is real, as the *lux* operon is not present in all strains of *V. cholerae* and has been found to be transferred horizontally, so loss and gain of the *lux* operon has happened evolutionarily.

Analysis of the nucleotide and amino acid coding sequences of *luxA*, the genetic determinant of the active subunit of luciferase, demonstrate that there is little sequence heterogeneity (Figure 5.10 and 5.11) even though there is considerable variation in the level of expression of luminescence. Only one of the six *lux* operon genes was analyzed, so sequence differences could exist in one of the other *lux* genes.

Three of the twelve *V. cholerae* UM strains, 4071, 4072, and 4075, did not have the same 5' and 3' genetic arrangement as that of UM4057 (Figure 5.13 and 5.14). All three of these strains are dark, exhibiting no detectable light, and in light of these results can be explained by possible loss of *lux* mRNA promoter site.

Table 5.4. Summary of results obtained for Group I *V. cholerae* strains concerning the nature of the luminescence defect.

Strain UM-#	Lum. level	Autoinducer level	Ald. limit[a]	*lux* genes present	*luxA* poly-morphisms[b]	5' region	3' region
4071	Class 0	Normal	ND	6/6	11121	-	-
4072	Class 0	Normal	ND	6/6	11121	-	-
4075	Class 0	Normal	133	6/6	22211	-	-
4082	Class 0	Normal	0	6/6	ND	+	+
4056	Class I	Normal	32	6/6	11121	+	+
4091	Class I	Normal	30	6/6	11111	+	+
4103	Class I	Normal	18	6/6	21112	+	+
4157	Class II	Normal	5	6/6	11111	+	+
4057	Class III	Normal	ND	6/6	21112	+	+
4086	Class III	Normal	0	6/6	ND	+	+
4102	Class IV	Normal	ND	6/6	ND	+	+
4089	Class V	Normal	ND	6/6	11121	+	+

[a]Numbers represent fold-change compared to luminescence levels prior to addition of decanal
[b]Presented as allelic profiles, where 1 represents the most common allele or SNP at a given position and 2 represents the alternate allele. It should be noted that there was not a third allele in any case. Profile is from 5' to 3' in *luxA*.

An effort was made to try to explain the nature of the defect for all of the luminescence defective *V. cholerae* UM strains. A summary of the results is given in Table 5.4. It is apparent that these defects are not easily explained and are likely caused by more than one factor. For all strains, autoinducer production level is normal and all *lux* genes were present. The extent of aldehyde limitation was variable among the defective strains, indicating that this factor is part of the cause of the defect, but other factors are involved as well. Analysis of the 5' and 3' flanking regions of the *lux* operon indicate that an altered 5' and or 3' flanking region can influence luminescence (UM4071, 4072, and 4075). However, a dark strain has the same gene arrangement (UM4082) as the normal luminescent strains. Surprisingly, UM4075, which has an altered 5' and 3' genetic arrangement, is also rescued

by exogenous aldehyde, explained by a second *lux* promoter site upstream of *luxA*, so that *luxABEG* are translated normally, but *luxC* and *luxD* are not. These two genes are important in aldehyde production (along with *luxE*), so loss or altered regulation would be identified as aldehyde limitation. Nevertheless, the level of luminescence expression post-aldehyde addition is still very low, even though it represents a 133-fold change. In conclusion, the various luminescent strains of *V. cholerae* and the gradient in luminescent expression can best be attributed to multiple factors, acting in concert or individually, most likely as a pleiotropic influence on the luminescent phenotype.

Chapter 6 FUNCTION OF LUMINESCENCE IN THE NON-SYMBIOTIC MARINE BACTERIUM *V. CHOLERAE*

6.1 Introduction

6.1.1 Habitats of luminescent bacteria

Luminescent bacteria can occur in a variety of marine habitats. Members of over 30 genera, covering 11 families of teleost fish, harbor luminescent bacteria, usually in specialized light organs; however, not all luminescent species of fish require bacteria to produce light. Two out of 19 luminous species of squid have been found to use luminescent bacteria to produce light, while most produce light themselves; i.e., without a microbial symbiont. *V. fischeri* is the most specialized and extensively studied case of this symbiosis, occurring in relationships with squids, such as the Hawaiian sepiolid squid, *Euprymna scolopes* (Makemson, 1986), and pinecone fish, *Monocentris japonica* (Ruby and Nealson, 1976). In many cases, the light organ symbiont can be visualized using microscopy; however, it cannot be cultured in the laboratory, indicating a highly specialized niche, such as symbionts of anomalopid and ceratoid fish. For symbiotic species of luminescent bacteria, it is assumed that this lifestyle acts as selective pressure, maintaining the phenotype.

In addition to occurring in symbiotic relationships, many marine luminescent bacteria can be found in the gut of fish and other marine animals, where they often occur at high densities (Ruby and Morin, 1979). It is proposed that extracellular chitinase production by the bacteria is important for digestion of plankton species. Often, more than one species is present, but the luminescent flora is dominated by one particular species, based on the likely temperature range of the fish involved. Infected animals release luminescent fecal pellets, and contain viable luminescent bacteria (Raymond and DeVries, 1976; Warner et al., 1979;

and Ruby and Morin, 1979). In addition to the gut of fish, luminescent bacteria can be found on the surface or in the hemolymph of certain crustaceans. These infections are not species specific and are correlated with higher temperature, which is explained by a higher number of bacteria during these times (Harvey, 1952).

As opposed to the lifestyles described above, all luminescent bacteria are found free-living in seawater. There is evidence of a dynamic seasonal distribution of luminescent species based on temperature range (Ruby and Nealson, 1978). Luminescent *V. cholerae* offers an ideal model to study the function of luminescence in a non-symbiotic or parasitic habitat, as it is only found free-living in seawater or attached to marine surfaces, such as copepods (Huq et al., 1990; Tamplin et al., 1990), oysters (Hood et al., 1981; Murphee and Tamplin, 1991), and even water hyacinths (Spira et al., 1981).

6.1.2 Ecological functions of the *lux* operon

One of the main focuses of investigations of the luminescent phenotype has been to determine the ecological significance of light emission. Initially, three types of functions were favored to explain the incidence of luminescence: to aid in predation; to avoid predation; and for interspecific communication (Morin et al., 1975). For luminescent bacteria, these descriptions are difficult to apply, although light organ symbiosis fulfills the first two of the three functions. Light organ symbiosis is largely viewed as a highly specific niche. The high content of luminescent bacteria in the gut of marine animals emitting light indicate that this habitat is preferred to free-living, as there is undoubtedly more available nutrient in the former habitat. During their free-living existence, luminescent bacteria do not produce light (Booth and Nealson, 1975) as autoinduction regulation allows them to occupy this habitat without diverting energy to this metabolism.

An extended time in the free-living stage would require some other role of light emission in the marine environment in order for the luminescent phenotype to be conserved. In the case of *V. cholerae,* this is especially true, since there is no described symbiosis, at least to date. One hypothesis that is based on the three functions described above, but modified for the bacterial world, is to aid in propagation. If luminescent *V. cholerae* can attach to a food source of a higher organism, such as a copepod, and emit light, then the food will be ingested and the organism will reach the gut. Several "alternate" roles of luminescence expression have been postulated to explain the conservation of this trait in a non-symbiotic niche or non-symbiotic species.

One possibility is that the luminescence pathway can function as an alternative pathway for electron flow. Alternate pathways for electron flow have been described for *E. coli* (Haddock and Jones, 1977), *Klebsiella aerogenes* (Harrison and Pirt, 1967), and *V. natriegens* (Linton et al., 1977). This hypothesis comes from an early observation that luminescence can function at extremely low oxygen concentrations (Hastings, 1952), in which electron flow cannot proceed by cytochromes. By functioning as a terminal oxidase, luciferase and the luminescent pathway would allow re-oxidation of reduced co-enzymes permitting continuation of growth (Makemson and Hastings, 1986). This would give luminescent bacteria a competitive advantage over non-luminescent species, since they would possess increased metabolic capacities.

Another hypothesis is that luciferase evolved in ancient aquatic bacteria to aid in the detoxification of molecular oxygen, which was accumulating due to an increase in photosynthesis activity (McElroy and Seliger, 1962). These ancient bacteria are hypothesized to have lived under anaerobic conditions, and as such, increasing oxygen

content would have been toxic. Currently, the luminescent pathway could function in the removal of toxic oxygen species, such as superoxide and other radicals (Dunlap, 1991). One study involving *V. fischeri* supports this hypothesis. Dark mutants of strain MJ-1 were more sensitive to hydrogen peroxide than the wild-type parental strain (Dunlap, 1991). Szpilewska et al. (2003) also found that luciferase aided *V. harveyi* in detoxification or reactive oxidative species. And Lyzen and Wegrzyn (2005) recently found that dark mutants of *V. fischeri* and *P. leiognathi* are more sensitive to hydrogen peroxide and other oxidative stresses.

One hypothesis that has been proposed recently, namely that luminescence plays a role in DNA repair, was found by chance experimentation. Czyz et al. (2000, 2003) observed that dark mutants of *V. harveyi* were sensitive to UV radiation. They propose that production of internal light allows DNA repair, in a process termed photoreactivation. Kozakiewicz et al. (2005) later found that chemically induced dark mutants of *V. fischeri, P. phosphoreum* and *P. leiognathi* also are more sensitive to UV radiation, compared to luminescent parentals.

6.1.3 Allelic exchange

To investigate the function of luminescence in *V. cholerae*, a *luxA* chromosomal mutant was produced by allelic exchange via the conditional, or "suicide" plasmid, pVCD442. Allelic exchange to create chromosomal mutations using conditional plasmids involves two homologous recombination, "cross-over," steps. First, a plasmid containing a mutated target allele is mobilized into a recipient cell. Under conditions that prevent replication of the plasmid, but select for the plasmid (usually antibiotic resistance), the plasmid is integrated into the bacterial chromosome by homologous recombination between

the cloned, mutated fragment and the wild-type target. The gene duplication is segregated by a second homologous recombination of the flanking repeat regions of the genes, leaving one copy on the chromosome. These double events are usually rare, so the counter-selectable marker, *sacB* from *B. subtilis*, has been cloned into pCVD442. Expression of this gene is toxic to Gram-negative bacteria when grown in the presence of sucrose. Sucrose resistant colonies which have also lost the antibiotic resistance of the plasmid, in this case ampicillin, have the mutated copy of the gene. Insertional mutation of the gene with an antibiotic resistance marker different from that of the suicide vector allows selection of the mutated chromosomal copy, greatly reducing the number of clones needed to be screened.

6.2 Materials and Methods

6.2.1 Bacterial strains, plasmids, and media

Luminescent *V. cholerae* strain UM4057 was used as the source of *luxA* and strains UM4086, RC457, and RC582 were used as recipients of mutated *luxA*. The plasmids used in this study are presented in Table 6.1. *E. coli* S-17 λpir, containing the *tra* gene, was used as a host to propagate plasmids pCVD442 and pKO1 and to transfer pKO1 by conjugation in *V. cholerae*. *E. coli* DH5α was the host strain for the other plasmids used. Luria Bertani (LB) medium was used to propagate bacteria. Ampicillin and kanamycin were added to LB at a final concentration of 150 μg/ml and 50 μg/ml, respectively. Sucrose was added to LB plates (without sodium chloride) at a final concentration of 10%.

Table 6.1. Plasmids used in this study.

Plasmid	Key features	Source
PGEMblunt	Circularized version of pGEMT-easy, Ampicillin selection, unique *Eco*RV site	Promega
PCG4057luxA	PGEMblunt with *luxA* inserted at *Eco*RV site, leaving unigue *Eco*RV in *luxA*	This study
PUC18K3	Kanamycin cassette flanked by *Sma*I sites, ATG GAA CCC	Menard et al., 1993
PCG4057luxAKm	PCG4057luxA with *aphA-3* inserted into *luxA Eco*RV site (nt 654)	This study
PCVD442	Suicide vector for allelic exchange; MCS of plasmid pGP704	Donneberg and Kaper, 1991
PKO1	*luxA* with inserted *aphA-3* and some flanking pGEM-Teasy MCS inserted into *Sal*I/*Sph*I sites of pCVD442	This study

6.2.2 Construction of a nonpolar *luxA* kanamycin insertion mutant of *V. cholerae* by allelic exchange

There were fours steps involved in the construction of the *V. cholerae luxA* mutant. The process is presented schematically in Figure 6.1. Each is described below. It should be noted that careful and accurate confirmation of each step was performed using either PCR, restriction enzyme digestion, and sequencing, or a combination of the three protocols.

Cloning luxA into ampicillin-resistant plasmid. Since the *aphA*-3 cassette, inferring resistance to kanamycin, was used to disrupt *luxA*, the *luxA* gene from *V. cholerae* UM4057 was cloned into the ampicillin resistant plasmid, pGEMblunt. An 827-bp fragment of *luxA* was amplified from *V. cholerae* UM4057 genomic DNA using *Pfu* polymerase. The blunt-ended fragment was cloned into pGEMblunt, which had been linearized by digesting the vector with *Eco*RV and de-phosphorylating with Antarctic phosphatase, using T4 DNA ligase. The resulting plasmid, pCG4057luxA, was transformed into *E. coli* DH5α on LB

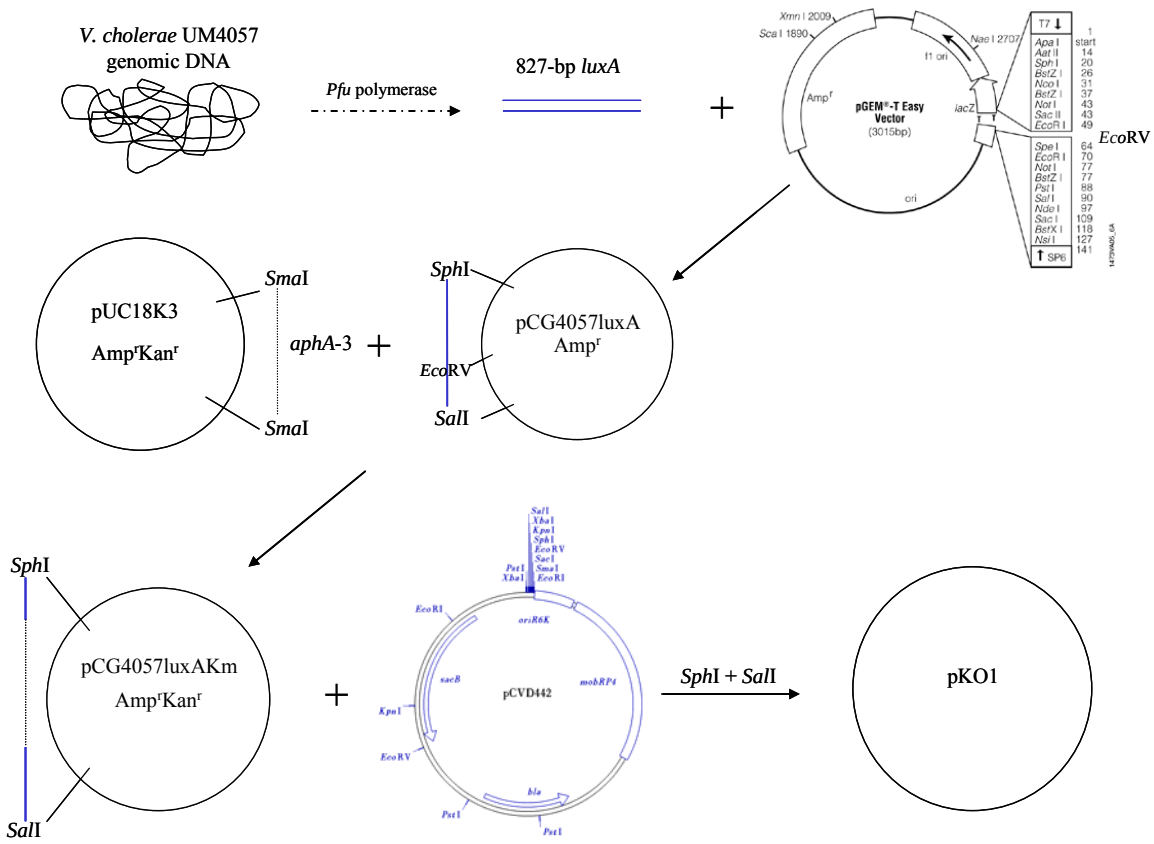


Figure 6.1. Schematic representation of the production or vector pKO1, the first three of four steps used in allelic exchange procedure to generate *luxA* chromosomal mutation in *V. cholerae*. Refer to Table 1 for additional information on each plasmid used. Solid arrows indicate ligation and cloning steps.

plates amended with ampicillin. The plasmid was extracted from the host cells, using the QIAprep spin miniprep kit (Qiagen). The inserted fragment was confirmed by restriction digestion with *Not*I and PCR amplification with *luxA* PCR primers (108F/757R); the *Eco*RV insertion site is flanked by *Not*I sites on both sides.

Insertion of kanamycin cassette into luxA. The previous cloning step, insertion of *luxA* into pGEMblunt, "knocked-out" the unique *Eco*RV site on the plasmid. The *luxA* fragment contains a unique *Eco*RV site located at nt 562 (out of 827). The *Eco*RV site in

luxA was chosen as the site for *aphA*-3 cassette insertion, since the cassette can be cut out of plasmid pUC18K3 with *Sma*I, another blunt-end restriction endonuclease.

Plasmid pUC18K3 was propagated and extracted using the QIAprep spin miniprep kit (Qiagen). The plasmid was digested with *Sma*I to excise the *aphA*-3 cassette containing a 3' start codon with appropriate spacer to ensure nonpolarity (GAA CCC). Plasmid pCG4057luxA was digested with *Eco*RV and dephosphorylated with Antartic phospatase. The *aphA*-3 cassette was ligated into pCG4057luxA with T4 DNA ligase and the resulting plasmid, pCG4057luxAKm, was transformed into *E. coli* DH5α on LB plates amended with ampicillin. Insertion was confirmed by subculturing onto LB plates amended with ampicillin and kanamycin. Plasmid, PCG4057luxAKm, was extracted using the QIAprep spin miniprep kit (Qiagen). The orientation of the *aphA*-3 insert was confirmed by sequencing using flanking M13 forward and reverse sequencing primer binding sites.

Insertion of mutated luxA gene into pCVD442. Plasmid pCVD442 contains 5 unique restriction sites, *Xba*I, *Sal*I (cannot be used with *Xba*I), *Sph*I, *Sac*I, and *Sma*I. The mutated *luxA* gene on plasmid pCG4057luxAKm contains unique *Apa*I, *Aat*II, *Sph*I, *Nco*I, and *Sac*II, sites located on one side of the mutant gene fragment (5') and unique *Spe*I, *Pst*I, *Sal*I, *Nde*I, *Sac*I, and *Nsi*I sites located on the other side (3'). In addition, three enzymes, *Not*I, *Eco*RI, and *BstZ*I contain sites on both sides of the mutated gene. Both plasmids, pCVD442 and pCG4057luxAKm, were digested with *Sph*I and *Sal*I. In addition, digested pCVD442 plasmid was dephosphorylated with Antartic phospatase. Both digestions were analyzed by gel electrophoresis, excised and purified. The cut vector and insert were ligated using T4 DNA ligase. The resulting plasmid, pKO1, was electroporated (see below) into host *E. coli*

S-17 λpir, and the host cells were plated onto LB agar plates amended with ampicillin. Transformation of the vector with insert was confirmed by whole-colony PCR (see below).

Mating of luminescent V. cholerae with E. coli S-17 λpir pKO1. Mating and selection of *luxA* mutant *V. cholerae* were performed as follows. Single colonies of luminescent *V. cholerae* UM4086, RC457, and RC582 were inoculated into culture flasks containing LB broth and incubated overnight at 37°C. A single colony of *E. coli* S-17 λ*pir* pKO1 was inoculated into a culture flask containing LB broth amended with ampicillin and incubated overnight at 37°C. Twenty-five μl of luminescent *V. cholerae* was spotted onto a LB agar plate and allowed to dry. Then, *E. coli* S-17 λ*pir* pKO1 was spotted on top of the *V. cholerae* spot and the plate was incubated overnight at 37°C. The growth from the co-culture spots was scraped off the LB agar plate and resuspended into LB broth. Five 10-fold serial dilutions were made and plated onto LB amended with kanamycin. At this point, the kanamycin resistant *V. cholerae* cells have two copies of *luxA*, one wild-type and one mutant. Two colonies per cross were picked and inculated into culture tubes containing 1 ml of LB broth and incubated at 37°C, for 8 hours, with shaking. Culture growth was diluted with 5 10-fold serial dilutions and 100 μl of each tube was plated onto LB and LB amended with sucrose (and containing no salt) agar plates. The plates were incubated at 30°C overnight. Sucrose-resistant colonies were replica-plated onto LB amended with ampicillin and LB amended with kanamycin agar plates. Ampicillin-sensitive, kanamycin-resistant colonies were subcultured, and confirmed by PCR to be the correct mutation.

6.2.3 Growth characteristics of *luxA* mutants

The growth curve profile of three *luxA* mutants, UM4086 Δ*luxA*, RC582 Δ*luxA*, and RC457 Δ*luxA*, was compared wtih the wild-type parental strains in MB over a 24-hour period. Additionally, luminescence was measured.

6.2.4 Microcosm survival of *luxA* mutants

Overnight cultures grown in Marine broth of three pairs of wild-type and *luxA* mutant strains of *V. cholerae* were diluted into fresh media and incubated until cells reached mid exponential phase (~OD_{660} = 0.5). Cells were harvested by centrifugation and washed twice in 1% Instant Ocean. Aliquots of washed cells were resuspended into 10 ml 1% Instant Ocean and their density standardized (OD_{660} = 0.125) by addition of media or washed cells. Cells were inoculated into 1L 1% instant ocean microcosm flasks at a 1:100 dilution so that a cell density of approximately 1 x 10^7 cells/ml was achieved. For each wild-type or *luxA* mutant strain, six replicate flasks were prepared each time the experiment was performed. Three replicate microcosms were stored at 4°C and three replicate microcosms were maintained at room temperature. Colony-forming units on MA were measured over a three-month period for 4°C microcosms, until the cells reached the VBNC state, and over a one to two year period for room temperature microcosms.

6.2.5 Biofilm production of *luxA* mutants

Overnight growth in Marine broth 2216 (Difco) of three pairs of wild-type and *luxA* mutant strains of *V. cholerae* was diluted into fresh media at a ratio of 1:1000. The cultures were incubated at 30°C, with shaking (200 rpm) for 7 hours. The cultures were inoculated into fresh media in wells of polystyrene tissue culture plates. The cultures were incubated at 30°C for 24 hours. At 12, 18 and 24 hours, biofilm production in each well was measured as

follows. Wells were emptied by dumping the unattached culture growth. Wells were stained with crystal violet and incubated for 10 minutes. The wells were emptied and treated with DMSO to release crystal violet incorporated into attached cells. The released crystal violet/DMSO was quantified by measuring the absorbance at 575 nm (OD_{575}). Negative controls containing only media were used to estimate the background level of crystal violet.

6.2.6 Copepod attachment of *luxA* mutants

Overnight growth in Marine broth of three pairs of wild-type and *luxA* mutant strains of *V. cholerae* were diluted into fresh media at a ratio of 1:1000. The cultures were incubated at 30°C, 200 rpm for 7 hours. Cells were harvested by centrifugation and washed twice in sterile, filtered (0.22 µm) Baltimore Harbor water. Aliquots of washed cells were resuspended into 150 ml 1% Instant Ocean and their density standardized (OD_{660} = 0.125) by addition of media or washed cells. Cells were either used immediately or incubated at room temperature to enhance starvation.

Copepods were collected from Baltimore Harbor, MD by filtering water samples through a plankton net with a 20 µm cut-off size reservoir. Copepods were washed with 1-2 L or sterile, filtered Baltimore Harbor water collected and prepared the previous day. For each strain tested, washed or starved cells of wild-type and *luxA* mutant culture were added to washed copepods and incubated for various times at room temperature to promote attachment of the bacteria to the chitin surface. Additionally, control replicates containing only bacteria or only copepods were prepared.

After incubation, aliquots from each sample were collected and processed to determine the number of bacteria that attached to the copepods. From vessels containing copepods or copepods and bacteria, the copepods were collected by filtering through a 20 µm

sieve and washed to remove unattached bacteria. The copepods were enumerated for each sample processed, homogenized using a glass tissue grinder, diluted and spread-plated onto Marine agar or LB agar plates. Samples containing only bacteria were diluted and spread-plated onto Marine agar or LB agar plates. Plates were incubated at 30°C and RNA colony blots were prepared and hybridized with Vchomim1276 probe, as described in Chapter 3.

6.2.7 Growth of *luxA* mutants under different oxygen tensions.

Overnight growth in Marine broth of three pairs of wild-type and *luxA* mutant strains of *V. cholerae* were diluted into fresh media at a ratio of 1:1000. The cultures were incubated at 30°C, 200 rpm for 7 hours. Cells were diluted 1:1000 into fresh Marine broth in flasks and incubated at room temperature or 30°C in one of four conditions: with shaking (200 rpm), statically, in a microaerophilic chamber, and in an anaerobic chamber. Culture growth was assayed at various time points over 24 hours by measuring the absorbance of the cultures at 600 nm (OD_{600}).

6.2.8 Effect of reactive oxidative species on *luxA* mutants

Overnight growth in Marine Broth of three pairs of wild-type and *luxA* mutant strains of *V. cholerae* were diluted into fresh media at a ratio of 1:1000. When the culture growth reached an OD_{600} value of approximately 0.2 (3-4 hours post dilution), various reactive oxidative species were added to the cultures and culture density was measured over 2-3 hour period. Hydrogen peroxide was added at a final concentration of 1 mM, ferrous sulfate at a final concentration of 0.2 mM, and *t*-butyl hydroperoxide at a final concentration of 0.3 mM.

6.2.9 DNA repair ability of *luxA* mutants.

Overnight growth in Marine broth of three pairs of wild-type and *luxA* mutant strains of *V. cholerae* was diluted up to $1:10^7$, using 10-fold serial dilutions in Marine broth, and

inoculated into fresh media at a ratio of 1:10. The cultures were grown for 4 hours at 30°C, with shaking. The cultures were subjected to UV irradiation at various doses, and 10-fold serial dilutions were prepared for MPN determination. The MPN tubes were incubated for 2 hours in the dark or in the presence of fluorescent lighting. Following these treatments, 100-µl aliquots from each tube were spread-plated onto Marine agar plates to determine initial survival rate. The MPN tubes were incubated for another 22 hours, at which time; the culture density was determined by measuring absorbance at 600 nm. Long term survival determined by MPN count, was monitored after an additional 48 hour incubation at 30°C.

6.3 Results

6.3.1 Construction of a nonpolar *luxA* kanamycin insertion mutant of *V. cholerae* by allelic exchange

Nonpolar *luxA* mutants of *V. cholerae* were successfully produced for three strains, RC457, RC582, and UM4086. Pfu polymerase was used to amplify a 827-bp *luxA* fragment from *V. cholerae* UM4057, whose *lux* operon was previously sequenced. The *luxA* fragment was cloned into vector pGEMblunt (Table 6.1), and confirmed by PCR; the results are shown in Figure 6.2A. To disrupt expression of the *luxA* gene, the kanamycin cassette gene, *aphA*-3, from *Enterococcus faecalis* was inserted into a unique *Eco*RV site located close to the middle of the gene. Figure 6.2B shows successful ligation of *aphA*-3 into pCG4057luxA, resulting in a larger product (1502 instead of 650 bp) when *luxA* PCR, using primers VCluxA108F/757R, was performed on plasmid pCG4057luxAKm (Table 6.1). In addition, clones for which insertion of *aphA*-3 was successful were sequenced to ensure that the kanamycin gene inserted in the correct orientation. The *aphA*-3 disrupted *luxA* fragment was

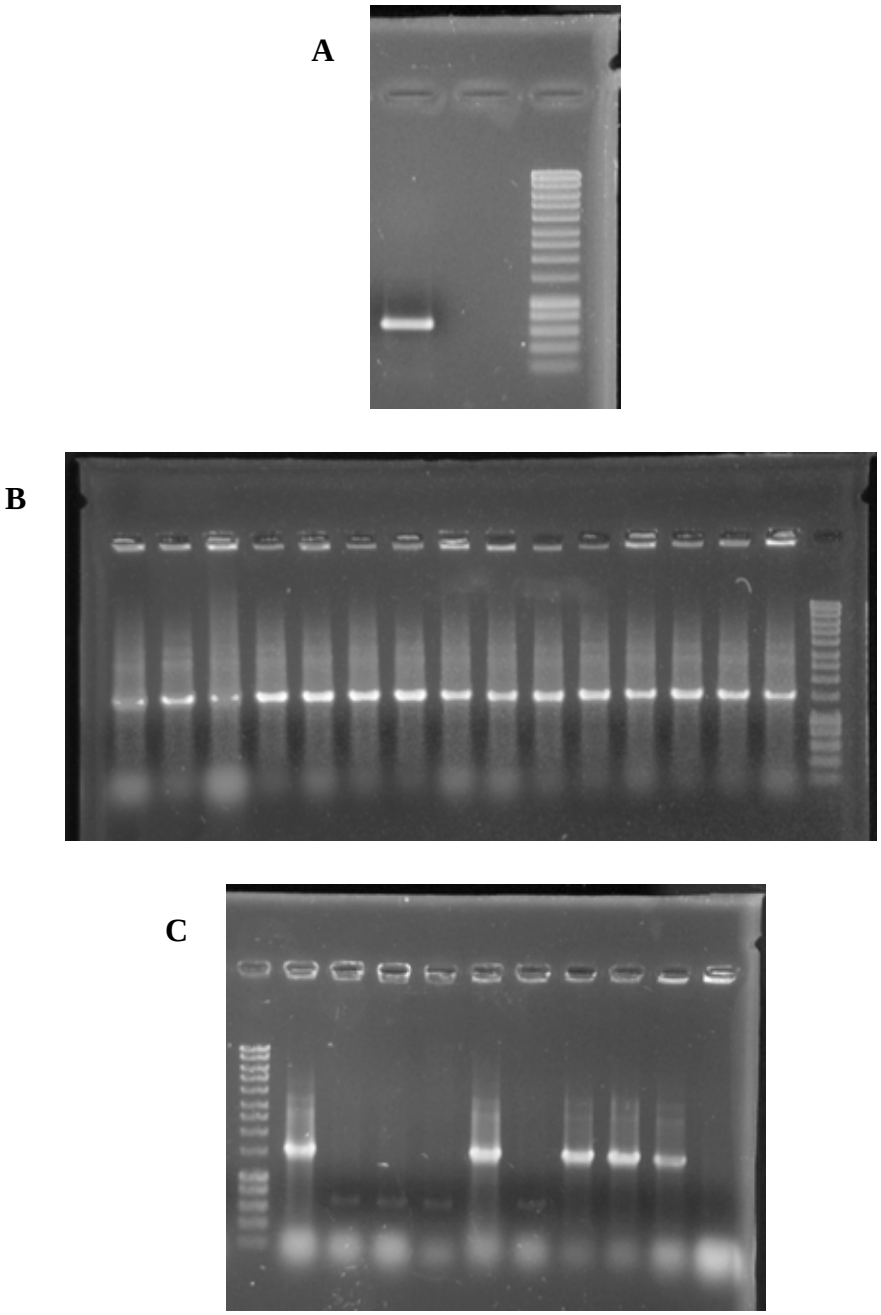


Figure 6.2. PCR confirmation, using *luxA* primers VluxA108F/757R of various steps in production of vector pKO1: A., Step 1, production of plasmid pCG4057luxA (lane 1), containing *luxA* from *V. cholerae* UM4057, B., Step 2, insertion of kanamycin cassette into *luxA* (numerous clones shown, lanes 1-15), and C., Step 3, insertion of mutated *luxA* gene into pCVD442 to yield pKO1 (lanes 2, 6, 8-10). The DNA ladder used (Hyperladder I, Bioline) gives bands at (from top to bottom) 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2.5kb, 2kb, 1.5kb, 1kb, 800bp, 600bp, 400bp, and 200bp.

then cloned into plasmid pCVD442, resulting in plasmid pKO1. The identity of plasmid pKO1 was confirmed by PCR (Figure 6.2C).

Plasmid pKO1 was efficiently mobilized into *V. cholerae* by the direct conjugation, since *E. coli* S17 λ*pir* contains the *tra* gene. Growth in the presence of sucrose was used to induce loss of the integrated plasmid and one of the two *luxA* alleles. Unfortunately, sucrose selection is not effective and yields only 10% fewer colonies and, in most cases, the wild-type and not the mutated allele is retained. This results in screening numerous colonies in order to find a *luxA* mutant. Once identified by subculturing on LB amended with kanamycin and LB amended with ampicillin (mutant is Amp^S/Kan^R), the clones were tested using *luxA* PCR. Figure 6.3 shows the different PCR products from wild-type parental and *luxA* mutant strains for two of the three strains used. Loss of light production for each strain was confirmed using the luminescence bioassay (Chapter 2).

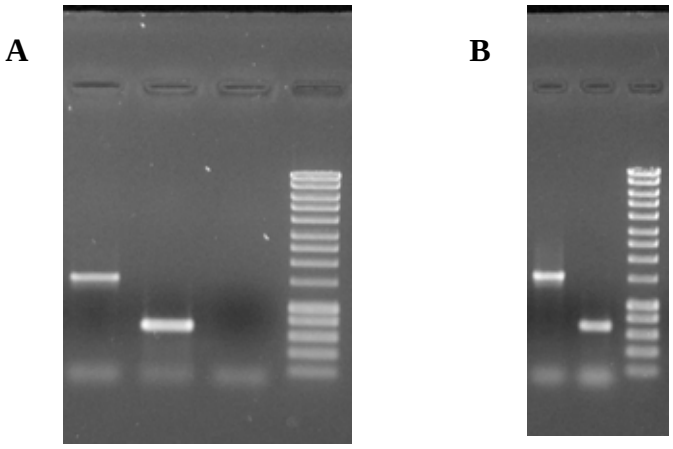


Figure 6.3. PCR confirmation of *luxA* mutants of *V. cholerae* RC457 (A) and UM4086 (B). A. Lane 1, RC457 ΔluxA; lane 2., RC457; lane 3 negative control; lane 4 Hyperladder I. B. Lane 1, UM4086 ΔluxA; lane 2., UM4086; lane 3., Hyperladder I.

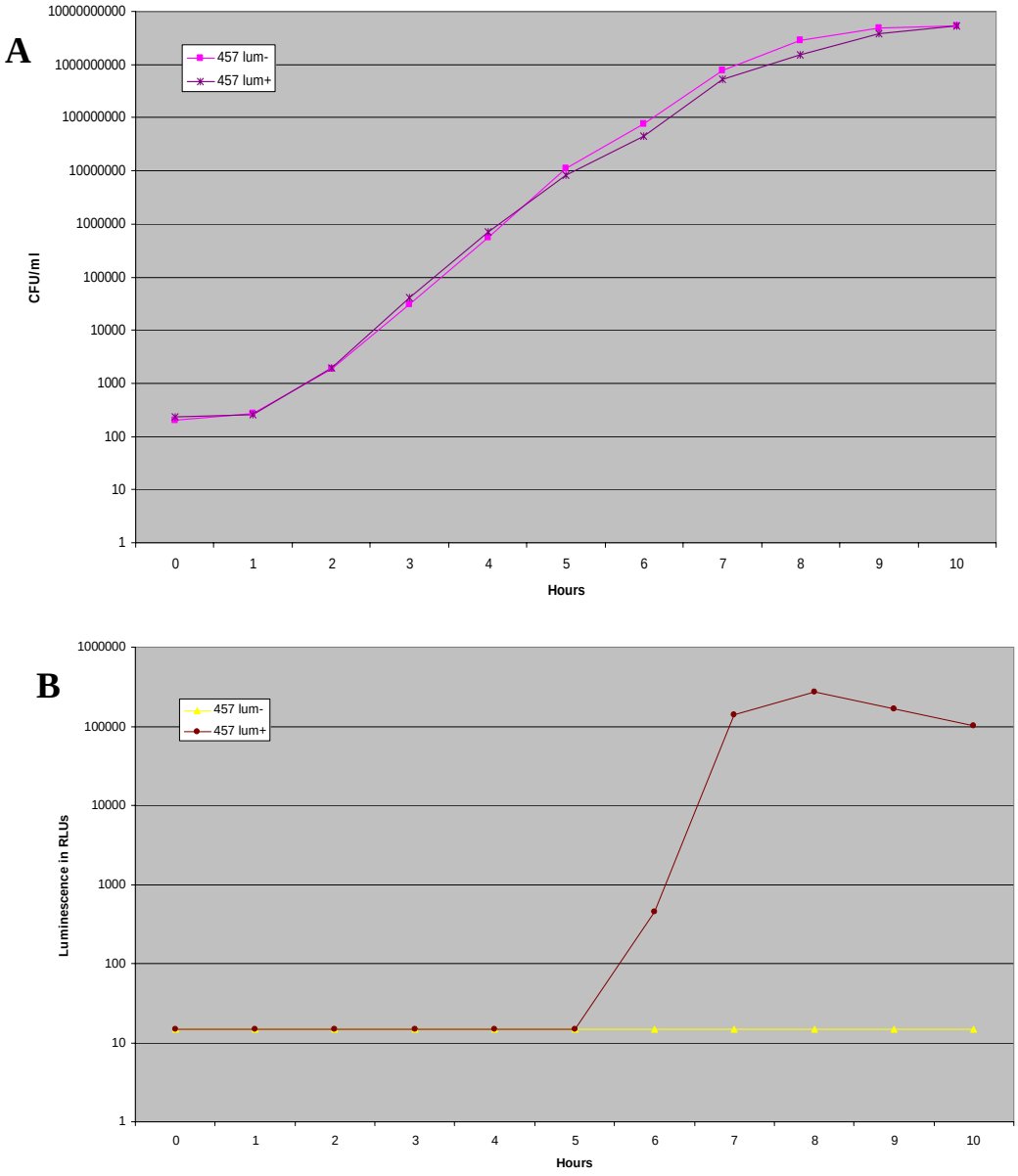


Figure 6.4. Growth (A) and luminescence (B) of *V. cholerae* RC457, and RC457 Δ*luxA* over a 10 hour period.

6.3.2 Growth characteristics of *luxA* mutants

In order to ensure that any difference found between the wild-type parent and *luxA* mutant was a result of loss of light production only, and not from some other defect incurred

during mutation production, several growth and survival characteristics of three luxA mutant strains were compared to those of the corresponding wild-type parent. First, growth curve experiments were conducted during 24 hours for each strain. Figure 6.4 shows the result of a typical experiment, in this case, strain RC457 with only the first 10 hours shown. For all three strains, there was a slight increase in growth by the *luxA* mutant, compared to the wild-type parent during the time points when luminescence was first expressed until it stabilized. In Figure 6.4A, this corresponds to 5 to 8 hours post dilution. From Figure 6.4B, it is clear that this period corresponds to the period of induction and rapid increase of luminescence expression. At all other time points, cell density was equivalent.

6.3.3 Starvation survival and entry into VBNC

To assess whether the *luxA* mutation affects starvation and low-temperature survival, room temperature and 4°C microcosms of the mutant and wild-type parental strains were prepared. When maintained in starvation conditions at room temperature, colony forming unit counts decreased rapidly within the first two weeks, but then stabilized (Figure 6.5A). Remarkably, these microcosms maintained a cell density of approximately 10^4 cells/ml for one year (Figure 6.5B). Some differences can be seen in Figure 6.5, however, the variability was primarily due to variability in microcosm preparation conditions and not light emission.

VBNC induction microcosms yielded results similar to starvation microcosms for the three luxA mutant strains. The results for RC457 and UM4086 are shown in Figure 6.6. Some variability existed, but was largely due to error during microcosm preparation.

6.3.4 Biofilm formation of *luxA* mutants

In addition to light emission, the *lux* regulatory pathway of *V. cholerae* controls biofilm formation, HA protease production, and virulence in pathogenic strains. To test

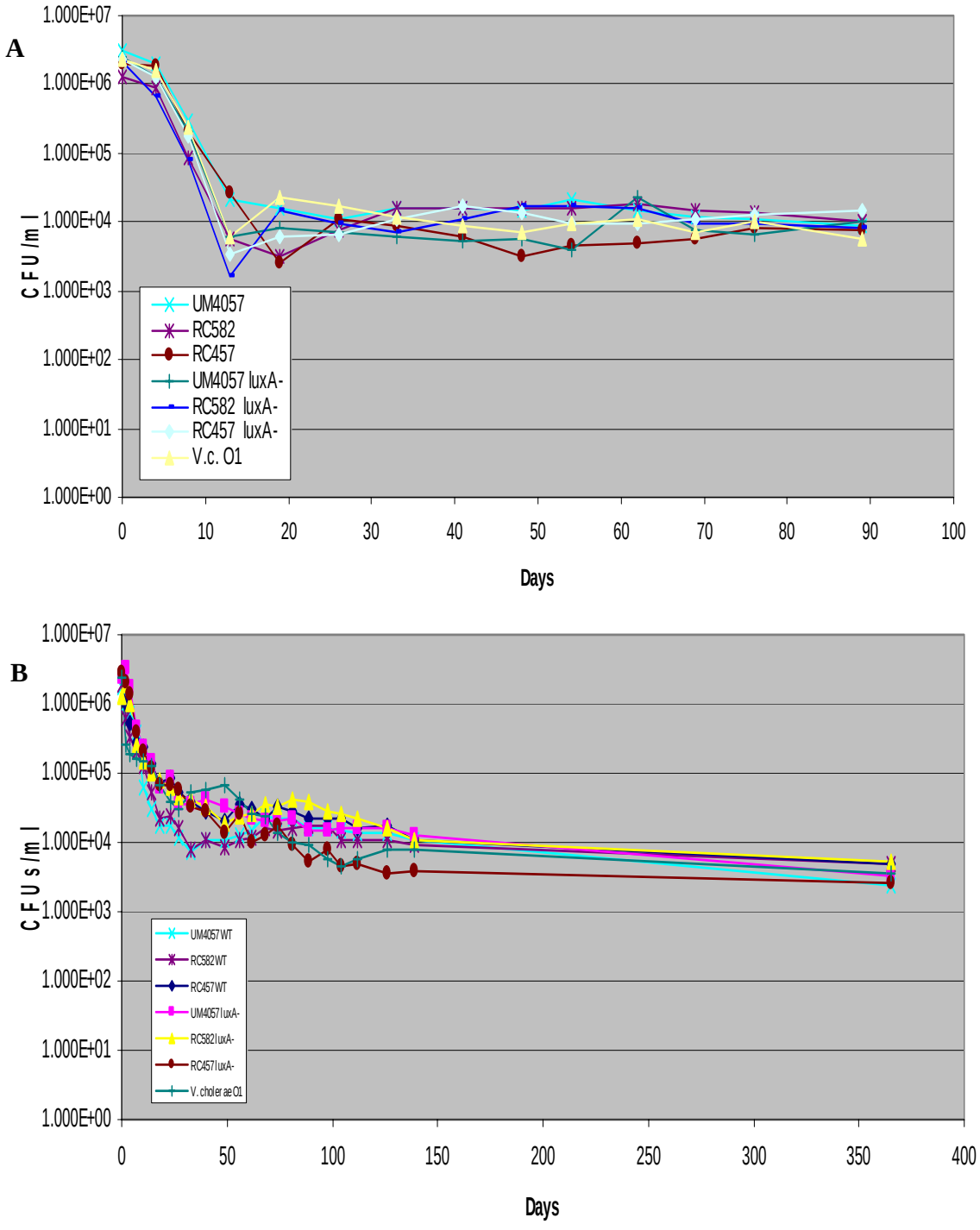


Figure 6.5. Survival of luminescent wild-type and *luxA* mutants of *V. cholerae* in starvation conditions, 1% instant ocean microcosm, over a period of 90 days (A) and 365 days (B).

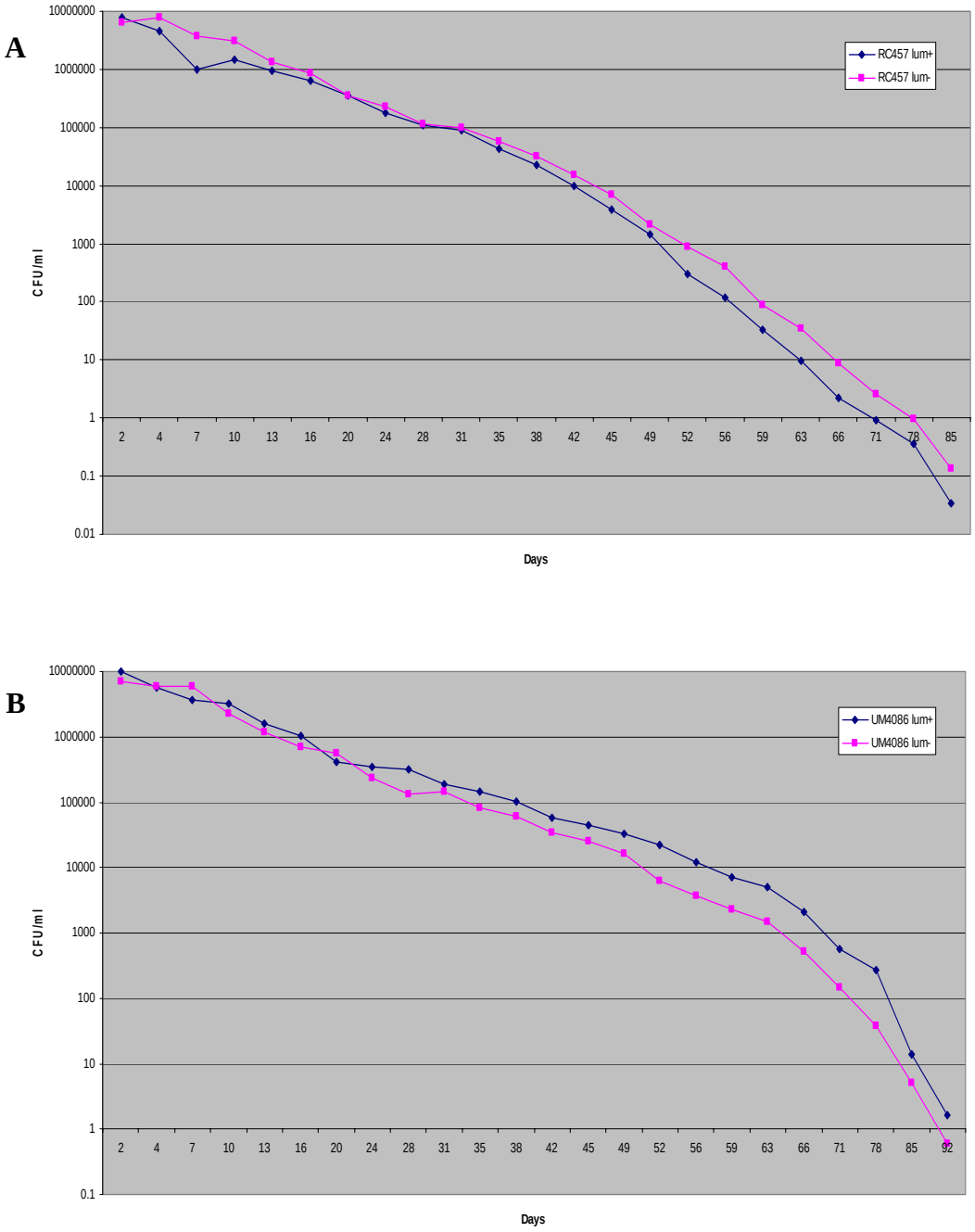


Figure 6.6. Survival of luminescent wild-type and *luxA* mutants of *V. cholerae* in VBNC induction conditions, 1% instant ocean microcosm at 4°C, for strain RC457 (A) and UM4086 (B).

equivalent to the parental strains (Figure 6.7). Longer incubation time, e.g., 24 hours, allowed production of a thicker biofilm, but did not significantly change the difference in biofilm formation between the wild-type parent and *luxA* mutant. Interestingly, thicker biofilm was formed in Marine Broth than in LB broth (Figure 6.7). Other media were not tested, as the cause of this difference was unclear.

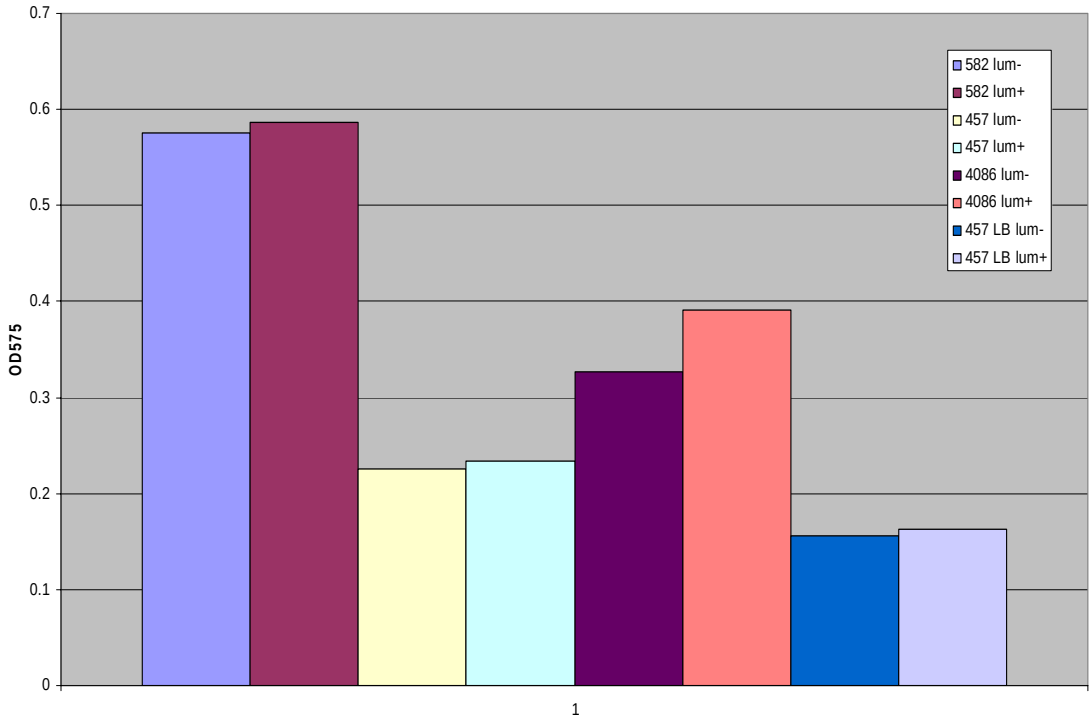


Figure 6.7. Biofilm formation of luminescent wild-type and *luxA* mutants of three strains of *V. cholerae*, RC457, RC582, and UM4086 in Marine broth after 12 hours. Biofilm formation in LB broth for strain RC457 after 12 hours is shown for comparison of media conditions. n=6.

6.3.5 Copepod attachment

Attachment to chitinous zooplankton, such as copepods, has been shown to be an important habitat for *V. cholerae* (Huq et al., 1990; Tamplin et al., 1990). To determine if loss of light emission affects this attachment, wild-type parents and *luxA* mutants of three strains of *V. cholerae* were added to copepods, which had been washed to remove unattached or weakly-attached bacteria. Preliminary experiments and control samples containing only copepods revealed that even after washing copepods with sterile, natural water, many bacteria remain attached to the copepods, ranging from 1×10^3 to 1×10^4 CFU per copepod.

The *V. cholerae* inoculum ranged from 5×10^6 to 5×10^7 cells per ml. Spread plates of bacteria only controls, which were used to enumerate the bacterial inoculum, served as positive control for the RNA colony blot hybridization protocol (Figure 6.8A and 6.8B) to enumerate *V. cholerae* colonies from copepod and copepod and attached *V. cholerae* samples.

Wild-type parents and *luxA* mutants attached to copepods at a similar rate (Figures 6.8C and D, Figure 6.9A and B), although the dark strains attached at a more variable rate compared to the luminescent strains. Enumeration of attached *V. cholerae* ranged from 1×10^4 to 5×10^4 CFUs per copepod, yielding attachment rates of 0.3 to 0.05% of the inoculum. This value varied with length of time that the copepods were incubated with the bacteria, with higher values obtained with longer incubation times. However, incubation times longer than two days were not permissible, since copepod mortality rates increased dramatically after the first 24-36 hours.

Control samples of copepods without *V. cholerae* inoculum revealed a significant number of indigenous *V. cholerae* cells attached to the zooplankton (Figure 6.9C). The

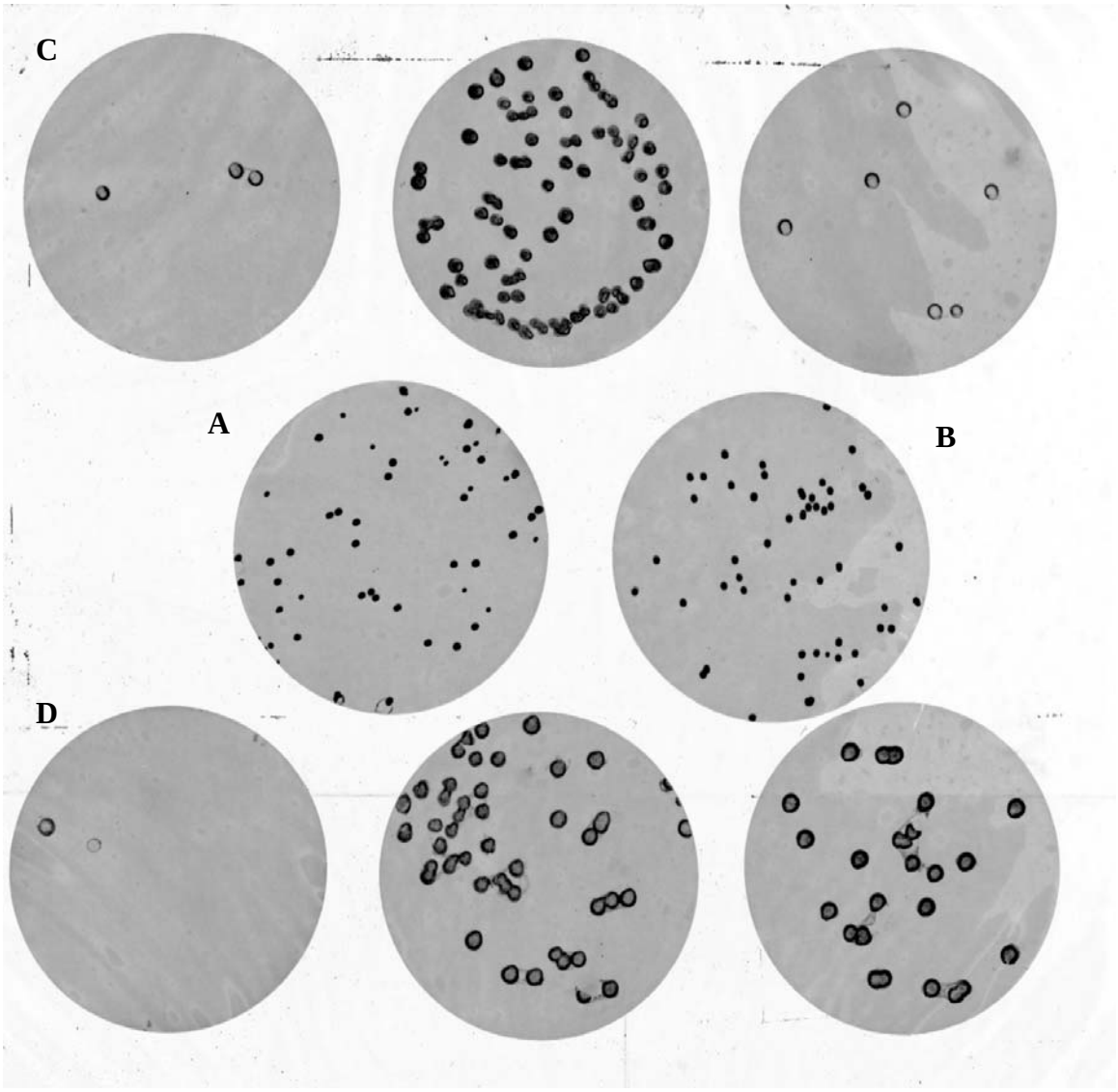


Figure 6.8. *V. cholerae*-specific RNA colony blot hybridization of wild-type and *luxA* mutants of *V. cholerae* RC457. A. Bacterial inoculum without copepods of luxA mutant; B. Bacterial inoculum without copepods of wild-type luminescent; C. Replicates of *luxA* mutants attached to copepods, D. Replicates of wild-type luminescent attached to copepods. Spread plates were done on Marine agar and colony blots were done after 24 hours (A and B) or 48 hours (C and D).

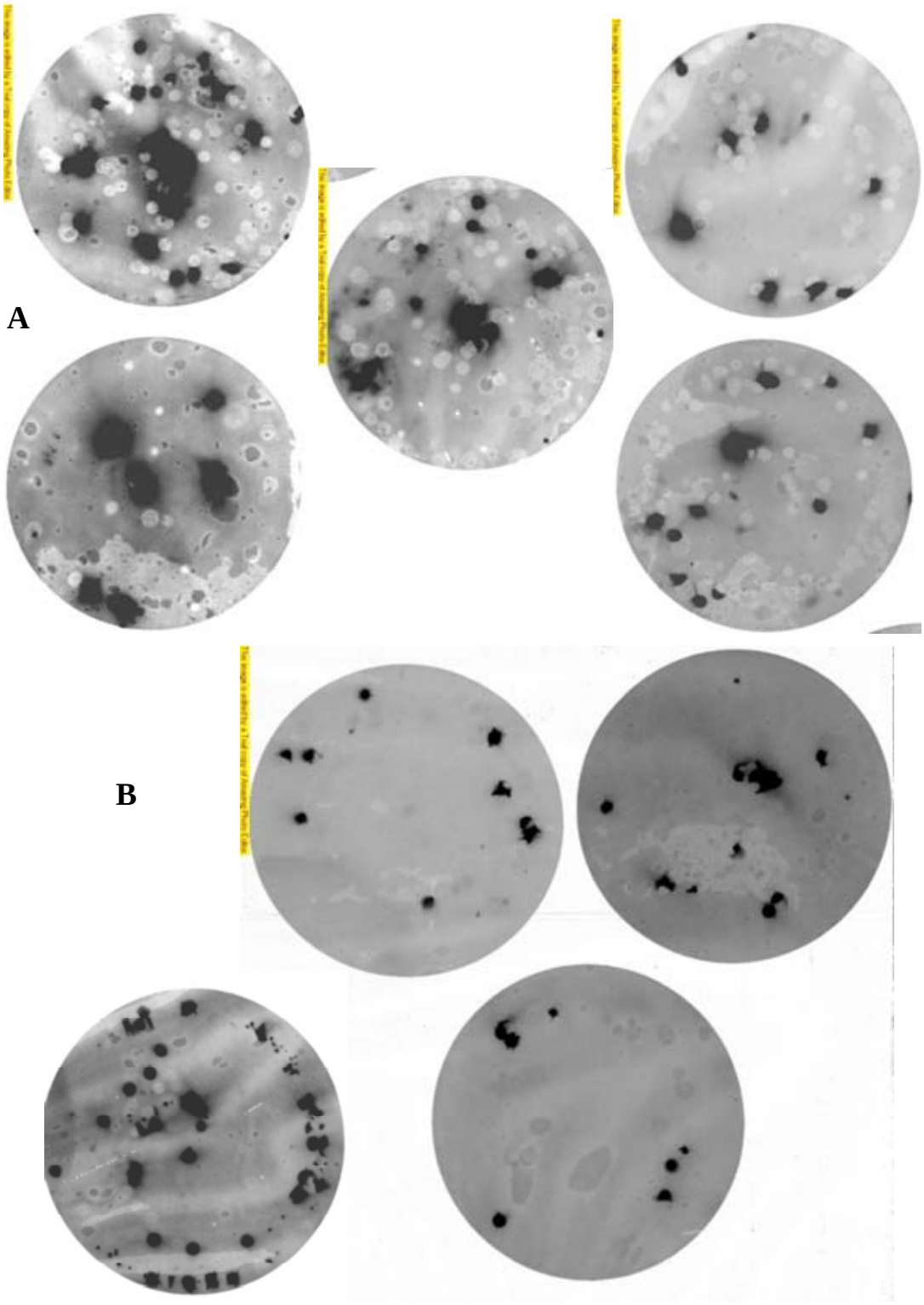


Figure 6.9. *V. cholerae*-specific RNA colony blot hybridization of wild-type and *luxA* mutants of *V. cholerae* RC582. A. Replicates of wild-type luminescent attached to copepods; B. Replicates of *luxA* mutants attached to copepods, C. Replicates of indigenous bacteria attached to copepods (no *V. cholerae* inoculum added). Spread plates were done on LB agar and colony blots were done after 48 hours.

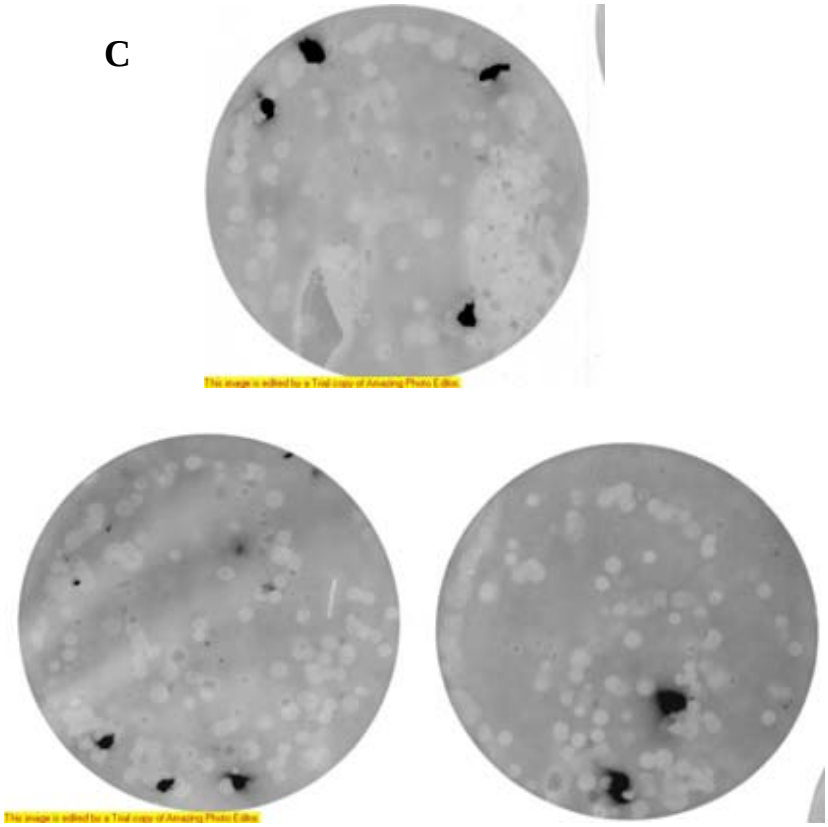


Figure 6.9. *V. cholerae*-specific RNA colony blot hybridization of copepod-associated wild-type and *luxA* mutants of *V. cholerae* RC582 (continued).

natural, attached *V. cholerae* population numbered 100-400 CFUs per copepod, indicating that 1-4% of the attachment rate estimate is actually attributable to indigenous *V. cholerae*.

6.3.6 Growth of *luxA* mutants under different oxygen tensions

To test whether luminescence expression provides a growth advantage under different oxygen tensions, wild-type parents and *luxA* mutants of three strains of *V. cholerae* were incubated under 4 different oxygen tensions and growth was measured at various time points.

Growth of luminescent strains was significantly higher than *luxA* mutants when grown under microaerophilic conditions and significantly lower when grown under anaerobic conditions (Figures 6.10 and 6.11). For one strain, this pattern was not as strong (Figure 6.12), and in most cases the pattern was only applicable to early time points, or low cell

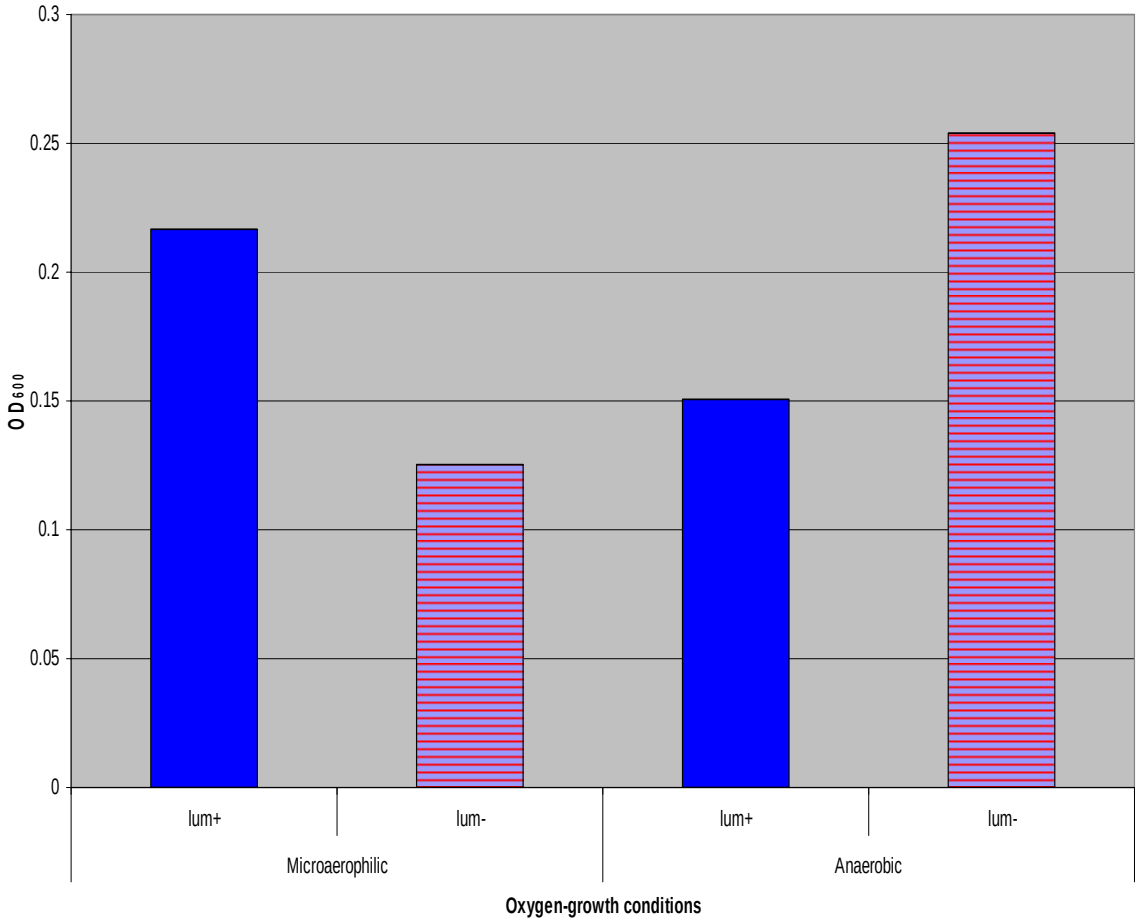


Figure 6.10. Growth of wild-type and *luxA* mutants of *V. cholerae* RC582 in microaerophilic and anaerobic conditions, after 12 hours.

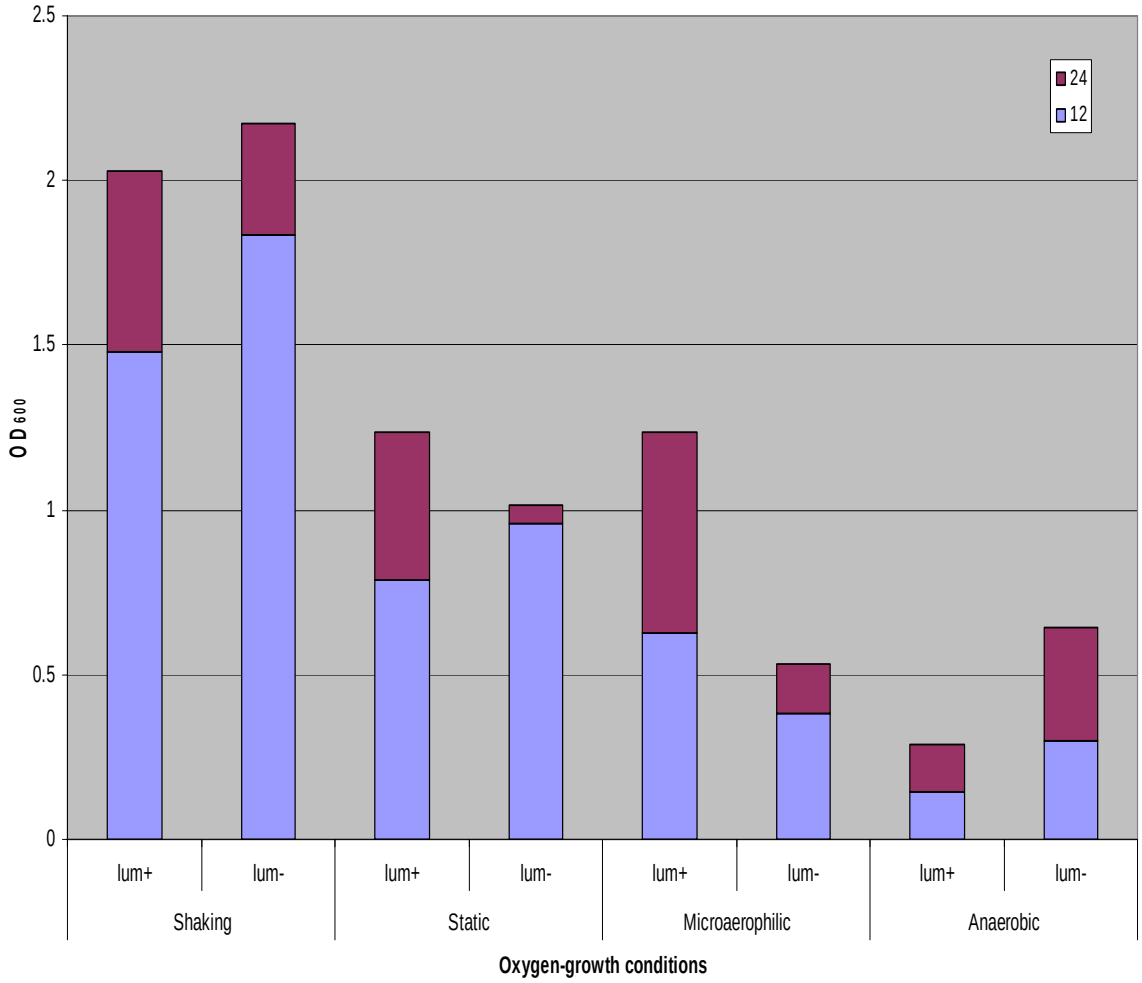


Figure 6.11. Growth of wild-type and *luxA* mutants of *V. cholerae* RC457 in high oxygen (shaking), normal oxygen (static), low oxygen (microaerophilic) and anaerobic conditions, after 12 hours and 24 hours.

density. The cultures appeared to overcome the differential growth pattern when the incubation time was extended to 24 hours. Not surprisingly, conditions with high aeration produced highest growth (Figure 6.11). Interestingly, microaerophilic conditions produced culture growth equivalent to static conditions for luminescent strains (Figure 6.11). For *luxA* mutants, the microaerophilic condition yielded the poorest culture growth (Figure 6.11).

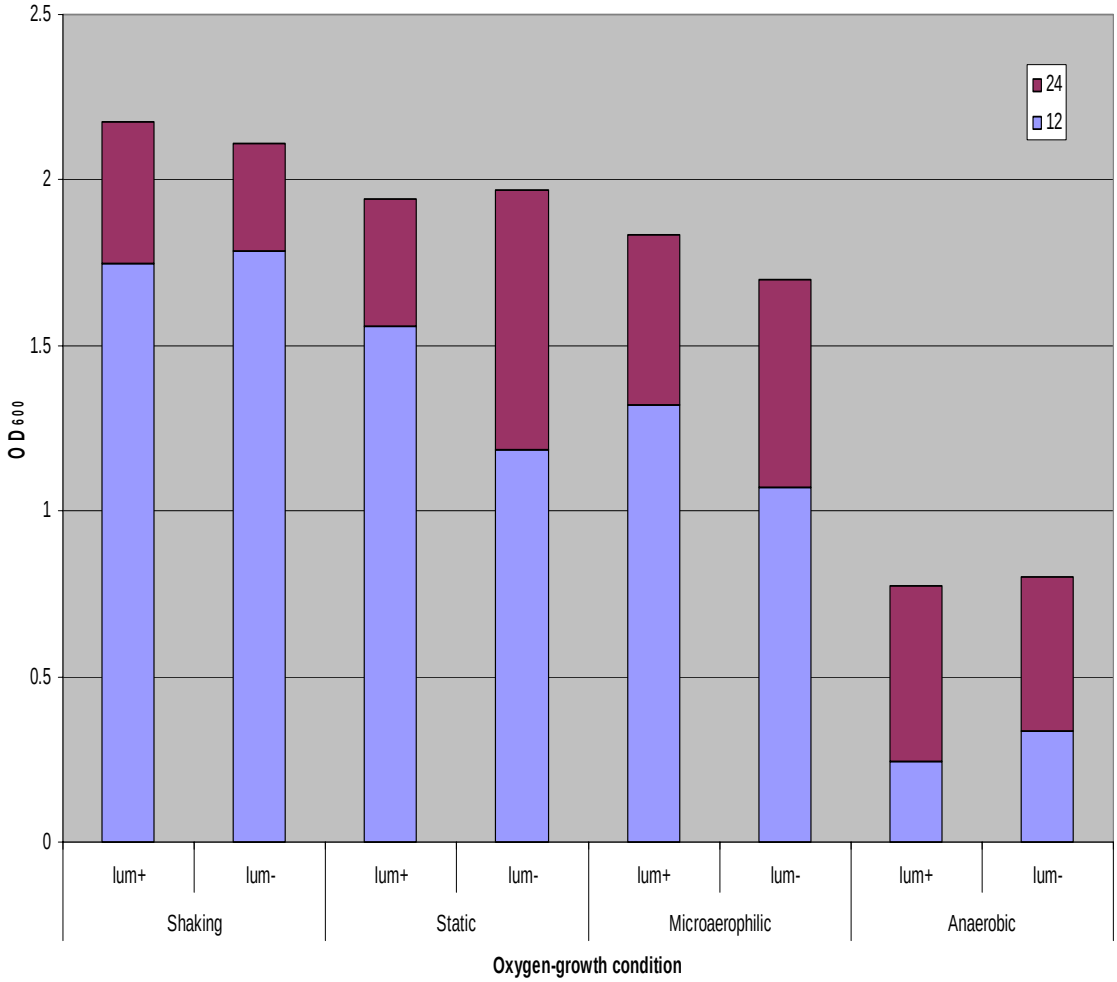


Figure 6.12. Growth of wild-type and *luxA* mutants of *V. cholerae* UM4086 in high oxygen (shaking), normal oxygen (static), low oxygen (microaerophilic) and anaerobic conditions, after 12 hours and 24 hours.

6.3.7 Effect of reactive oxidative species on *luxA* mutants

Growth of luminescent strains of *V. cholerae* was significantly higher than *luxA* mutants when grown in the presence of certain reactive oxidative species, namely hydrogen peroxide and *t*-butyl hydroperoxide. Addition of hydrogen peroxide (Figure 6.13C and

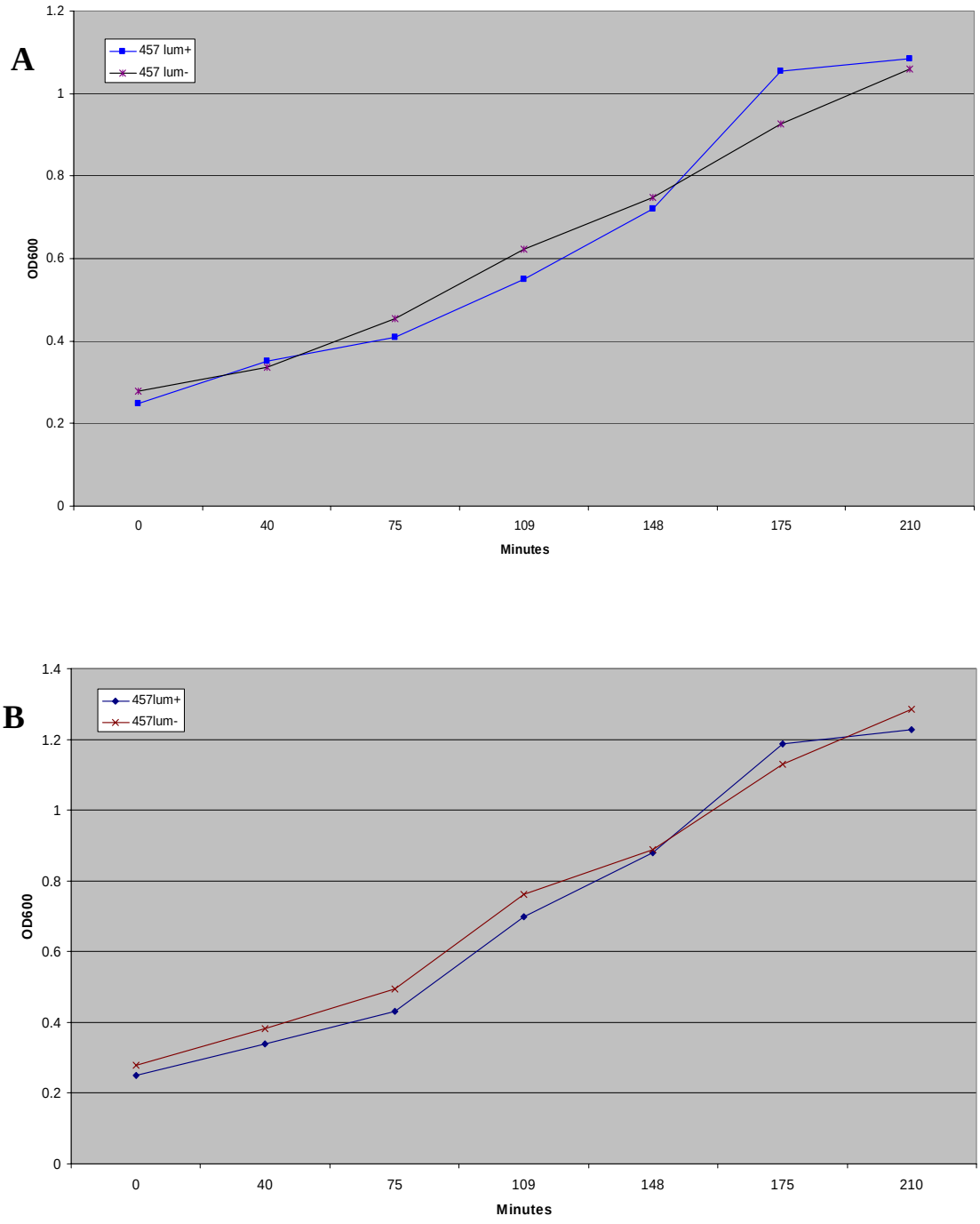


Figure 6.13. Growth of wild-type and *luxA* mutants of *V. cholerae* RC457 after addition (T=0) of ferrous sulfate (B), hydrogen peroxide (C), or sterile water (A, negative control). Cells were grown for 3-4 hours prior to addition of reactive oxidative species.

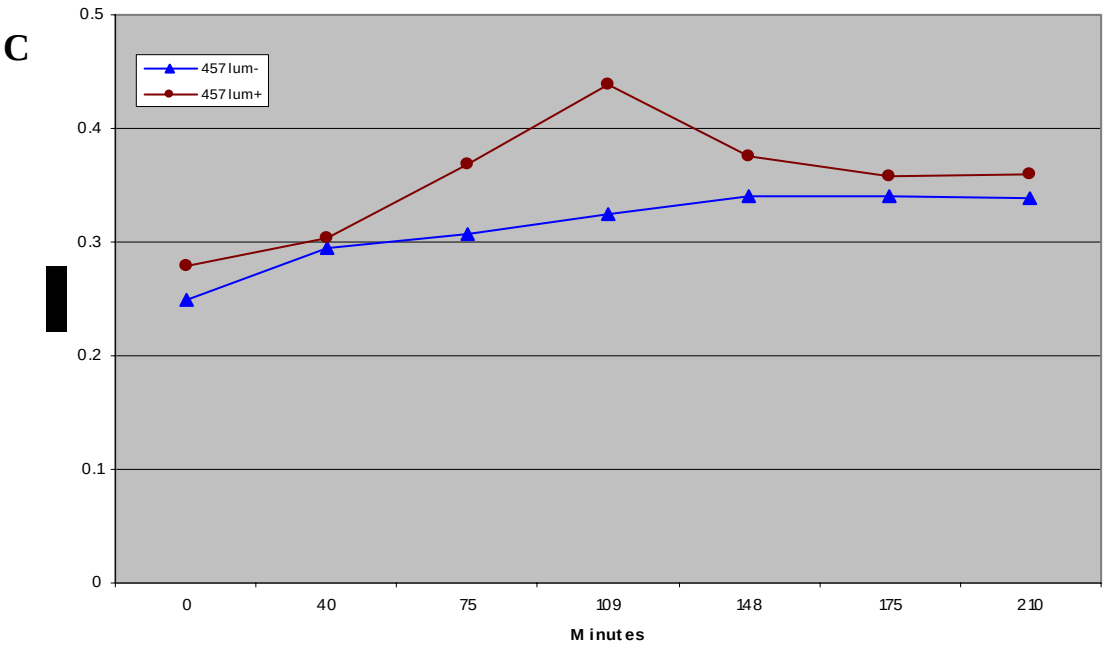


Figure 6.13. Growth of wild-type and *luxA* mutants of *V. cholerae* RC457 after addition of ferrous sulfate and hydrogen peroxide (continued).

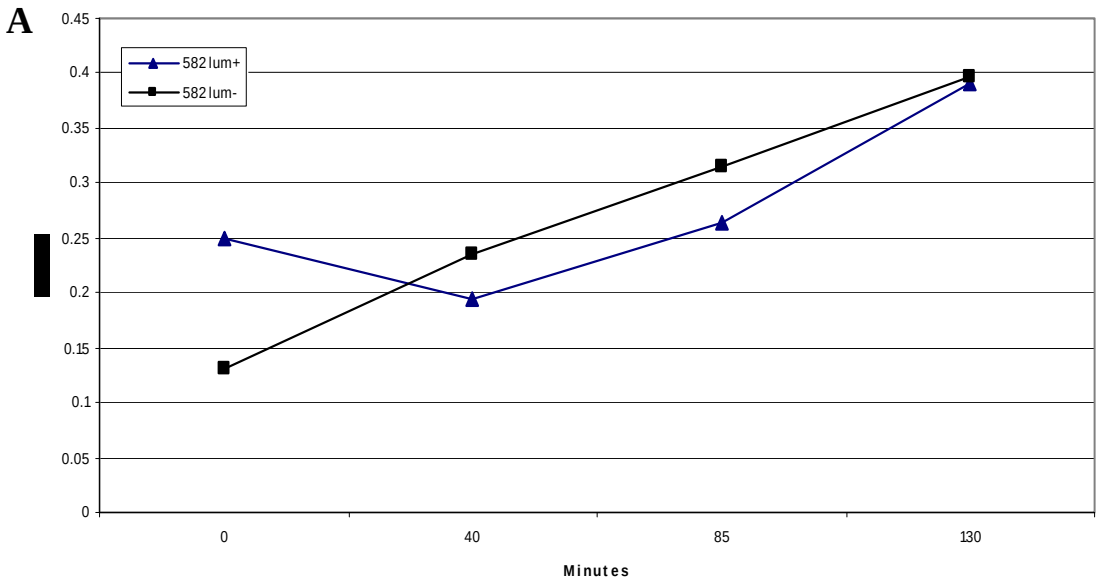
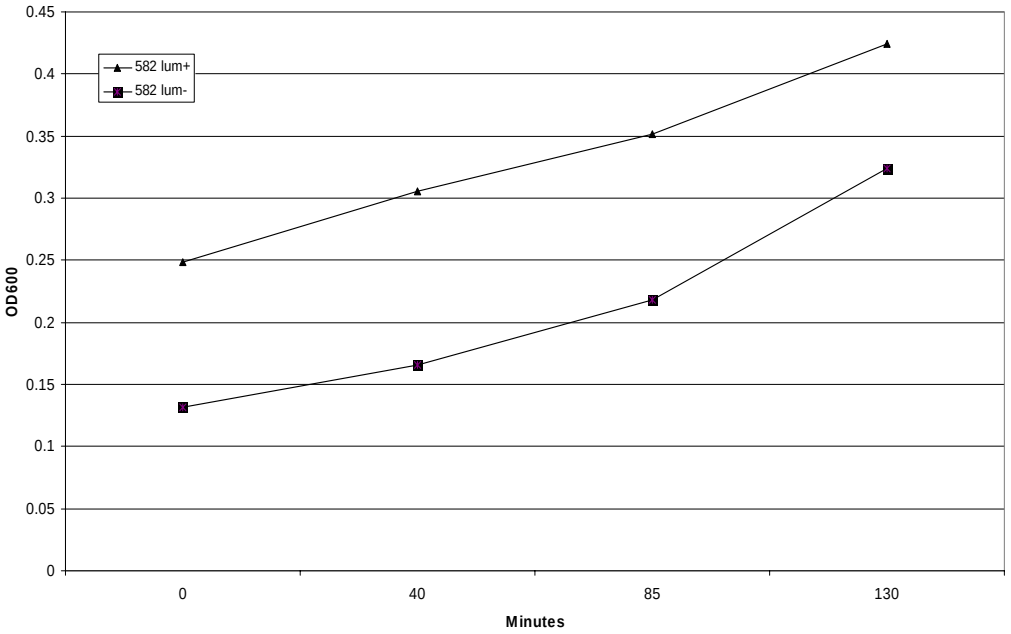


Figure 6.14. Growth of wild-type and *luxA* mutants of *V. cholerae* RC582 after addition (T=0) of ferrous sulfate (B), hydrogen peroxide (C), or sterile water (A, negative control). Cells were grown for 3-4 hours prior to addition of reactive oxidative species.

B



C

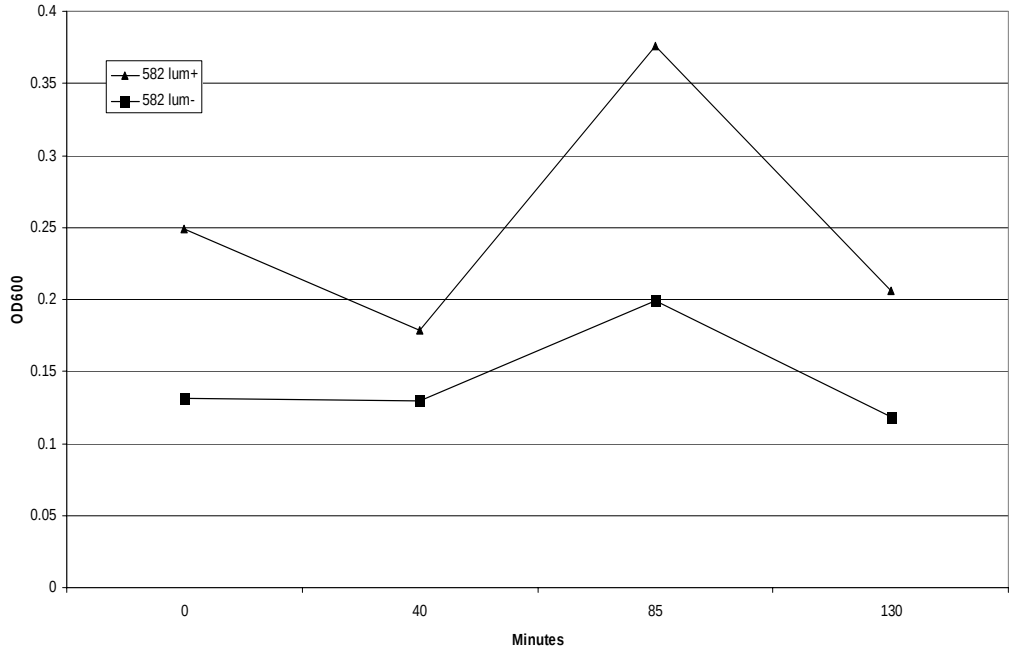


Figure 6.14. Growth of wild-type and *luxA* mutants of *V. cholerae* RC582 after addition of ferrous sulfate and hydrogen peroxide (continued).

6.14C) and *t*-butyl hydroperoxide (data not shown) significantly reduced the growth rate compared to negative controls (Figure 6.13A and 6.14C), which received sterile water. The reaction to addition of iron (II) sulfate of luminescent parents and *luxA* mutants of *V. cholerae* was equivalent, with cell growth only slightly impaired (Figures 6.13B and 6.14B). Interestingly, the growth advantage seen in luminescent *V. cholerae* when hydrogen peroxide was added, corresponded to an OD value at which cells begin expressing and rapidly increase expression of luminescence (Figure 6.13C and 6.14C versus Figure 5.3A-C, Chapter 5).

6.3.8 DNA repair ability of *luxA* mutants

To test whether luminescence expression plays a role in stimulation of DNA repair, *luxA* mutants and wild-type strains of *V. cholerae* were grown to various cell densities, subjected to increasing doses of UV irradiation, placed in dark storage or in the presence of fluorescent lights for 2 hours, and assayed for survival. Previous studies used only one cell density, approximately 1 x 10^8 cells/ml, the midpoint of luminescence expression, and did not include *V. cholerae*. In this study, cells densities of 4 x 10^3 to 1 x 10^9 cells/ml were used to determine if bioluminesce plays a role in DNA repair and whether cells have to be induced prior to a UV dose. Additionally, three different *V. cholerae* strains were used, with varying maximal levels of luminescence expression. For strain RC457, which expresses luminescence at a normal to high level, clear patterns were found. For the other two strains, which are defective to various degrees in their luminescence expression levels, the patterns were not as clear or pronounced, and the results from these experiments are not presented.

For cell cultures which were already emitting light at the time of the UV dose, luminescent strains had a higher survival rate compared to *luxA* mutants, especially when placed in the presence of fluorescent light (Figure 6.15A). For cultures not yet expressing

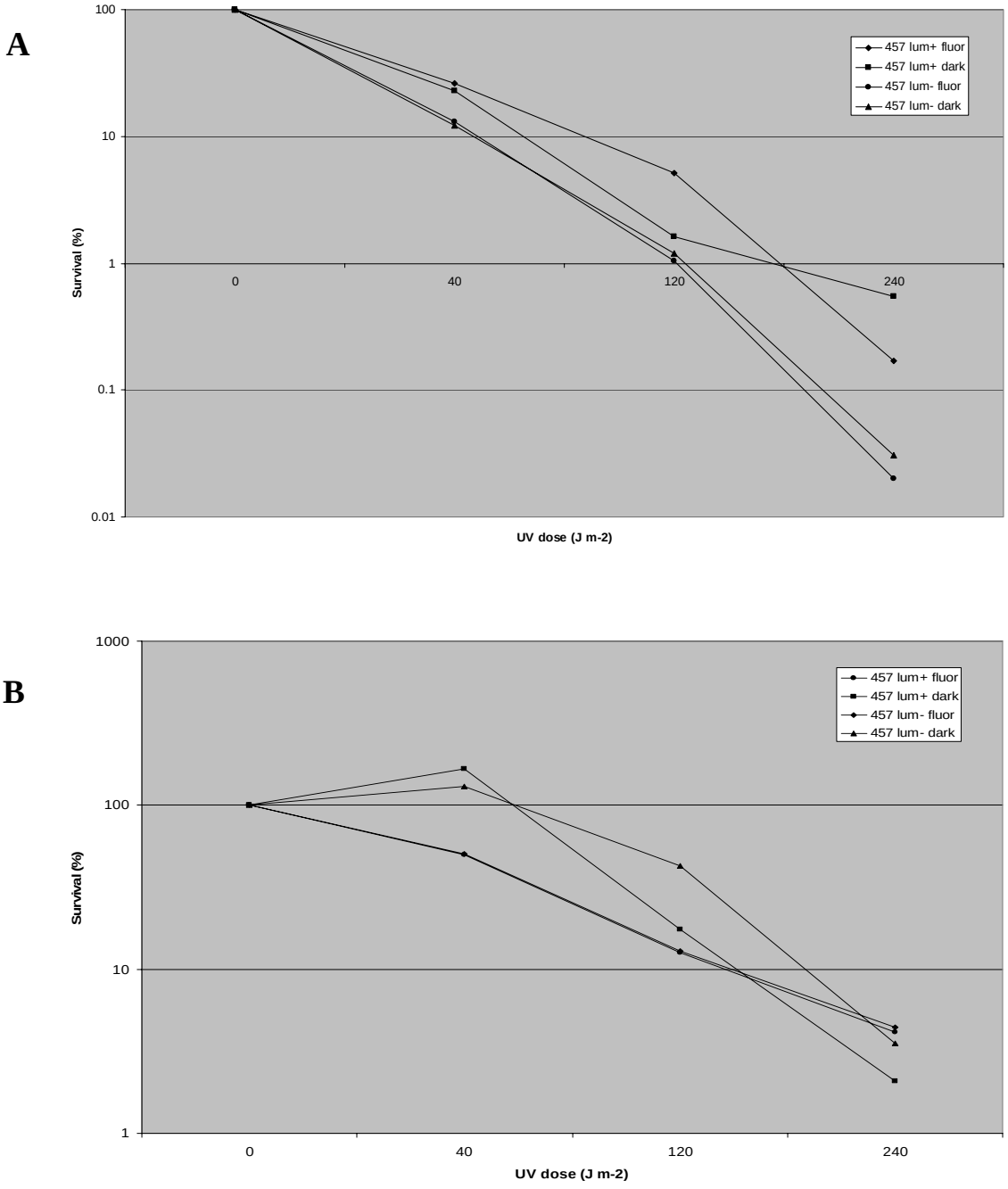


Figure 6.15. Reaction of wild-type and *luxA* mutants of *V. cholerae* RC457, after increasingly higher doses of UV irradiation. Starting cell densities were approximately 1 x 109 cells/ml (A), 1 x 107 cells/ml (B), and 1 x 105 cells/ml (C). Following UV dose, MPN tubes were placed in the dark or in the presence of fluorescent lights for 2 hours to aid photoreactivation.

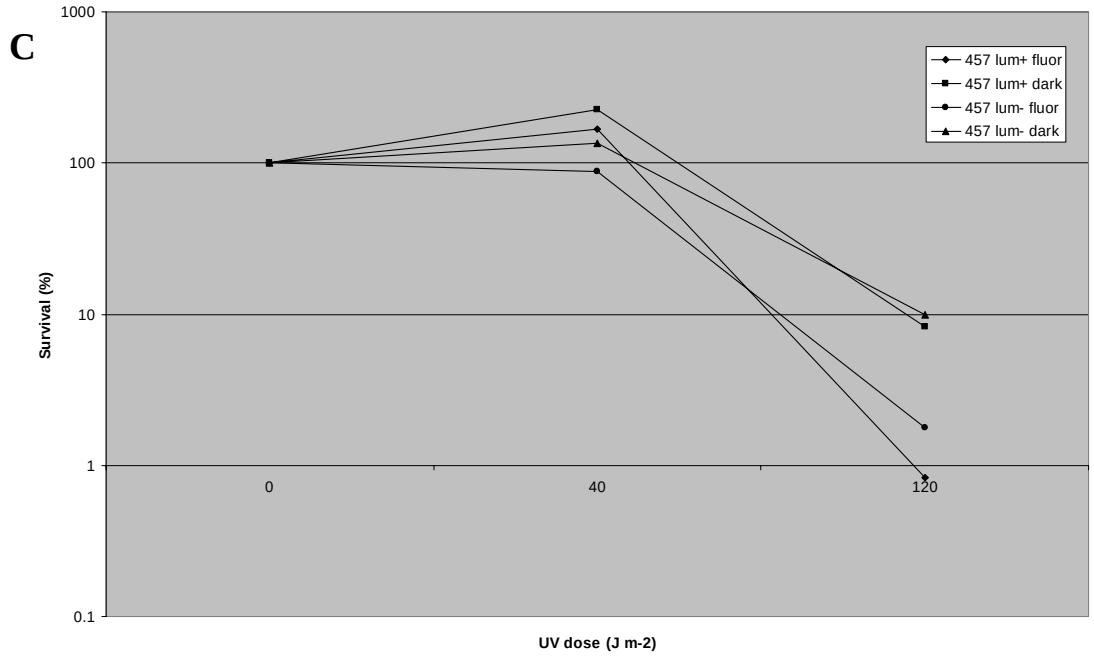


Figure 6.15. Reaction of wild-type and *luxA* mutants of *V. cholerae* RC457 after increasingly higher doses of UV irradiation (continued).

luminescence, dark storage immediately following a UV dose, yielded higher survival rates (Figure 6.15B and C). For high doses of UV irradiation, dark storage immediately following the UV treatment yielded higher survival rates, while for low doses, luminescent strains usually yielded higher survival rates. Results of MPN analysis of irradiated *luxA* and wild-type strains indicate a general trend in which luminescent strains have a higher long-term survival rate when treated with fluorescent light for two hours immediately after the UV dose was administered, while *luxA* mutants had a higher survival rate when placed in the dark. Typically, luminescent strains stored in the dark had the lowest survival rate.

6.4 Discussion

6.4.1 Characterization of *luxA* mutants of *V. cholerae*

Three different strains of *V. cholerae* were used to generate *luxA* mutants, RC457, RC582 and UM4086. Strains RC457 and 582 were isolated from the Chesapeake Bay, MD, from the same geographical site exactly one year apart. They differ in only two of 23 biochemical properties (acid production from mannitol and methyl red). They are not clones, based on ERIC-PCR fingerprinting (Choopun, 2004). Strain RC457 expresses luminescence at a normal level, while strain RC582 expresses luminescence at only 13.5% of the level of RC457. Strain UM4086 was isolated from an oyster in Florida, and has an even bigger defect in luminescence expression than RC582. It expresses luminescence at 1.6% of the level of RC457.

The experimental approach to use allelic exchange generated *luxA* mutants, rather than naturally occurring non-luminescent strains, to compare with luminescent strains of *V. cholerae,* was chosen to ensure that any differences were caused by loss of light emission, exclusively. Phenotypically, *luxA* mutants should have growth profiles similar to their wild-type luminescent parent, since despite the luminescence reaction has been eliminated, the *lux* operon was still transcribed in these strains at the induced level. This was evident, as seen in Figure 6.4A, which shows the growth curve for strain RC457. The dark mutant had a slightly, but not significantly higher, cell density during the three hour period when luminescence expression began and increased rapidly. Outside of this time period, the cell densities were identical. This slight decrease in cell growth seen in the *luxA* mutant is then a direct measure of the cost of light emission, in terms of cellular resources, less energy necessary for protein expression of the operon. The autoinduction control and strong

feedback repression (Chapter 5) enable luminescent species to sense whether light emission should be switched on or off. From Figure 6.4A, it is clear that there is very little loss in environmental fitness to maintain this phenotype.

The experiments performed above were conducted in Marine broth over only a 24-hour time period. Starvation (1% instant ocean, room temperature) and VBNC induction (1% Instant Ocean, 4°C) microcosms were used to investigate the impact on loss of light production on long term survival (Figure 6.5 and 6.6). The target starting inoculum for all microcosms was 1 x 10^7 cells/ml, a cell density at which luminescence expression is about to commence for strains that emit light at a normal level. There is an inherent amount of experimental variability in these two types of experiments. Although the procedure is exactly replicated and the cell inoculum spectrophotometrically determined each time, the standard error was relatively high. There was no significant difference between *luxA* mutants and wild-type luminescent parents for either type of microcosm. There was a general pattern found for all strains, however. For starvation microcosms, cell density decreased rapidly during the first two weeks, followed by a short transitional period and then a long period of stabilized cryptic growth which lasted a year or more (Figure 6.5). During this cryptic growth, cells are possibly maintained by the recycling of cellular components of dead cells. Another interesting characteristic of this type of microcosm is a predictable shift in colony morphology from larger translucent or transparent colonies to smaller, opaque colonies, with some strains shifting beyond this to a rugose phenotype. Although this colonial morphology phenomenon, including the rugose phenotype, has been well documented, it is still not well understood.

There was also no difference in VBNC induction microcosms (Figure 6.6). It is interesting to note that some microcosms simply "die" for no apparent reason when prepared as described in the Materials and Methods. That is, their cell density follows the general pattern seen in Figure 6.6 for the first 2-4 days and then the cell density drastically drops to undetectable limits within 1-2 days. The reason is not clear. Another interesting trend is that VBNC induction microcosms show a very gradual decrease in cell density that is quite different from starvation microcosms (Figures 6.5 and 6.6). At earlier time points (1-10 days), VBNC induction microcosms yielded higher cell densities than starvation microcosms. After 30-45 days, starvation microcosms stabilized at around 10^4 cells/ml, while VBNC induction microcosms continued to decrease in cell density until they become non-culturable.

6.4.2 Loss of light production does not affect other quorum sensing targets

The *lux* regulon of *V. cholerae* controls multiple phenotypes, in an assumed concerted fashion, including protease production and genes important in biofilm formation. To ensure that later experiments accurately assessed the impact of loss of light emission and not a change in autoinduction regulation, the biofilm formed by *luxA* mutants was compared to those of their wild-type luminescent parental strains. The biofilm formation of the wild-type luminescent and luxA mutant strains was statistically identical (Figure 6.7). For uniformity with other experiments, Marine broth was used in biofilm quantification experiments. Previous researchers used LB broth for biofilm production assays (Watnick and Kolter, 1999). When the two medium were compared for one strain, RC457, the biofilm production in Marine broth was higher than in LB broth. This is somewhat surprising since LB broth has a higher nutrient content, indicating that low nutrient levels may induce biofilm formation.

Copepod attachment of vibrios is now viewed as an important survival strategy. Attachment to the chitinous surface is analogous to biofilm formation on polystyrene or other surfaces, and since all vibrios produce chitinase, the substrate is a source of nutrient. The attachment to zooplankton by luminescent bacteria is proposed as a positive selective force for conservation of the phenotype, since "visible" zooplankton would be preyed upon by higher animals such as fish, and the gut tract of higher marine animals is a preferred habitat for marine enterobacteria, such as *V. cholerae*.

Wild-type luminescent and *luxA* mutant strains of *V. cholerae* attach to copepods at a similar rate (Figures 6.8 and 6.9). Despite the copepods having been washed and stored in sterile, filtered natural water, many indigenous bacteria remain attached to the zooplankton (Figure 6.9C). Unfortunately, washing cannot be done in a vigorous fashion, as it results in high zooplankton mortality. Development of the *V. cholerae*-specific RNA colony blot hybridization protocol in Chapter 3 allowed enumeration of attached *V. cholerae,* despite the measure of a large number of indigenous bacteria. Even though the *V. cholerae* inoculum was high, attachment rates were only slightly higher, compared to indigenous bacteria (Figure 6.9).

Development and testing of the *V. cholerae* specific RNA colony blot hybridization protocol is described in Chapter 3, but the experiments on copepod attachment performed above served as an additional evaluation. Knowing that attachment to chitinous zooplankton is a preferred habitat, isolation methods often include sampling water and plankton fractions and then performing a traditional enrichment and culture based method (Huq et al., 2006). Results from these experiments offer an alternative method, namely spread-plating, preparing RNA colony blots and hybridizing with a *V. cholerae*-specific oligonucleotide probe.

Although still reliant upon cultivation, there is less emphasis on screening a large number of isolates using tedious and laborious biochemical tests, instead relying on molecular techniques.

Using this technique, the number of attached indigenous *V. cholerae* cells per copepod can be enumerated. The colony blots from Figure 6.9 are from a 100-fold dilution of a 1-ml copepod homogenate, which was made with 10-15 copepods on average. Using some simple calculations, a *V. cholerae* per copepod load can be estimated, approximately 200-300 per copepod. A single copepod represents approximately 0.56 mm^2 of surface area, using previously described average dimensions (Conway, 2006). This estimate agrees with estimates produced using flow cytometry of water samples treated with the same probe (Heidelberg et al., 2002).

6.4.3 Ecological function of luminescence in *V. cholerae*

As stated in the Introduction, several functions have been hypothesized for luminescence expression to explain its conservation in the *Vibrionaceae*. Support for two of these hypothesized functions, DNA repair and response to reactive oxidative species, has recently been described (Szpilewska et al., 2003; Lyzen and Wegrzyn, 2005; Czyz et al., 2000; and Kozakiewicz et al., 2005). Thus far these investigations have focused primarily on *V. harveyi,* using natural or chemically induced *lux* mutants. This study is the first to investigate the function of luminescence in *V. cholerae*. Inclusion of these two testable hypotheses will reveal whether these functions are either somewhat species specific, only important to traditional symbiotic luminescent species, or if they are a general consequence prevalent in all luminescent species. Additionally, the hypothesis that luminescence

expression aids in survival in low oxygen environments was tested, as it seems feasible that present-day luminescent species to encounter this niche.

Wild-type luminescent strains had a significantly higher growth rate under microaerophilic conditions compared to *luxA* dark mutants, and a significantly lower growth rate under anaerobic conditions (Figures 6.10 and 6.11). The extent of the differential growth advantage appears to be correlated with levels of luminescence expression and growth time, with strain RC457 and RC582 demonstrating a larger advantage under microaerophilic conditions than UM4086, which is highly defective in its luminescence expression level. The finding that dark mutants had a higher growth rate under anaerobic conditions is explained by the fact that the luminescent strains are slightly oxygen adapted, given the cultivation technique. The differential growth advantage under microaerophilic conditions was lost when the cultures were grown for 24 hours; i.e., to a higher cell density (Figures 6.11 and 6.12). This is most likely explained by the strong repression of stationary phase conditions, seen in growth curves of aerated cultures.

Wild-type luminescent strains had a significantly higher survival and/or growth rate when reactive oxidative species were added to the culture prior to the onset of luminescence expression (Figures 6.13 and 6.14). As in the case of the results above for growth in different levels of oxygen tension, the advantage was more pronounced for strains RC457 and RC582 compared to UM4086. The advantage was also pronounced in experiments in which hydrogen peroxide or *t*-butyl hydroperoxide was added, as opposed to ferrous sulfate, which did not appear to be advantageous for either phenotype. The toxicity of hydrogen peroxide was evident and this could not be overcome by luminescent strains when luminescence expression is repressed (Figures 6.13C and 6.14C). The results from experiments involving

ferrous sulfate are complicated by the fact that iron level of the surrounding medium affects luminescence expression by some indirect mechanism (Dunlap, 1991). It is interesting to note that all vibrios produce a catalase and superoxide dismutase. Additionally, *V. cholerae* gene1560 is a proposed catalase/perooxidase and there is also an superoxide dismutase and catalase gene located downstream of the *lux* operon in the N16961 El Tor genome.

The affect of luminescence expression on DNA repair was largely dependent on the UV dose and the stage of growth of the culture (Figure 6.15). Luminescent strains had a significant survival advantage when the UV dose was given at a time when the cell density was high, correlating to luminescence expression, or when the UV dose was the lowest. When the UV dose was given at a high cell density, favoring luminescence expression, survival rates were higher among luminescent strains when treated with fluorescent lighting, as opposed to dark storage, which presumably is important in the process of photoreactivation (Kato et al., 1997). In contrast, higher UV doses or doses given at lower cell densities, especially when followed by dark storage, resulted in *luxA* dark mutants having a survival advantage.

Based on the results of these three latter experiments, it is clear that the *lux* phenotype can provide a survival advantage under certain conditions. This apparently slight advantage could translate into a distinct environmental advantage when competing with other bacteria for resources. It is not clear whether the results presented here summarize the total advantage of light emission by luminescent bacteria, or if other conditions exist in which this phenotype can convey fitness advantage. With the coupling of autoinduction and stationary phase repression, clearly luminescent bacteria appear to have devised a means to maintain the *lux* phenotype that allows bacteria to effectively exploit multiple habitats.

Chapter 7 SUMMARY

The major objective of the study was to determine the significance of the *lux* operon in *Vibrio cholerae,* an inhabitant of estuarine and marine ecosystems. Findings of the study concerning ecological function, incidence, and expression are summarized, with key contributions and accomplishments highlighted and the direction of future work described.

Based on the results of this study, it is concluded that luminescence provides an advantage to wild-type luminescent strains of *V. cholerae,* compared to *luxA* mutants under microaerophilic growth conditions. In addition, light production provides some advantage, but less significant, in ability to respond to UV damage and reactive oxidative species at those cell concentrrations at which luminescence is expressed.

To test for advantages of light expression accurately, chromosomal *luxA* mutants were generated by employing allelic exchange with the suicide vector pCVD442 containing a mutated *luxA* gene fragment. This approach was preferred, rather than employing a naturally-occurring, non-luminescent strain, in order to ensure differences arising in subsequent experiments would be directly ascribable to loss of light emission and not other, unknown, genetic differences. Results of several experiments confirmed that *luxA* mutants generated in this study were not altered in traits other than light production. Specifically, differences were not observed in growth rate, survival under starvation conditions, or existence in the VBNC state. The luminescent phenotype is controlled by quorum sensing. That is, it is regulated by cell concentration. The experiments carried out in this study were designed to assess the effect of loss of light production on growth and growth-cycle related events. As was expected, significant differences were not observed. Those experiments summarized in Figure 6.4, comparing growth rate of wild-type luminescent and *luxA* mutant

strains, revealed negligible differences in growth rate, explained by the fact that the *lux* operon continues to be expressed in the *luxA* mutant, the mutation being non-polar and cellular resources, in the form of protein production, continuing to be utilized. The slight decrease in cellular growth shown in Figure 6.4 represents the cost of energy for luminescence expression.

The consequence of loss of light production on another quorum-sensing controlled phenotype, namely biofilm formation, was assessed. Microtiter plates and laboratory media yielding high concentrations of cells in culture showed no difference between wild-type luminescent strains and *luxA* mutants, with respect to biofilm formation. Furthermore, the prescence of copepods or exposure to starvation conditions yielded no differences in ability to attach and colonize the chitin surfaces.

Other potential ecological roles of light production merit investigation. In this study, three of many possible roles for light production and these were selected based on historical and emerging hypotheses. The most interesting of the potential hypotheses, that light production may be beneficial in the propagation of a luminescent bacterial species, might be tested in a complex, aggregate/multi-animal model. However, the phenomenon of light emission in bacterial species, in general, merits re-evaluation based on findings of this study and recently published new information in the literature. Additional hypotheses, no doubt, will be formed as more data are accumulated. Three strains of *V. cholerae* were used in this study, based on the intensity of luminescence of the parental strain, in addition to phenotypic, genotypic, and ERIC-PCR analyses. Additional strains or *lux* mutants derived using other methods would contribute significantly to understanding of the role of luminescence.

The occurence of bioluminescence in non-O1/non-O139, O1, and O139 *V. cholerae* was determined using a luminescence bioassay developed in this study and the results were confirmed employing molecular biology tools and improved genetic markers. Luminescence was not found to occur in the clinical or environmental isolates of *V. cholerae* serogroup O1 or O139, or in *V. mimicus* strains. However, luminescence is a common phenotype of *V. cholerae* non-O1/non-O139, notably in selected environments. In the Chesapeake Bay, luminescence was found to occur in approximately 50% of the strains tested, with the *lux* operon detected in approximately 60% of the *V. cholerae* strains. However, the incidence of luminescence in Bangladesh isolates was only 6%, with the *lux* operon detected in 11% of the isolates, when only non-O1/non-O139 *V. cholerae* isolates were examined.

Results of a previous study (West et al., 1986) showed 10% (11/115) of the *V. cholerae* strains tested were luminescent. However, those isolates had been collected from various geographic locations and were not analyzed in a census mode. A portion of the non-luminescent isolates in this study were from an earlier study (Palmer and Colwell, 1991) and, were included for purposes of comparison. Results from all three studies were combined, the over-all incidence rate of luminescence was approximately 17%, and the *lux* operon was detected in 20% of the isolates comprising the total set of strains. Clearly, the occurence of both detectable luminescence and the *lux* operon among isolates of *V. cholerae* varies significantly, depending on a variety of factors and habitat plays a major role.

Analysis of phenotypic and genotypic traits of each of the isolates included in this study and the environmental parameters measured at the time of sampling in the case of the Chesapeake Bay isolates, i.e., Group II strains, revealed an association of luminescence with a laterally transferred heat stable enterotoxin (*stn*) and an outer membrane protein (*ompU*)

and revealed a complex interaction with the water sample fraction throughout the year. Future work is planned to obtain detailed analysis of the physio-chemical parameters of each of the two habitats analyzed in this study in order to identify environmental factors or ranges of environmental factors that may select for the luminescence phenotype. Obviously, such information for other geographic areas would be useful in this context.

The *lux* operon of *V. cholerae* UM4057 was sequenced and found to comprise six genes, *luxCDABEG.* The organization of the *lux* operon of *V. cholerae* is concluded to be most similar to *P. leiognathi* and *S. hanadei*. However, the nucleotide sequence was judged to be most similar to *V. harveyi* and *P. luminescens*. Sequence similarity of *V. cholerae* and *V. harveyi* and *P. luminescens,* with respect to the *lux* operon, suggests one of these two species served as the donor of the *lux* operon in the terrestrial luminescent bacterial species. Given the natural habitat and salt requirements of both species, it would be more probable that *V. cholerae* served as the donor. Examination of the *lux* operon of *V. cholerae* and flanking regions revealed matching direct-reverse repeat sequences, indications of mobility, at locations 5' of *luxC* and within *luxE*, such that the main composition of the gene (79%) would be included.

Analysis of the G+C% content and codon usage patterns of the luminescent bacterial species, for which the entire *lux* operon was sequenced, including *V. cholerae* UM4057, was done to determine the origin of the *lux* operon, that is, whether it arose from a common ancestor or was laterally transferred. Results of the analyses suggest that the *lux* operons in these species have been residents in the genomes for a long time, perhaps passed down from an ancient marine ancestor and lost in non-luminescent members of this phylogenetic group,

considering the G+C% content compared to the entire genome, except in the case of *P. luminescens*, which most probably acquired the *lux* operon by lateral gene transfer.

To understand the significance of the occurence of luminescence and to explain "dark" strains, that is, strains which contain components of the *lux* operon but are not visibly luminescent, the expression profile patterns of luminescence in *V. cholerae* were analyzed. Luminescence was expressed at different levels, independent of cell concentration, with the majority of the luminescent strains producing light approximately 10% of *V. fischeri* and *V. harveyi* luminescence. In *V. cholerae*, onset of luminescence is indicative of maximal expression level. As expected, luminescence in *V. cholerae* was found to be controlled by quorum-sensing regulation and a strong repressor of luminescence was observed.

Dark or K variants occur naturally among luminescent strains of *V. cholerae* and were readily produced by modifying selected laboratory culture conditions. Analysis of the nature of the K variant defect among isolates of Group I strains revealed two possible causes, at least for some of the isolates. Three strains showed altered 5' and 3' flanking regions, potentially significant since altered 5' regions lead to altered regulation via loss of active promoter/operator region. Several of the isolates were aldehyde limited, leading to the dark phenotype, but the extent to which the defect could be rescued varied from strain to strain, indicating that this may be only part of the explanation for the observed defect. In all cases, the *lux* operon was found to be intact, with only small differences in nucleotide sequence, suggesting the defect occurred via most likely altered regulation. The interplay of multiple input signals and sensors with the central regulatory genes located at several unlinked locations in the two chromosomes of *Vibrio cholerae* make it difficult to pinpoint the exact

cause of altered light production. Therefore, future work will focus on determining the causes of sub-normal luminescence expression.

In addition to the findings described above, two new colony blot hybridization methods for isolation and detection of *V. cholerae* were developed during the course of this study. These two methods were designed to bypass the burden of traditional culture methods by replacing the battery of biochemical tests typically used to identify *V. cholerae* with a simple DNA-DNA or DNA-RNA hybridization. They can be adapted for use in isolation of *V. cholerae* strains by preserving master plates or replica-plating membranes prior to colony lysing and nucleic acid immobilization, or enumeration, since the enrichment step can be eliminated.

In conclusion, the significant occurrence of the luminescence phenotype for V. cholerae in specific environments, the correlation of luminescence with certain traits and environmental parameters, and the potential ecological advantages suggested from the results of this study indicate that luminescence is a significant and important ecological characteristic for *V. cholerae*. Nevertheless, autoinduction regulation, strong repression, and occurrence of many expression defects also indicate that those environmental conditions favoring luminescence expression are not always present, rendering the benefits of bacterial luminescence dependent on environmental conditions.

References

Alam, M, N.A. Hasan, A. Sadiquue, N.A. Bhuiyan, K.U. Ahmed, S. Nusrin, G.B. Nair, A.K. Siddique, R.B. Sack, D.A. Sack, A. Huq, and R.R. Colwell. Seasonal cholera caused by *Vibrio cholerae* serogroups O1 and O139 in the coastal aquatic environment of Bangladesh. *Appl. Env. Microbiol*. 72:4096-4104.

Andrews, S.C., D. Shipley, J.N. Keen, J.B.C. Findlay, P.M. Harrison, and J.R. Guest. 1992. The hemoglobin-like protein (HMP) of *E. coli* has ferrisiderophore reductase activity and it's C-terminal domain shores homology with ferredoxin $NADP^+$ reductases. *FEBS Lett*. 302:247-252.

Antoine, R. and C. Locht. 1992. Isolation and molecular characterization of a novel broad-host-range plasmid from *Bordetella bronchiseptica* with sequence similarities to plasmids from Gram-positive organisms. *Mol. Microbiol*., 6:1785-1799.

Ast, J.C., and P.V. Dunlap. 2004. Phylogenetic analysis of the *lux* operon distinguishes two evolutionarily distinct clades of *Photobacterium leiognathi*. *Arch. Microbiol*. 181:352-361.

Baldwin, T.O., J.H. Devine, R.C. Heckel, J.-W. Lin., and G.S. Shadel. 1989a. The complete nucleotide sequence of the *lux* regulon of *Vibrio fischeri* and the *luxABN* region of *Photobacterium leiognathi* and the mechanism of control of bacterial bioluminescence. *J. Biolumin. Chemilumin.* 4:326-341.

Baldwin, T.O., L.H. Chen, L.J. Chlumsky, J.H. Devine and M.M. Ziegler. 1989b. Site-directed mutagenesis of bacterial luciferase: analysis of the "essential" thiol. *J. Biolum. Chemilum*. 4:40-48.

Bassler, B.L., E.P. Greenberg, and A.M. Stevens. 1997. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *J. Bacteriol*. 179:4043-4045.

Bassler, B.L., M. Wright, R.E. Showalter, and M.R. Silverman. 1993. Intercellular signaling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. *Mol. Microbiol*. 9:773-786.

Beijerinck, M. W. 1889. *Neerl. Sci. Exact. Naturelles* 23:401-415.

Beijerinck, M. W. 1916. Die Leuchbacterien der Nordsee im August und September. *Folia Mikrobiol. Delft* 4:15-40.

Boettcher, K.J. and E.G. Ruby. 1990. Depressed light emission by symbiotic *Vibrio fischeri* of the sepiolid squid *Euprymna scolopes*. *J. Bacteriol*. 172:3701-3706.

Booth, C.K., and K.H. Nealson. 1975. Light emission by luminous bacteria in the open ocean. *Biophys. J*. 15:65a

Boyd, E.F., K.E. Moyer, L. Shi and M.K. Waldor. 2000. Infectious CTXΦ and the Vibrio pathogenicity island prophage in *Vibrio mimicus*: evidence for recent horizontal transfer between *V. mimicus* and *V. cholerae*. *Inf. Immun*. 68:1507-1513.

Cámara, M., A. Hardman, P. Williams, and D. Milton. 2002. Quorum sensing in *Vibrio cholerae*. *Nat. Genet*. 32:217-218.

Chaiyanan, S., S. Chaiyanan, C. Grim, T. Maugel, A. Huq, and R.R. Colwell. 2006. Ultrastructure of coccoid viable but non-culturable *Vibrio cholerae*. *Env. Microbiol.* (in press).

Chang, B., S. Halgamuge, and S-L. Tang. 2006. Anaylysis of SD sequences in completed microbial genomes: Non-SD-led genes are as common as SD-led genes. *Gene* 373:90-99.

Chen, X., S. Schauder, N. Potier, A.V. Dorsselaer, I. Pelczer, B.L. Bassler, and F.M. Hughson. 2002. Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* 415:545-549.

Choopun, N. 2004. The population structure of *Vibrio cholerae* in Chesapeake Bay. Ph.D. dissertation, University of Maryland, College Park, MD.

Chun, J., A. Huq and R.R. Colwell. 1999. Analysis of 16S-23S rRNA intergenic spacer regions of *V. cholerae* and *Vibrio mimicus*. *Appl. Environ. Microbiol.* 65:2202-2208.

Cline, T.W., and J.W. Hastings. 1971. Temperature-sensitive mutants in the bacterial bioluminescence system. *Proc. Natl. Acad. Sci. USA* 68:500-504.

Cline, T.W., and J.W. Hastings. 1974. Bacterial bioluminescence in vivo: control and synthesis of aldehyde factor in temperature-conditional luminescence mutants. *J. Bacteriol*. 118:1059-1066.

Coffey, J.J. 1967. Inducible synthesis of bacterial luciferase: specificity and kinetics of induction. *J. Bacteriol.* 94:1638-1647.

Colepicolo, P., K.-W. Cho, G.O. Poinar, and J.W. Hastings. 1989. Growth and luminescence of the bacterium *Xenorhabdus luminescens* from a human wound. *Appl. Environ. Microbiol*. 55:2601-2606.

Conway, D.V.P. 2006. Identification of the copepodite developmental stages of twenty-six North Atlantic copepods. Occasional Publications. Marine Biological Associations of the United Kingdom (21), 28 pp.

Cousineau, J. and E.A. Meighen. 1976. Chemical modification of bacterial luciferase with ethoxyformic anhydride: evidence for an essential histidyl residue. *Biochemistry* 15:4992-5000.

Czyz, A., B. Wrobel, and G. Wegrzyn. 2000. *Vibrio harveyi* bioluminescence plays a role in stimulation of DNA repair. *Microbiology-Uk* 146:283-288.

Czyz, A., K. Plata, and G. Wegrzyn. 2003. Stimulation of DNA repair as an evolutionary drive for bacterial luminescence. *Luminescence* 18:140-144.

Dahlberg, C., M. Bergström, and M. Hermansson. 1998. In situ detection of high levels of horizontal plasmid transfer in marine bacterial communities. *Appl. Env. Microbiol*., 64:2670-2675.

Datta-Roy, K., K. Banerjee, S. P. De, and A. C. Ghose. 1986. Comparative study of expression of hemagglutinins, hemolysins, and enterotoxins by clinical and environmental isolates of non-O1 *Vibrio cholerae* in relation to their enteropathogenicity. *Appl. Environ. Microbiol*. 52:875-879.

Devine, J. H., C. Countryman, and T. O. Baldwin. 1988. Nucleotide sequence of the *luxR* and *luxI* genes and structure of the primary regulatory region of the *lux* regulon of *Vibrio fischeri* ATCC 7744. *Biochemistry* 27:837-842.

Devine, J.H., G.S. Shadel and T.O. Baldwin. 1989. Identification of the operator of the *lux* regulon from the *Vibrio fischeri* strain ATCC 7744. *Proc. Natl. Acad. Sci*. 86:5688-5692.

Donnenberg, M.S. and J.B. Kaper. 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. *Infect. Immun*. 59:4310-4317.

Doolittle, W.F. 1999. Phylogenetic classification and the universal tree. *Science* 284:2124-2129.

Doudoroff, M. 1938. Lactoflavin and bacterial luminescence. *Enzymologia* 5:239-243.

Dunlap, P.V. 1989. Regulation of luminescence by cyclic AMP in *cya*-like and *crp*-like mutants of *Vibrio fischeri. J. Bacteriol*. 171:1199-1202.

Dunlap, P.V. 1991. Organization and regulation of bacterial luminescence genes. Photochem. Photobiol. 54:1157-1170.

Eberhard, A. 1972. Inhibition and activation of bacterial luciferase synthesis. *J. Bacteriol.* 109:1101-1105.

Eberhard, A., A.L. Burlingame, C. Eberhard, G.L. Kenyon, K.H. Nealson, and N.J. Oppenheimer. 1981. Structural identification of autoinducer *of Photobacterium fischeri* luciferase. *Biochemistry* 20:2444-2449.

Engebrecht, J. and M. Silverman. 1984. Identification of genes and gene products necessary for bioluminescence. *Proc. Natl. Acad. Sci*. 81:4154-4158.

Engebrecht, J., and M. Silverman. 1987. Nucleotide sequence of the regulatory locus controlling expression of bacterial genes for bioluminescence. *Nucleic Acids Res*. 15:10455-10467.

Engebrecht, J., K.H. Nealson, and M. Silverman. 1983. Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri. Cell* 32:773-781.

Fidopiastis, P.M., H. Sorum, and E.G. Ruby. 1999. Cryptic luminescence in the cold-water fish pathogen *Vibrio salmonicida*. *Arch. Microbiol*. 171:205-209.

Fuqua, C., S.C. Winans, and E.P. Greenberg. 1996. Census and consensus in bacterial ecosystems: The LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu. Rev. Microbiol*. 50:727-751.

Giegerich, R., F. Meyer, and C. Schleiermacher. 1996. *GeneFisher* - Software Support for the Detection of Postulated Genes. Proceedings of the Fourth International Conference on Intelligent Systems for Molecular Biology, AAAI Press (ISSN 57735-002-2).

Gilson, L., A. Kuo, and P.V. Dunlap. 1995. AinS and a new family of autoinducer synthesis proteins. *J. Bacteriol*. 177:6946-6951.

Greenberg, E.P. 1997. Quorum sensing in gram-negative bacteria. *ASM News* 63:371-377.

Greenberg, E.P., J.W. Hastings, and S. Ulitzur. 1979. Induction of luciferase synthesis in *Beneckea harveyi* by other marine bacteria. *Arch. Microbiol*. 120:87-91.

Hada, H.S., J. Stemmler, M.L. Grossbard, P.A. West, C.J. Potrikus, J.W. Hastings, and R.R. Colwell. 1985. Characterization of non-O1 serovar *Vibrio cholerae* (*Vibrio albensis*). *System. Appl. Microbiol*. 6:203-209.

Haddock, B.A., and C.W. Jones. 1977. Bacterial respiration. *Bacteriol. Rev.* 41:47-99.

Hammer, B.K., and B.L. Bassler. 2003. Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Molecular Microbiology* 50:101-114.

Harrison, D.E.F., and S.J. Pirt. 1967. The influence of dissolved oxygen concentration on the respiration and glucose metabolism of *Klebsiella aerogenes* during growth. *J. Gen. Microbiol*. 46:193-211.

Hastings, J.W. 1952. Oxygen concentration and bioluminescence intensity. I. Bacteria and fungi. *J. Cell. Comp. Physiol.* 39:1-30.

Hastings, J.W. and G. Mitchell. 1971. Endosymbiotic bioluminescent bacteria from the light organ of the ponyfish. *Biol. Bull*. 141:261-268.

Hastings, J.W., and K.H. Nealson. 1977. Bacterial bioluminescence. *Ann. Rev. Microbiol*. 31:549-595.

Haygood, M.G., and K.H. Nealson. 1985. Mechanisms of iron regulation of luminescence in *Vibrio fischeri. J. Bacteriol*. 162:209-216.

Heidelberg J.F., J.A. Eisen, W.C. Nelson, R.A. Clayton, M.L. Gwinn, R.J. Dodson, D.H. Haft, E.K. Hickey, J.D. Peterson, L. Umayam, S.R. Gill, K.E. Nelson, T.D. Read, H. Tettelin, D. Richardson, M.D. Ermolaeva, J. Vamathevan, S. Bass, H. Qin, I. Dragoi, P. Sellers, L. McDonald, T. Utterback, R.D. Fleishmann, W.C. Nierman, and O. White. 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* 406:469-470.

Heidelberg, J. F. 1997. Seasonal abundance of bacterioplankton populations of *Bacteria,* gamma proteobacteria, *Vibrio/Photobacterium, Vibrio vulnificus, Vibrio cholerae/Vibrio mimicus,* and *Vibrio cincinnatiensis* associated with zooplankton in the Choptank River, Maryland. Ph.D. dissertation. University of Maryland, College Park.

Heidelberg, J.F., K.B. Heidelberg, and R.R. Colwell. 2002. Bacteria of the γ-subclass *Proteobacteria* associated with Zooplankton in Chesapeake Bay. *Appl. Env. Microbiol*. 68:5498-5507.

Henry, J. P., and A.M. Michelson. 1970. Studies of bioluminescence. Regulation of bacterial bioluminescence. *CR Acad. Sci*. 270:1947-1949.

Hood, M.A., G.E. Ness, and G.E. Rodrick. 1981. Isolation of *Vibrio cholerae* serotype O1 from the eastern oyster, *Crassostrea virginica. Appl. Env. Microbiol.* 41:559-560.

Huq, A., C. Grim, R.R. Colwell, and G.B. Nair. 2006. Detection, isolation, and enumeration of *Vibrio cholerae* from the environment. In: *Current Protocols in Microbiology* (R. Coico, T. Kowalik, J. Quarles, B. Stevenson, R. Taylor, eds.), John Wiley and Sons, Inc., pp. 6A.5.1-38.

Huq, A., R.R. Colwell, R. Rahman, A. Ali, M.A. Chowdhury, S. Parveen, D.A. Sack, and E. Russek-Cohen. 1990. Detection of *Vibrio cholerae* O1 in the aquatic environment by fluorescent monoclonal antibody and culture method. *Appl. Env. Microbiol.* 5:2370-2373.

Illarionov, B.A., V.M. Blinov, A.P. Donchenko, M.V. Protopopova, V.A. Karginov, N.P. Mertvetsov, and J.I. Gitelson. 1990. Isolation of bioluminescent functions from *Photobacterium leiognathi*: analysis of *luxA, luxB, luxG* and neighboring genes. *Gene* 86:89-94.

Jensen, M.J., B.M. Tebo, P. Baumann, M. Mandel, and K.H. Nealson. 1980. Characterization of *Alteromonas hanedai* (sp. Nov.), a nonfermentative luminous species of marine origin. *Curr. Microbiol*. 3:311-315.

Jobling, M.G., and R.K. Holmes. 1997. Characterization of *hapR*, a positive regulator of the *Vibrio cholerae* HA/protease gene *hap*, and its identification as a functional homologue of the *Vibrio harveyi luxR* gene. *Mol. Microbiol*. 26:1023-1034.

Kalendar, R. 2006. FastPCR, PCR primer design, DNA and protein tools, repeats and own database searches program. (www.biocenter.helsinki.fi/bi/programs/fastpcr.htm).

Kaper, J.B., S.L. Moseley and S. Falkow. 1981. Molecular characterization of environmental and nontoxigenic strains of *Vibrio cholerae*. *Infect. Immun*. 32:661-667.

Kaplan, H.B. and E.P. Greenberg. 1985. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. *J. Bacteriol*. 163:1210-1214.

Karaolis, D.K.R., J.A. Johnson, C.C. Bailey, E.C. Boedeker, J.B. Kaper and P.R. Reeves. 1998. A *Vibrio cholerae* pathogenicity island associated with epidemic and pandemic strains. *Proc. Natl. Acad. Sci. USA* 95:3134-3139.

Karlin, S., A.M. Campbell, and J. Mrazek. 1998. Codon usage in different gene classes of the *Escherichia coli* genome. *Mol. Microbiol*. 29:1341-1355.

Kato, R., K. Hasegawa, Y. Hidaka, S. Kuramitsu, and T. Hoshino. 1997. Characterization of a thermostable DNA photolyase from an extremely thermophilic bacterium, *Thermus thermophilus* HB27. *J. Bacteriol*. 179:6499-6503.

Keynan, A., and J.W. Hastings. 1961. The isolation and characterization of dark mutants of luminescent bacteria. *Biol. Bull*. 121:375.

Knight, T., and N. Papadakis. 1999. AF170104: *Vibrio fischeri lux* operon *Sal*I digest. NCBI GenBank database, direct submission.

Koch, W., W. Payne, B. Wentz and T. Cebula. 1993. Rapid PCR method for detection of *Vibrio cholerae* in foods. *Appl. Environ. Microbiol.* 59:556-560.

Kozakiewicz, J., M. Gajewska, R. Lyzen, A. Czyz, and G. Wegrzyn. 2005. Bioluminescence-mediated stimulation of photoreactivation in bacteria. *FEMS Microbiol. Lett*. 250:105-110.

Lawrence, J.G. and H. Ochman. 1997. Amelioration of bacterial genomes: rates of change and exchange. *J. Mol. Evol.* 44:383-397.

Lawrence, J.G. and H. Ochman. 1998. Molecular archaeology of the *Escherichia coli* genome. *Proc. Natl. Acad. Sci. USA* 95:9413-9417.

Lawrence, J.G. and J.R. Roth. 1996. Selfish operons: horizontal transfer may drive the evolution of gene cluster. *Genetics* 143:1843-1860.

Lee, C.Y., R. Szittner, and E.A. Meighen. 1991. The *lux* genes of the luminous bacterial symbiont, *Photobacterium leiognathi*, of the ponyfish. *Eur. J. Biochem*. 201:161-167.

Lin, J.W., Y.F. Chao, and S.F. Weng. 1998. Characteristic analysis of the *luxG* gene encoding the probable flavin reductase that resides in the *lux* operon of *Photobacterium leiognathi*. *Biochem. Biophys. Res. Commun*. 46:446-452.

Louis, V.R., E. Russek-Cohen, N. Choopun, I.N.G. Rivera, B. Gangle, S.C. Jiang, A. Rubin, J.A. Patz, A. Huq, and R.R. Colwell. 2003. Predictability of *Vibrio cholerae* in Chesapeake Bay. *Appl. Environ. Microbiol*. 69:2773-2785.

Lyzen, R., and G. Wegrzyn. 2005. Sensitivity of dark mutants of various strains of luminescent bacteria to reactive oxygen species. *Arch. Microbiol*. 183:203-208.

Mahillon, J. and M. Chandler. 1998. Insertion sequences. *Microbiol. Mol. Biol. Revs*. 62:725-774.

Makemson, J.C. 1973. Control of *in vivo* luminescence in psychrophilic marine photobacterium. *Arch. Microbiol*. 93:347-358.

Makemson, J.C. 1986. Luciferase-dependent oxygen consumption by bioluminescent vibrios. *J. Bacteriol*. 165:461-466.

Mancini, J., M. Boylan, R. Soly, S. Ferri, R. Szittner, and E. Meighen. 1989. Organization of the *lux* genes of *Photobacterium phosphoreum*. *J. Biolum. Chemilum*. 3:201-205.

Mancini, J., M. Boylan, R.R. Soly, A.F. Graham, and E. Meighen. 1988. Cloning and expression of the *Photobacterium phosphoreum* luminescence system demonstrate a unique *lux* gene organization. *J. Biol. Chem*. 263:14308-14314.

Martin, M., R. Showalter, and M. Silverman. 1989. Identification of a locus controlling expression of luminescence genes in *Vibrio harveyi*. *J. Bacteriol*. 171:2406-2414.

McElroy, W.D., and H.H. Seliger. 1962. Origin and evolution of bioluminescence. In: *Horizons in Biochemistry* (M. Kasha and B. Pullman, eds.), Academic Press, NY, pp. 91-101.

Meighen, E.A., and R.B. Szittner. 1992. Multiple repetitive elements and organization of the *lux* operons of luminescent terrestrial bacteria. *J. Bacteriol*. 174:5371-5381.

Meighen, E.A., M.Z. Nicoli, and J.W. Hastings. 1971. Hybridization of bacterial luciferase with a variant produced by chemical modification. *Biochemistry* 10:4062-4068.

Menard, R., P.J. Sansonetti, and C. Parsot. 1993. Nonpolar mutagenesis of the *ipa* genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. *J. Bacteriol*. 175:5899-5906.

Miller, M.B., K. Skorupski, D.H. Lenz, R.K. Taylor, and B.L. Bassler. 2002. Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*. *Cell* 110:303-314.

Miyamoto, C., P.V. Dunlap, E.G. Ruby, and E.A. Meighen. 2003. LuxO controls *luxR* expression in *Vibrio harveyi*: evidence for a common regulatory mechanism in *Vibrio*. *Mol. Microbiol*. 48:537-548.

Montilla, R., M.A.R. Chowdhury, A. Huq, B. Xu and R.R. Colwell. 1996. Serogroup conversion of *Vibrio cholerae* non-O1 to *V. cholerae* O1: effect of growth state of cells, temperature, and salinity. *Can. J. Microbiol*. 42:87-93.

Moore, D. 1996. Purification and concentration of DNA from aqueous solutions. In: *Current Protocols in Molecular Biology* (F.A. Ausubel, R. Brent, R.E. Kingston, D.D. Mooore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) John Wiley & Sons, New York, pp.2.4.1-2.4.5.

Morin, J.G., A. Harrington, N. Krieger, K.H. Nealson, T.O. Baldwin, and J.W. Hastings. 1975. *Science* 190:74-76.

Müller-Breitkreutz, K. and U. K. Winkler. 1993. VIIth International Symposium on Bioluminescence and Chemiluminescence *Banff, Alberta, Canada* 14-18 March 1993. *J. Biolumin. Chemilumin*. 8:108-11.

Murphee, R.I., and M.L. Tamplin. 1991. Uptake and retention of Vibrio cholerae O1 in the eastern oyster, *Crassostrea virginica. Appl. Env. Microbiol.* 61:656-660.

Nealson, K.H. 1977. Autoinduction of bacterial luciferase: occurrence, mechanism, and significance. *Arch. Microbiol*. 112:73-79.

Nealson, K.H. and J.W. Hastings. 1979. Bacterial bioluminescence: its control and ecological significance. *Microbiol. Revs*. 43:496-518.

Nealson, K.H., A. Eberhard, and J.W. Hastings. 1972. Catabolite repression of bacterial bioluminescence: functional implications. *Proc. Natl. Acad. Sci. USA* 69:1073-1076.

Nealson, K.H., and A. Markovitz. 1970. Mutant analysis and enzyme subunit complementation in bacterial bioluminescence in *Photobacterium. J. Bacteriol*. 104:300-312.

Nealson, K.H., T. Platt, and J.W. Hastings. 1970. The cellular control of the synthesis and activity of the bacterial luminescent system. *J. Bacteriol*. 104:313-322.

Nei, M., J.C. Stephens, and N. Saitou. 1985. Methods for computing the standard errors of branching points in an evolutionary tree and their application to molecular data from humans and apes. *Mol. Biol. Evol.* 2:66-85.

Nicoli, M.Z., and J.W. Hastings. 1974. Bacterial luciferase: the hydrophobic environment of the reactive sulfhydryl. *J. Biol. Chem*. 249:2393-2396.

Oliver, J.D., D.M. Roberts, V.K. White, M.A. Dry, and L.M. Simpson. 1986. Bioluminescence in a strain of the human pathogenic bacterium *Vibrio vulnificus*. *Appl Environ Microbiol*. 52:1209–1211.

Palmer, L.M., and R.R. Colwell. 1991. Detection of luciferase gene sequence in nonluminescent *Vibrio cholerae* by colony hybridization and polymerase chain reaction. *Appl. Env. Microbiol*. 57:1286-1293.

Ramaiah, N., J. Chun, J. Ravel, W.L. Straube, R.T. Hill, and R.R. Colwell. 2000. Detection of luciferase gene sequences in nonluminescent bacteria from the Chesapeake Bay. *FEMS Microbiol. Ecol*. 33:27-34.

Rozen, S., and H.J. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* Humana Press, Totowa, NJ, pp 365-386.

Ruby, E.G., and K.H. Nealson. 1976. Symbiotic association of *Photobacterium fischeri* with the marine luminous fish *Monocentris japonica*; a model of symbiosis based on bacterial studies. *Biol. Bull*. 151:574-586.

Ruimy, R., V. Breittmayer, P. Elbaze, B. Lafay, O. Boussemart, M. Gauthier, and R. Christen. 1994. Phylogenetic analysis and assessment of the genera *Vibrio, Photobacterium, Aeromonas*, and *Plesiomonas* deduced from small-subunit rRNA sequences. *Int. J. Syst. Bacteriol*. 44:416-426.

Schaefer, A.L., T.A. Taylor, J.T. Beatty, and E.P. Greenberg. 2002. Long-chain acyl-homoserine lactone quorum-sensing regulation of *Rhodobacter capsulatus* gene transfer agent production. *J. Bacteriol*. 184:6515-6521.

Shadel, G.S. and T.O. Baldwin. 1990. Control of the *lux* regulon of *Vibrio fischeri*. *J. Biolumin. Chemilumin*. 5:99-106.

Shewan, J.M., and M. Veron. 1974. Genus *Vibrio*, In: Buchanan, R.E., Gibbons, N.E. (Eds): *Bergey's Manunal of Determinative Bacteriology*, Baltimore, Williams and Wilkins, Co, pp.340-344.

Shimomura, O., F.H. Johnson, and H. Morise. 1974. The aldehyde content of luminous bacteria and of an "aldehydeless" dark mutant. *Proc. Natl. Acad. Sci*. USA 71:4666-46669.

Sokal, R.R., and F.J. Rohlf. 1994. *Introduction to Biostatistics*. Freeman: San Francisco, CA.

Soly, R.R., J.A. Mancini, S.R. Ferri, M. Boylan, and E.A. Meighen. 1988. A new *lux* gene in bioluminescent bacteria codes for a protein homologous to the bacterial luciferase subunits. *Biochem. Biophys. Res. Commun*. 155:351-358.

Spira, W.M., A. Huq, Q.S. Ahmed, and Y.A. Saeed. 1981. Uptake of *Vibrio cholerae* biotype eltor from contaminated water by water hyacinth (*Eichornia crassipes*). *Appl. Env. Microbiol*. 42:550-553.

Steidle, A., M. Allesen-Holm, K. Riedel, G. Berg, M. Givskov, S. Molin, and L. Eberl. 2002. Identification and characterization of an *N*-acylhomoserine lactone-dependent quorum-sensing system in *Pseudomonas putida* strain IsoF. *Appl. Environ. Microbiol.* 68:6371-6382.

Sugihara, J., and T.O. Baldwin. 1988. Effects of 3' end deletions from the *Vibrio harveyi luxB* gene on luciferase subunit folding and enzyme assembly: Generation of temperature-sensitive polypeptide folding mutants. *Biochem*. 27:2872-2880.

Swartzman, E., C. Miyamoto, A. Graham, and E. Meighen. 1990a. Delineation of the transcriptional boundaries of the *lux* operon of *Vibrio harveyi* demonstrates the presence of two new *lux* genes. *J. Biol. Chem*. 265:3513-3517.

Swartzman, E., S. Kapoor, A. F. Graham, and E. A. Meighen. 1990b. A new *Vibrio fischeri lux* gene precedes a bidirectional termination site for the *lux* operon. *J. Bacteriol.* 172:6797-6802.

Swift, S. M., J.P. Throup, P. Williams, G.P.C. Salmond and G.S.A.B. Stewart. 1996. Quorum sensing: a population-density component in the determination of bacterial phenotype. *Trends Biochem. Sci*. 21:214-219.

Szpilewska, H., A. Czyz, and G. Wegrzyn. 2003. Experimental evidence for the physiological role of bacterial luciferase in the protection of cells against oxidative stress. *Curr. Microbiol*. 47:379-382.

Tamplin, M.L., A.L. Glauzens, A. Huq, D.A. Sack, and R.R. Colwell. 1990. Attachment of *Vibrio cholerae* serogroup O1 to zooplankton and phytoplankton of Bangladesh waters. *Appl. Env. Microbiol*. 56:977-980.

Thomas, G.M., and G.O. Poinar, Jr. 1979. *Xenorhabdus* gen. non., a genus of entomopathogenic, nematophilic bacteria of the family *Enterobacteriaceae*. *Int. J. Syst. Bacteriol*. 29:352-360.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 24:4876-4882.

Timmins, G.S., S.K. Jackson, and H.M. Swartz. 2001. The evolution of bioluminescent oxygen consumption as an ancient oxygen detoxification mechanism. *J. Mol. Evol*. 52:321-332.

Ulitizur, S. and J.W. Hastings. 1979. Control of aldehyde synthesis in the luminous bacterium *Beneckea harveyi*. *J. Bacteriol*. 137:854-859.

Vincze, T., J. Posfai, and R.J. Roberts. 2003. NEBcutter: a program to cleave DNA with restriction enzymes. *Nucleic Acids Res*. 31:3688-3691.

Visick, K.L., J. Foster, J. Doino, M. McFall-Ngai, and E.G. Ruby. 2000. *Vibrio fischeri lux* genes play an important role in colonization and development of the host light organ. *J. Bacteriol.* 182:4578-4586.

Waldor, M.K. and J.J. Mekalanos. 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* 272:1910-1914.

Watnick, P.I., and R. Kolter. 1999. Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Mol. Microbiol*. 34:586-595.

West, P.A., P.R. Brayton, T.N. Bryant, and R.R. Colwell. 1986. Numerical taxonomy of vibrios isolated from aquatic environments. *Intl. J. Sys. Bacteriol*. 36:531-543.

Wilson, K. 1994. Preparation of genomic DNA from bacteria. In: *Current Protocols in Molecular Biology* (F.A. Ausubel, R. Brent, R.E. Kingston, D.D. Mooore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) John Wiley & Sons, New York, pp.2.4.1-2.4.5.

Wilson, T. and J.W. Hastings. 1998. Bioluminescence. *Annu. Rev. Cell Dev. Biol*. 14:197-230

Wright, A., G. Micelli, W. Landry, J. Christy, W. Watkins and G. Morris, Jr. 1993. Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. *Appl. Environ. Microbiol.* 59:541-546.

Wright, A., Y. Guo, J. Johnson, J. Nataro, G. Morris, Jr. 1992. Development and testing of a nonradioactive DNA oligonucleotide probe that is specific for *V. cholerae* cholera toxin. *J. Clin. Microbiol*. 30:2302-2306.

Zavil'gel'skii, G.B., A.P. Zarubina, and I.V. Manukhov. 2002. Sequencing and comparative analysis of the *lux* operon of *Photorhabdus luminescens* strain ZM1: ERIC elements as putative recombination spots. *Mol. Biol. (Mosk.)* 36:792-804.

Zhu, J., and J. J. Mekalanos. 2003. Quorum sensing-dependent biofilms enhance colonization in *Vibrio cholerae*. *Developmental Cell* 5:647-656.

Zhu, J., M.B. Miller, R.E. Vance, M. Dziejman, B.L. Bassler, and J.J. Mekalanos. 2002. Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. *Proc. Natl. Acad. Sci.* 99:3129-3134.