Identification of a constitutively active variant of LuxO that affects production of HA/protease and biofilm development in a non-O1, non-O139 *Vibrio cholerae* O110.

Key words: Haemagglutinin; virulence; quorum sensing; *hapA*

Saumya Raychaudhuri[1 *], Vibhu Jain[1] and Mitesh Dongre

Institute of Microbial Technology, Molecular Biology Division, Chandigarh, India 160036

Address for Correspondence [*] - Saumya Raychaudhuri,

Institute of Microbial Technology,

Sector –39A, Chandigarh – 160036,

India.

Fax-91-172-2690585/ 2690632;

Phone: 91-172-2690004 extension-463

email: saumya@rocketmail.com, saumya@imtech.res.in,

[1] S.R. and V.J. contributed equally to work

Abbreviations

SDS-PAGE=Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, PCR= Polymerase chain reaction, CT= Cholera toxin, TCP= Toxin co-regulated pilus, TSB= Tryptic soya broth.

Abstract

Pathogenesis of *Vibrio cholerae* depends on the concerted action of numerous virulence factors that includes a secreted hemagglutinin (HA) protease. Recent studies have evidenced that the expression of these virulence factors as well as the genes responsible for biofilm development is subject to control by quorum sensing in this organism. At low cell density, LuxO, the pivotal regulator of quorum sensing circuit has been shown to be phosphorylated at aspartate – 47. Working in concert with sigma-54, LuxO-P activates the downstream repressor, which turned out to be four sRNAs (Lenz et al., 2004, Cell 118, 69-82). Subsequently, these sRNAs form complex with sRNA chaperone, Hfq. The Hfq-sRNA complex causes the destabilization of *hapR* mRNA transcript. HapR is a positive regulator of *hapA* that encodes HA/protease. At high cell density, dephosphorylation of LuxO impairs its function to activate the expression of sRNA, which in turn promotes HapR expression and cause protease production. It has been demonstrated that conversion of aspartate to glutamate (D47E) renders the LuxO molecule active without being phosphorylated. This variant of LuxO is referred as constitutively active LuxO or con-LuxO (Freeman and Bassler, 1999, Mol Microbiol 31, 665-677). Other than D47E, mutation at L104Q also develops con-LuxO (Vance et al., 2003, Infect. Immun. 71, 2571-2576). The purpose of this study was to investigate the cause of protease negative phenotype of a non-O1, non-O139 strain of *Vibrio cholerae* O110. In the process of exploring the nature of the phenotype, a constitutively active variant of LuxO molecule was characterized which represses protease production and enhances biofilm formation by this strain. Unlike *luxU*, disruption of *luxO* restored the protease production, which showed the constitutively active nature of LuxO protein in this strain.

1. Introduction

Vibrio cholerae is the causative agent of cholera, a form of diarrhoea, which continues to be one of the major causes of morbidity and mortality in the developing world. Studies on the pathogenesis of *V. cholerae* led to the identification of several critical virulence factors such as cholera toxin (CT), responsible for profuse watery diarrhoea, a hallmark symptom of the disease and the toxin co-regulated pilus (TCP), essential for colonization in the human small intestine. Besides CT and TCP, *V. cholerae* secretes a major Zn-dependent metalloprotease, hemagglutinin/protease (HA/protease) encoded by *hapA*. Owing to have mucin degrading ability, this protease helps vibrios to overcome the protective mucus barrier, enhances the access of bacteria towards the gastrointestinal epithelium and consequently, promotes bacterial colonization in the epithelia. This protease also facilitates the dissemination of vibrios in stool. Importantly, HA/protease contributes to the reactogenecity of the live attenuated vaccine (Silva and Benitez, 2004).

V. cholerae has the capacity to survive in diverse estuarine environments as well as in human host. Both in fresh and marine aquatic settings, biofilm development appears to be crucial for the survival as well as sustenance of *V. cholerae* during and after epidemic period (Kierek and Watnick, 2003). Recent studies have evidenced that the expression of these virulence factors as well as genes responsible for biofilm development is subject to control by quorum sensing in *V. cholerae* (Zhu et al., 2002, Hammer and Bassler, 2003, Zhu and Mekalanos, 2003).

The mechanistic model for quorum sensing in *V. cholerae* mirrors that of *V. harveyi*. Like *V. harveyi*, *V. cholerae* possesses multiple quorum sensing circuits, which work in parallel to regulate virulence and biofilm development in this organism

(Miller et al., 2002, Hammer and Bassler, 2003). So far, two quorum-sensing systems have been characterized at the molecular level, each unit consists of a unique autoinducer synthase and its cognate sensor (Miller et al., 2002). *V. cholerae* has an additional system (system 3) that remains to be identified, whereas all three quorum-sensing systems in *V. harveyi* have been characterized (Henke and Bassler, 2004). Genetic analysis indicates that sensory information from all systems converges at the central molecule LuxO by a shared phosphorelay protein, LuxU (Lilley and Bassler, 2000). At low cell density (i.e., in the absence of autoinducers), LuxU activates LuxO in both *V. cholerae* and *V. harveyi* through phosphorylation. This phosphorylated LuxO (LuxO-P) together with σ^{54} turns on the expression of a downstream repressor. In a series of elegant genetic experiments, Lenz et al., demonstrate that the repressor is the sRNA chaperone Hfq and four sRNAs (Lenz et al., 2004). Mechanistically, the LuxO-P-σ^{54} complex drives the expression of the loci encoding all four sRNA and repression occurs via Hfq-sRNA-mediated destabilization of the *hapR* mRNA transcript. HapR is an essential activator of the *hapA* protease gene and also down regulates the expression of *tcpPH*, by repressing the transcription of *aphA* (Vance *et al*., 2003). On the other hand, at high cell density (i.e., in the presence of autoinducers), LuxO is dephosphorylated and becomes inactive. Inactive dephospho-LuxO permits *hapR* expression, thus cause protease production.

Being a central molecule of quorum-sensing circuit, a great deal of work has therefore been dedicated to understanding the function of LuxO. The molecule belongs to Ntrc family of two-component response regulator, which also works in concert with an alternative sigma factor, σ^{54} (Lilley and Bassler, 2000). Sequence analysis indicates that it contains a conserved aspartate residue (Asp-47) that is critical for its signaling function (Freeman and Bassler, 1999). Signal transduction is proposed to occur via a series of phosphorylation and

dephosphorylation reaction. Inactivation of LuxO function occurs by dephosphorylation at Asp-47. Based on genetic analysis, two classes of mutant LuxO are obtained. One category of LuxO molecules harbor a specific mutation at a particular residue(s) that lock the protein in a constitutively active form mimicking phosphorylated-LuxO while a diverse set of mutation in the same residue or different residues convert the protein into an inactivated form simulating dephosphorylated – LuxO. For example, mutant proteins mimicking unphosphorylated LuxO (D4K, D47A, D47N and K97A) inactivated its activator function and caused constitutive luminescence in *V. harveyi* (Freeman and Bassler, 1999). On the contrary, mutant protein mimicking P-LuxO (D47E, F94W) resulted in constitutively active form, which repressed luminescence in *V. harveyi*. Because of its constitutive nature of activity, the mutant form D47E is referred as constitutively active- LuxO (Con-LuxO). Recently, Vance et al., reported the identification of a naturally occurring point mutation in LuxO protein of a protease-defective mutant of *V. cholerae* El Tor strain E7946. The mutant *luxO* allele produces a protein with a leucine-to-glutamate substitution at amino acid 104, which results a constitutively active LuxO (Con-LuxO), thus represses protease production and enhances biofilm formation by this strain (Vance et al., 2003).

So far, more than 200 serogroups of *V.cholerae* strains have been reported (Faruque et al., 2003, Dziejman et al., 2005). Only *V. cholerae* strains belonging to the O1 and O139 serogroups are thought to be capable of causing epidemic cholera. Strains belonging to serogroup other than O1 and O139 are collectively designated as non-O1, non-O139. Unlike its pathogenic counterpart (O1 and O139), most non-O1, non-O139 strains are nonpathogenic and ubiquitously found in the aquatic environment. Nevertheless, some non-O1, non-O139 strains are capable of causing sporadic cases of gastroenteritis and extraintestinal infections in human (Ou et al.,

2003). In the recent past, an upsurge in the incidence and emergence of drug resistant clinical as well as environmental non-O1, non-O139 strains has been reported (Thungapatra et al., 2002). Genome sequencing data also revealed that non-O1, non-O139 strains are quite divergent from O1 and O139 stains (Dziejman et al., 2005). As quorum sensing regulates virulence and environmental fitness of this bacterium, we wanted to explore the extent of this phenomenon in *V. cholerae* strain belonging to non-O1, non-O139 serogroup.

The study described in this paper was undertaken to examine the protease negative phenotype of a non-O1, non-O139 *V. cholerae* strain PL91. In the process of characterizing the nature of the phenotype, a novel variant of LuxO molecule was identified from this strain. Though various constitutively active LuxO molecules reported so far, results presented here revealed the identification of another naturally occurring constitutively active LuxO, which harbors a deletion of 12 amino acids within the D47 and F94 residues thus renders a protease negative strain. This strain also developed a robust biofilm, which appeared as a pellicle under appropriate condition. Upon disruption of *luxO*, this strain became protease positive, which showed the constitutive active nature of the LuxO protein in this strain. This is a first report that describes the molecular characterization of a key quorum sensing molecule LuxO, which turned out to be constitutively active form, from a non-O1, non-O139 strain of *V.cholerae*.

2. Materials and methods

2.1. Organisms and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All strains were propagated at 37°C in liquid with agitation or on solid (1.5 % agar) in

Luria Broth unless mentioned otherwise. When appropriate, the growth medium was supplemented with ampicillin (100 µg ml $^{-1}$) and /or streptomycin (10µg ml $^{-1}$). For expression of *hapR* gene, *V. cholerae* cells carrying the recombinant plasmid were grown in Luria broth containing ampicillin. The antibiotics were purchased from Sigma and Amersham. Media ingredients were purchased from Himedia.

2.2. DNA preparations and manipulations

Small and large-scale plasmid DNA was made by Qiagen kit. Chromosomal DNA from *V. cholerae* strain was prepared from overnight cultures according to previously described method (Majumder et al., 2005). Restriction endonucleases and T4 DNA ligase were obtained from New England Biolab. DNA fragments were purified using the Qiagen Qia Quick gel elution kit.

2.3. Protease assay

Protease activity was measured using an azocasein assay as described elsewhere (Benitez et al., 2001). Briefly, PL91, PL91-SVM, PL91-SC, N16961-C and N16961-R strains were grown in tryptic soy broth without dextrose (TSB) containing streptomycin (10µg ml $^{-1}$-100µg ml $^{-1}$) and ampicillin (100µg ml $^{-1}$) accordingly with agitation to stationary phase at 37°C. 100 µl of stationary phase culture supernatant was incubated with 100 µl azocasein (5mg ml $^{-1}$ in 100mM Tris pH 8.0) for 1 h at 37˚C. The reaction was stopped by the addition of 400µl of 10% tricholoroacetic acid. After centrifugation, the trichloroacetic acid supernatant was transferred to 700µl of 525 mM NaOH, and the optical density was determined at 442 nm. One azocasein unit was defined as the amount of enzyme producing an increase of 0.01 OD units per h.

2.4. Cloning of hapR in pKK1773R1

The *hapR* ORF was amplified and prepared for insertion into pKK177-3R1 by PCR. The N-terminal primer had a *Sma*I site adjacent to the initiating codon AUG, while in the reverse orientation C-terminal primer incorporated a *Hind*III after the terminator UAG. The amplified product was purified by gel electrophoresis, digested sequentially with *Sma*I and *Hind*III, and ligated with similarly digested and purified pKK177-3R1 for 16 h at 16°C. Transformation of Novablue cells was carried out by standard methods and yielded 7 positive clones out of 10 tested.

2.5. Electroporation of hapR recombinant construct in V. cholerae O11O

Overnight cultures of *V. cholerae* strains were subcultured 1:40 in 50 ml fresh SOB and grown for 3 h at 37°C. The cells were pelleted at 5000 rpm for 10 min at 4°C, followed by three times wash with ice-cold wash buffer (10% glycerol in water). The washed pellet was resuspended in 1-2 ml of wash buffer. Electroporation was carried out with a Bio-Rad Gene Pulser and Pulse Controller using cuvettes with a 0.2 cm gap distance and following settings: 1.51 KV, 25 μF and 200 Ω (Bio-Rad Laboratories, Inc, Hercules, Calif.). Cells were allowed to recover at 37°C for 3 h, in SOC, plated on L agar containing ampicillin (100μg ml^{-1}) and grown overnight at 37°C. A single colony from plates was checked for protease expression further as described in section 2.3.

2.6. Nucleic acid sequencing

The 1.332 kb *luxO* (ORF) PCR product generated by primers *luxO Sma*I and *luxO Hin*dIII using PL91 DNA as a template was directly sequenced by the ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin Elmer, Norwalk, CT) and an

ABI 377 automated DNA sequencer in both directions. Sequence of internal region was determined using primers designed from within this sequenced region (Table 2).

2.7. Construction of pSVM and *pSC*

A pair of oligonucleotides designated as LuxO *Xba*I and LuxO *Eco*RI was designed to amplify a 743 bp fragment internal to the *luxO* gene (Table 2). PCR product was purified from 1% agarose gel using the Qia Quick gel elution kit (Qiagen), digested with *Xba*I and *Eco*RI (New England Biolabs) according to the instructions of the manufacturer and was cloned into similarly digested pGP704 at *Xba*I and *Eco*RI site to generate the recombinant plasmid pSVM. pSVM was maintained in *E. coli* SM10: λ pir as a host.

In a similar fashion of pSVM construction, a 224 bp internal fragment of *luxU* gene was amplified by oligonucleotides termed as LuxU *Xba*I and LuxU *Eco*RI. The PCR product was subsequently purified, digested with *Xba*I and *Eco*RI and was cloned into similarly digested pGP704 at *Xba*I and *Eco*RI site. The resulting recombinant plasmid was named pSC and maintained in *E. coli* SM10: λ pir as a host.

2.8. Bacterial conjugation

Conjugation was conducted between recipient streptomycin resistant *V. cholerae* strain (PL91) and donor *E. coli* SM10: λ pir transformed with p*SVM* as well as p*SC* (Waldor and Mekalanos, 1996, Majumdar et. al., 2005). *luxO* and *luxU* mutants of PL 91 were screened by streaking onto LA plates containing ampicillin (100μg ml^{-1}) and streptomycin (10 μg ml^{-1}).

2.9. DNA hybridization

Chromosomal DNA (1-2 μg) was digested with HindIII (New England Biolab) and the resulting fragments were transferred onto Hybond TM – N Nylon membrane (Amersham Life science) after electrophoresis on agarose gel (1%). PCR

products carrying *luxO* and *luxU* genes were labeled with [α ^{32}P] dATP by random priming kit (New England Biolab) and were used as probes. Southern blot experiments were performed under stringent conditions (Majumder et al., 2005).

2.10. Western blot

Western blot analysis of HA/protease production was done as described elsewhere (Benitez et al., 2001). Briefly, PL91 and PL91-SVM (Δ*luxO*) strains were grown overnight in Tryptic soya broth (TSB) medium (without glucose) with agitation. The cultures were centrifuged, and 2 ml of each supernatant was concentrated 10 fold by centrifuging it through Centricon-10 centrifuge filters. The samples were run on a SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amersham). HA/protease was detected by using a rabbit anti-Hap serum and peroxidase–conjugated goat anti-rabbit immunoglobulin G. Molecular masses were calculated with reference to SDS-PAGE molecular mass standards (Broad range) from Bio-Rad laboratories.

2.11. Biofilm assay

Biofilm development of PL91, PL91-SVM (Δ *luxO*) and PL91-SC (Δ*luxU*) strains were done as described elsewhere (Watnick et al., 1999). Briefly, overnight cultures of all strains were resuspended in Luria Broth containing 4 X 10^6 bacteria per ml. One ml of each suspension was incubated statically at 37°C in borosilicate glass tube for 24hr. Biofilm development was observed visually as a function of pellicle formation and photographed.

3. Results and Discussion

3.1. Protease assay and functional analysis of hapR of V. cholerae PL91

There was no protease activity in the cell free culture supernatant of PL91 (Fig. 1). The protease activity of PL91 was comparable to that of N16961, the later is a known HA/protease negative strain due to frameshift mutation in *hapR*. In the light of current knowledge, this phenotypic behavior could be explained in the following manners, i) existence of a non-functional HapR, a transcriptional activator of *hapA* which encodes HA/P protease (Zhu et al., 2002, Hammer and Bassler, 2003) ii) a problem in protease secretory machinery or iii) repression of *hapR* due to constitutively active LuxO (Vance *et al*., 2003). Earlier it has been reported that several toxigenic, biofilm developing *V. cholerae* strains including N16961 carry a natural frame shift or point mutations in *hapR*, which might lead to obtain such phenotypic behavior. This observation prompted us to evaluate the status of *hapR* in PL91. Together with PCR amplification and subsequent sequencing analysis revealed no frame-shift mutation (Gene bank accession number DQ112157) in *hapR* gene of PL91 as described in N16961. To determine the functionality, *hapR* of PL91 was cloned into pKK177-3R1 (pSR) and electroporated in both PL91 and N16961. The recombinant *V. cholerae* strains PL91-R and N16961-R were found to be protease positive by an azocasein protease assay as mentioned (Fig. 1). These results, in combination with sequencing data, ruled out the possibility of PL91 being a *hapR* mutant. This data also suggested that there is no defect in the protease secretory pathway in PL91.

3.2. Analysis of the nucleotide sequence of the luxO gene from V. cholerae strain PL91

The results presented in section 3.1 suggested that the cause of protease deficient phenotype of PL91 might result from a problem in the upstream of *hapR* in the quorum sensing circuit. One possibility could be the presence of a constitutively active allele of LuxO, which suppress *hapR* even at high cell density and thus affect the protease production. Recently it was shown that specific mutations in or around the critical residues of LuxO render its activity independent of phosphorylation and cell density (Freeman and Bassler, 1999, Vance et al., 2003). Such LuxO molecules (e.g. LuxO D47E and LuxO L104Q) harbor these novel mutations are referred as constitutively active (Con-LuxO) LuxO, which will remain active through out the growth phase. It has been reported that *V. cholerae* strain harbors con-LuxO became protease negative and developed a robust biofilm (Vance *et al*., 2003), which set the stage for us to investigate the *luxO* gene of PL91 at the sequence level. The sequence (GenBank accession number DQ002872) was found to have 97.5% identity with that from N16961. Further alignment analysis revealed the missing of a stretch of 12 amino acids (36 nucleotides) within D47 and F94 residues (Fig. 2). We also found that the residue L104 remains intact. Taken together, nucleotide sequencing data of *luxO* as well as reversal of protease negative phenotype by supplying its own *hapR* in trans suggested the possibility of a constitutively active form of LuxO, which is enough to cause protease deficient phenotype of PL91.

3.3. Insertional inactivation of the luxO gene in V. cholerae strain PL91

In order to ascertain that PL91 produces a constitutively active form of *luxO* that may be the cause of this protease negative phenotype, we disrupted *luxO* of PL91 by insertional mutation using suicide vector pGP704 as described elsewhere (Waldor and Mekalanos, 1996, Majumder et al., 2005). Towards this end, suicidal plasmid pSVM carrying the internal fragment of *luxO* was generated as described in section 2.7 and maintained into *E. coli* SM10: λ pir cells which served as donor cells in

subsequent conjugational transfer of this plasmid into *V. cholerae* strain PL91. Mutants were selected on streptomycin ampicillin plate. To further confirm the integration, Southern blot analysis was done with *Hin*dIII digested chromosomal DNA from *luxO* mutant. Integration of pSVM is expected to abolish the original 6.7 kb *Hin*dIII fragment present in the wild type cells and replace it with two *Hin*d*III* fragment of 5.7 and 1.8 kb which are expected to hybridized with labeled 1.332 kb *luxO* probe. We observed in case of wild type a band of 6.7 kb appeared as expected while in case of mutant, two bands corresponding to 5.7 kb and 1.8 kb hybridized with the probe, confirming the integration of pSVM (data not shown). We found that resultant PL91Δ*luxO* strain produces protease (Fig.1), which was further confirmed as HA/protease by western blot (Fig.3). This data clearly manifested that a constitutively active LuxO causing the protease deficient phenotype in this strain.

3.4. Insertional inactivation of the luxU gene in V. cholerae strain PL91

To examine that LuxO function of PL91 is dependent on LuxU (a phosphorelay protein that phosphorylates LuxO) or not, attempts were made to inactivate the chromosomal copy of *luxU* by insertional mutation using suicide vector pGP704 in a similar fashion like *luxO*. A suicidal plasmid pSC carrying the internal fragment of *luxU* was generated as described in section 2.7 and maintained into *E. coli* SM10: λ pir cells. The plasmid was then transferred to *V. cholerae* PL91 by conjugation and the transconjugants were selected on streptomycin (10μg ml^{-1}) ampicillin (100 μg ml^{-1}) plate. The integration was further confirmed by Southern blot analysis with *Bst*EII digested chromosomal DNA from *luxU* mutant. Integration

of pSC is expected to abolish the original 2.0 kb *Bst*EII fragment present in the wild type strain and replace it with *Bst*EII fragment of 1.9 and 4.0 kb which are expected to hybridized with labeled 0.34 kb *luxU* probe. We found wild type strain produced a 2.0 kb fragment as expected while two bands of 1.9 kb and 4.0 kb appeared in case of mutant, confirming the integration of pSC (data not shown). We observed that resultant PL91Δ*luxU* (PL91-SC) strain remains protease negative (Fig.1) and biofilm positive (Fig.4). This data also suggested that the function LuxO of PL91is independent of LuxU activity.

3.5 Western blot of the culture supernatant of PL91-SVM

Protease assay showed a significant enhancement of protease activity in the culture supernatant of PL91-SVM in comparison to wild type PL91 and PL91-SC (Fig. 1). To further confirm, the presence of HA/protease in the culture supernatant, western blot was done as described in section (2.10). Western blot data demonstrated the presence of HA/protease in the culture supernatant (Fig. 3), which appeared as molecular mass of 35 kD as described in literature. This data clearly manifested that PL91 is harboring a constitutively active LuxO, which causes the HA/protease deficient phenotype of this strain.

3.6. Biofilm assay

To evaluate the ability of biofilm formation of PL91, a static biofilm assay was done as described in section 2.11. We observed a thick pellicle appeared (Fig. 4) in case of PL91 and PL91-SC *(ΔluxU*), which was absent in PL91-SVM (*ΔluxO*).

Recent studies have witnessed a seismic shift of conception and understanding towards the mechanism of quorum sensing, a cell-to-cell signaling process that enables several bacteria including *Vibrio cholerae* to collectively control gene

expression, thereby synchronizing activities that are productive only at a high population density. A body of evidence expounded that an ultrasensitive switchlike mechanism is used by *V. cholerae* to control the quorum-sensing events. At high cell density, dephosphorylation of LuxO, the quorum-sensing regulator in this bacterium, results in the repression of biofilm related genes and derepression of *hapR*, a positive regulator for the production of HA/protease. Recently, it was shown that specific mutations in this LuxO molecule could alter its activity independent of cell density. These mutants were characterized and categorized as constitutively active LuxO (con-LuxO). Strains harbor such con-LuxO develop robust biofilm and show no protease activity in the culture supernatant.

In the current study, we pursued an understanding of an observation of a protease deficient, but biofilm proficient variant of a non-O1, non-O139 strain of *V. cholerae*, PL91. In an effort to understand, we provide the initial characterization of a novel variant of constitutively active LuxO, which turns out a necessary and sufficient cause of the protease negative phenotype of strain, PL91. Our results further complement and extend the current understanding of quorum-sensing signaling cascade in *V. cholerae*. The identification of another novel mutation that leads to a constitutive active form of LuxO will be invaluable in understanding the structural basis of LuxO activation. We surmised that the absence of 12 amino acids might introduce some structural changes that conferred constitutively active nature of the molecule. Our hypothesis is further corroborated by *luxU* data, which showed con-LuxO of PL91, though it contains asp-47 (D-47), still remains active at high cell density and maintains the protease negative phenotype of PL91-SC. The next question arises how these amino acids are important. At this stage, we have no idea whether these 12 amino acids (SIDTAVEAMRHG) could be replaced by any other amino acids or not. The sequential arrangement along with the stretch may be an

important factor to determine the function of the molecule. A detail study is required to ascertain the importance of these missing amino acids.

Earlier studies established that constitutively active LuxO proteins such as LuxO D47E and LuxO F94W are not functionally equivalent (Freeman and Bassler, 1999). The repressor phenotype of LuxO D47E is more pronounced than that of LuxO F94W. Similarly, ΔRR LuxO protein has a stronger repressor phenotype than does the LuxO D47E protein (Freeman and Bassler, 1999). It was also postulated that, in addition to phosphorylation, oligomerization could also be required for LuxO function (Freeman and Bassler, 1999). There could be a possibility of an alteration in the degree of oligormerization owing to different structural organization among different constitutive LuxO molecules, which may influence its function. In this context, the con-LuxO of PL91 is expected to provide a novel system for structure-function analysis of this molecule. Additional studies are needed to address this issue.

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Table 1.

Strains/Plasmids	Description	Source/reference
V. cholerae strains		
PL91	Non-O1, non-O139, Sergroup O110. Sm^r	Thungapatra et al., 2002.
N16961	El Tor, Ogawa, Sm^r	Andrew Camilli Tuft University.
PL91-SVM	Non-O1, non-O139, Serogroup O110, Sm^r, *luxO*:: pSVM, Ap^r	This study
PL91-Sc	Non-O1, non-O139, Serogroup O110, Sm^r, *luxU*:: pSC, Ap^r	This study
N16961-R	El Tor, Ogawa, Sm^r, carrying pSR	This study
N16961-C	El Tor, Ogawa, Sm^r, carrying pKK177-3R1	This study
PL91-R	Non-O1, non-O139, Serogroup O110, carrying pSR.	This study
PL91-C	Non-O1, non-O139, Serogroup O110, carrying pKK177-3R1.	This study
E. coli strains		
Nova blue	*E. coli* K-12, *recA endA*, $lacI^q$, *lacY*	Novagen
SM10 :λpir	*thi thr leu tonA lacY supE recA*:: RP4-2-Tc :: Mu, *λ-pir* R6K	Simon *et al*. (1983).
Plasmids		
pSVM	743 kb *Eco*RI-*Xba*I fragment from PCR amplification of pSRC with *Xba*I-LuxO and *Eco*RI-LuxO and cloned into pGP704 digested with same restriction enzymes.	This study
pSC	224 kb *Eco*RI-*Xba*I fragment from PCR amplification of genomic DNA with *Xba*I-LuxU and *Eco*RI-LuxU and cloned into pGP704 digested with same restriction enzymes	This study
pSR (hapR)	612 bp fragment of hapR (ORF) cloned into *Sma*I – *Hind*III site of pKK177-3RI.	This study
pGP704	Ap^r, *mob* RP4, ori R6K	Miller and Mekalanos (1988)
pKK177-3R1	Ap^r	Giesla Stroz, National Institute of Health.

Table 2. Primers used in this study

Primer name	Sequence
HapR *Sma*I (cloning and sequencing primer)	5'- TAACCCGGGATGGACGCATCAATC
HapR *Hind*III (cloning and sequencing primer)	5'-CCCAAGCTTCTAGTTCTTATAGATAC
HapR 361 (sequencing primer)	5'-TTATGTACCCACTCAATAAAAC
HapR M (sequencing primer)	5'-CTGCTGATCAGAAACATGTTTATG
LuxO 843 (luxO sequencing primer)	5'- AGACCTGTACTACCGCTTGTATGTG
LuxO *Hind*III (luxO cloning and sequencing primer)	5' CCCAAGCTTTTACCGTTCCTTCTC
LuxO *Sma*I (luxO cloning and sequencing primer)	5'-TCCCCCGGGATGGTAGAAGACAC
LuxO MS (luxO sequencing primer)	5'- TGAGTTGTTTGGTCACGTC
LuxO *Xba*I (cloning primer)	5'-CTAGTCTAGACGCAAAGCCTCGAAAC
LuxO *Eco*RI (cloning primer)	5'-CCGGAATTCAGAGGAGGAGGCAG
LuxU *Xba*I (cloning primer)	5'-CTAGTCTAGAGTGCCGATCTTGGTC
LuxU *Eco*RI (cloning primer)	5'- CCGGAATTCCCTCTAGCGAGATGTCCATC

Figure(s)

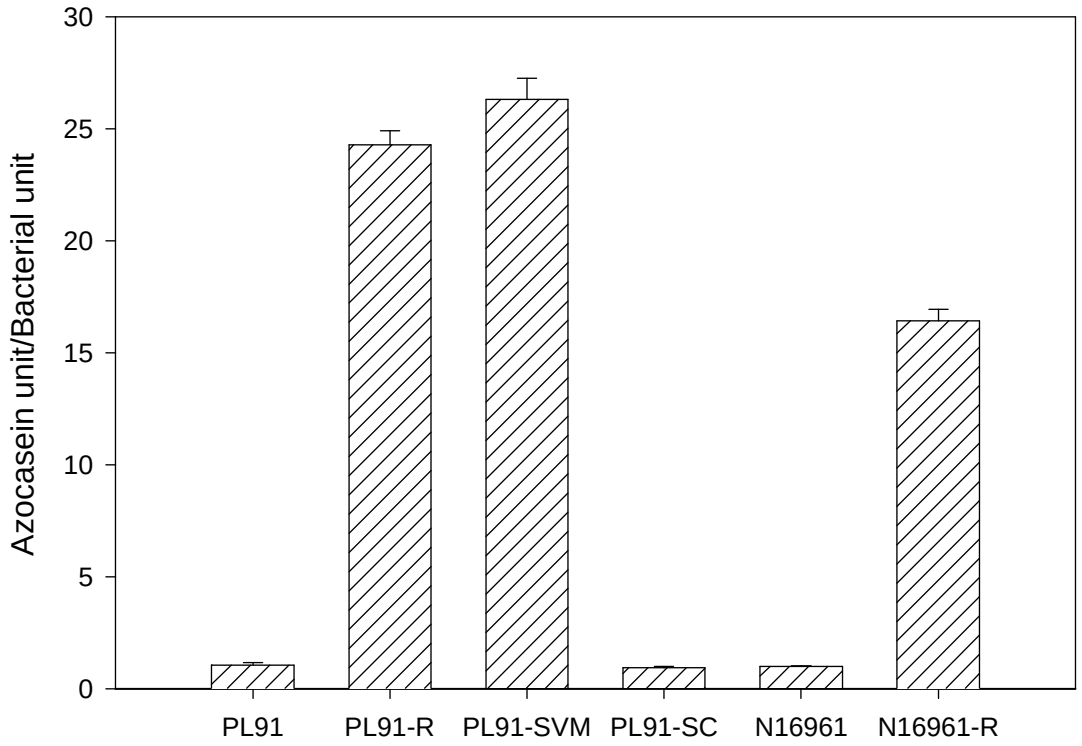


Fig1. Cell-free supernatants from cultures of indicated strains grown in TSB for 14 h at 37°C (200 rpm) were assayed for digestion of azocasein in triplicate. Enzyme activities are the mean of three independent cultures. Standard deviations are indicated with error bars.

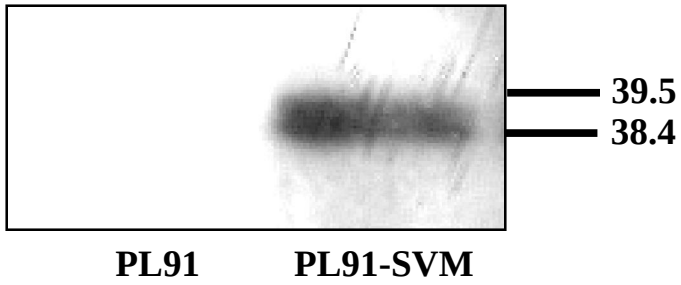
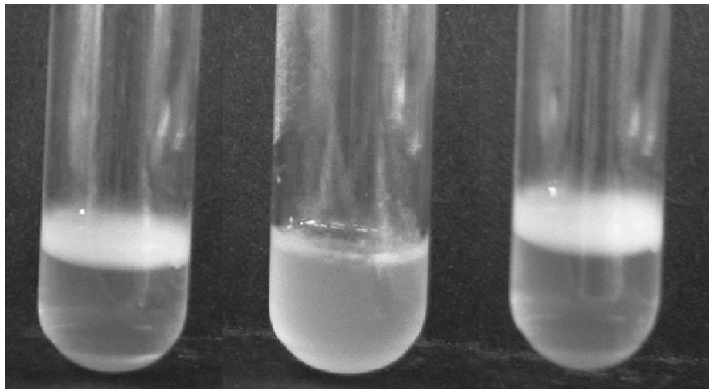


Fig 3. Sodium dodecyl sulfate –polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of Hap production. A 14-h culture of two indicated strains were centrifuged and 2 ml of each supernatant was concentrated 10-fold by centrifuging it through Centricon-10 centrifugal filters. The retentate was adjusted 0.02 ml aliquot was analyzed in an SDS-12% PAGE gel. The gel was subsequently transferred to a polyvinylidene difluoride membrane. Hap was detected by using a rabbit anti-Hap serum and peroxidase-conjugated goat anti rabbit immunoglobin G. Molecular masses were calculated with reference to SDS molecular mass standards (low range) from Bio-Rad laboratories.



PL91 PL91-SVM PL91-SC

Figure4: Biofilm assay of strain PL91, PL91-SVM and PL91-SC mutants was done. Overnight cultures of indicated strains were resuspended in 1 ml of LB (containing 4X 106 bacteria per ml) in borosilicate glass tubes. Bacteria were grown statically at 37℃ for 24 h. After 24 h, tubes were observed for biofilm formation and picture was taken by Canon digital camera.

Figure(s)

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CLUSTAL W (1.82) multiple sequence alignment

                    <                                                 o
V.c.91          MVEDTASVAALYRSYLTPLDIDINIVGTGRDAIESIGRREPDLILLDLRLPDMTGMDVLY 60
V.c             MVEDTASVAALYRSYLTPLDIDINIVGTGRDAIESIGRREPDLILLDLRLPDMTGMDVLY 60
V.h             MVEDTASVAALYRSYLTPLGIDINIVGTGRDAIESLNHRIPDLILLDLRLPDMTGMDVLH 60
                ******************.***************:.:* ******************:
                                                  •  •       *
V.c.91          AVKEKSPDVPIVFMTAHGS------------QDFLIKPCEADRLRVTVNNAIRKASKLKN 108
V.c             AVKEKSPDVPIVFMTAHGSIDTAVEAMRHGAQDFLIKPCEADRLRVTVNNAIRKASKLKN 120
V.h             AVKKSHPDVPIIFMTAHGSIDTAVEAMRHGSQDFLIKPCEADRLRVTVNNAIRKATKLKN 120
                ***:. *****:******            :************************:****

                                                          __________
V.c.91          DVDNK-NQNYQGFIGSSQTMQAVYRTIDSAASSKASIFITGESGTGKEVCAEAIHAASKR 167
V.c             DVDNK-NQNYQGFIGSSQTMQAVYRTIDSAASSKASIFITGESGTGKEVCAEAIHAASKR 179
V.h             EADNPGNQNYQGFIGSSQTMQQVYRTIDSAASSKASIFITGESGTGKEVCAEAIHAASKR 180
                :.**  *************** ************************************

V.c.91          GDKPFIAINCAAIPKDLIESELFGHVKGAFTGAATERQGAAEAADGGTLFLDELCEMDLD 227
V.c             GDKPFIAINCAAIPKDLIESELFGHVKGAFTGAATERQGAAEAADGGTLFLDELCEMDLD 239
V.h             GDKPFIAINCAAIPKDLIESELFGHVKGAFTGAANDRQGAAELADGGTLFLDELCEMDLD 240
                **********************************.:****** *****************

V.c.91          LQTKLLRFIQTGTFQKVGSSKMKSVDVRFVCATNRDPWKEVQEGRFREDLYYRLYVIPLH 287
V.c             LQTKLLRFIQTGTFQKVGSSKMKSVDVRFVCATNRDPWKEVQEGRFREDLYYRLYVIPLH 299
V.h             LQTKLLRFIQTGTFQKVGSSKMKSVDVRFVCATNRDPWKEVQEGRFREDLYYRLYVIPLH 300
                ************************************************************

V.c.91          LPPLRARGDDVIEIAYSLLGFMSKEEGKDFVRLSAEVVERFRQYEWPGNVRQLQNVLRNV 347
V.c             LPPLRARGDDVIEIAYSLLGFMSKEEGKDFVRLSAEVVERFRQYEWPGNVRQLQNVLRNV 359
V.h             LPPLRERGKDVIEIAYSLLGYMSHEEGKSFVRFAQDVIERFNSYEWPGNVRQLQNVLRNI 360
                ***** **.***********:**:****.***:: :*:***..****************:

V.c.91          VVLNEGREITLDMLPPPLNQMSAPINRALPLAHENKVSVHEIFPLWMTEKQAIEQAIEAC 407
V.c             VVLNEGREITLDMLPPPLNQMSAPINRALPLAHENKVSVHEIFPLWMTEKQAIEQAIEAC 419
V.h             VVLNNGKEITLDMLPPPLNQPVVRQSVAK-FIEPDIMTVSDIMPLWMTEKMAIEQAIQAC 419
                ****:*:************  .  . *  : . : ::* :*:******* ******:**

V.c.91          DGNIPRAATYLDVSPSTIYRKLQTWNEKVKEKEKER 443
V.c             DGNIPRAATYLDVSPSTIYRKLQTWNEKVKEKEKER 455
V.h             EGNIPRAAGYLDVSPSTIYRKLQAWNSKD-EKQNV- 453
                :******* **************:**.*  **::
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Fig 2. The multiple alignment of deduced amino acid sequence of LuxO protein from *V. cholerae* O110 PL91 (V.c.91),*V. cholerae* O1 N16961 LuxO (V.c.) and *V. harveyi* LuxO (V.h.) by using Clustal W (Thompson et al., 1994). The residues D-47, F-94 are indicated by open (o) and closed (•) circle. L104 is indicated by (*) asterisks, D4 is indicated by <, and glycine rich region is underlined.