Functional independence of a variant LuxO\textsubscript{PL91} from a non-O1 non-O139 \textit{Vibrio cholerae} over the activity of CsrA and Fis

Quorum sensing, a cell-to-cell communication process, has been found to regulate a plethora of cellular events in \textit{Vibrio cholerae}, including pathogenesis (Zhu \textit{et al.}, 2002). Recent work has established the complex architecture of the quorum sensing network of this bacterium, where multiple circuits work in parallel in response to population density. Genetic analysis reveals that sensory information from two parallel quorum sensing circuits (AI-2/LuxPQ and CAI-1/CqsS) converges into a central molecule, LuxO, through a shared phosphorelay protein, LuxU (Miller \textit{et al.}, 2002). At low cell density, LuxU activates LuxO through phosphorylation. This phosphorylated LuxO in concert with \(\sigma^{34}\) dictates the expression of cascades of small RNAs, which further cause the destabilization of \textit{hapR} mRNA transcript. HapR is a positive regulator of \textit{hapA}, which encodes haemagglutinin (HA)/protease. At high cell density, dephosphorylation of LuxO impairs its ability to activate the expression of small RNAs, which in turn promotes HapR expression and causes protease production (Lenz \textit{et al.}, 2004). In other words, the appearance of HA/protease, one of the developmentally regulated virulence factors, in the culture supernatant indicates the functional impairment of LuxO at high cell density.

In a recent effort, Bassler and co-workers have identified two additional systems of the quorum sensing network of \textit{V. cholerae} (Lenz \textit{et al.}, 2005; Lenz & Bassler, 2007). One such system is known as VarS/A-CsrA/BCD, which is constituted of a sensor kinase (VarS), a response regulator (VarA), an RNA-binding global regulatory protein (CsrA) and three small RNAs (CsrBCD). These small RNAs bind and antagonize the activity of CsrA. The functioning of the VarS/A two-component system depends on the growth phase. At low cell density, the two-component sensor kinase VarS remains inactive and does not phosphorylate the response regulator VarA. Unphosphorylated VarA is unable to turn on the expression of genes encoding CsrBCD small RNAs. As a consequence, CsrA is free to act on its cellular targets. In the quorum sensing network of \textit{V. cholerae}, CsrA delivers sensory information to LuxO without involving LuxU (Lenz \textit{et al.}, 2005). It has been proposed that CsrA maintains the active form of LuxO by a hitherto unknown mechanism. At high cell density, VarS becomes active and phosphorylates VarA. Phosphorylated VarA stimulates the expression of the genes that encode CsrBCD small RNAs. Subsequently, these small RNAs interfere with the activity of CsrA by titrating it away from its targets. As a result, CsrA can no longer promote the functioning of LuxO, which favours HapR production and HapR-mediated gene expression.

The key element of the fourth system is Fis, a small nucleoid protein. Recent studies have evidenced its role as a global regulator of exponential and stationary phase gene expression in many Gram-negative bacteria ( Ishihama 1999; Kelly \textit{et al.}, 2004). In \textit{Escherichia coli}, Fis was found to modulate transcription of genes encoding stable RNAs. Fis controls gene expression by altering the conformation of DNA through bending, as well as making contact with the \(\sigma^{34}\) subunit of RNA polymerase (Dorman & Deighan, 2003). Upon binding to a degenerate consensus sequence, it introduces a bend of between 40° and 90° (Hengen \textit{et al.}, 1997). Recently, Fis has been shown to control the expression of virulence genes in pathogenic strains of \textit{E. coli}, \textit{Shigella flexneri} and \textit{Salmonella typhimurium}. In addition to virulence factors, Fis also coordinates the expression of a number of housekeeping genes that are involved in metabolism, transport and flagellar biosynthesis in \textit{Salmonella typhimurium} (Kelly \textit{et al.}, 2004).

Importantly, a large member of the Fis regulon is required for survival in the gut, which further demonstrates the contribution of Fis in the host–pathogen interaction of \textit{Salmonella typhimurium}. The expression pattern of Fis is contingent on the growth phase: the level of this regulatory protein reaches a peak in the exponential phase and becomes undetectable during stationary phase under aerated condition (Walker \textit{et al.}, 2004). It is interesting to note that expression of Fis under non-aerated conditions continues in the stationary phase in the case of some \textit{E. coli} strains and \textit{Salmonella typhimurium} (O’Cro´inin & Dorman, 2007). It has also been demonstrated that alternate stress response sigma factor RpoS represses Fis expression and a reduced level of RpoS facilitates Fis expression under non-aerated condition (O’Cro´inin & Dorman, 2007). This observation is further expounded by a natural rpoS mutant of \textit{Salmonella typhimurium} strain LT2, where the level of Fis is significantly higher under non-aerated conditions than that observed in strain SL1344. In the case of \textit{V. cholerae}, expression of \textit{fis} is also regulated by growth phase, as described in other bacteria (Lenz & Bassler, 2007).

In the quorum sensing system of \textit{V. cholerae}, Fis works in parallel with LuxO to stimulate the expression of \textit{qrr}–4 small RNAs, thus controlling the production of HapR in a growth-dependent manner (Lenz & Bassler, 2007). Interestingly, Fis exhibits a gradation of affinity to the promoter regions of \textit{qrr} small RNAs and promotes maximum production of \textit{qrr} small RNA over others. In essence, Fis is a critical component of the quorum sensing network of \textit{V. cholerae} and maximal transcription of genes encoding \textit{qrr} small RNAs requires the involvement of LuxO-phosphate, \(\sigma^{34}\) and Fis. At present, the quorum sensing network of \textit{V. cholerae} reveals an intricate circuit that involves an interplay of two autoinducer systems, one growth-phase regulated VarS/A–CsrA/BCD unit and a small nucleoid protein Fis to control population-dependent gene...
expression. It is also interesting to note that neither of the two global regulators CsrA and Fis control the expression of LuxO (Lenz et al., 2005; Lenz & Bassler, 2007).

Being a central molecule of quorum sensing circuit, a great deal of work has been dedicated to understanding the function of LuxO. Sequence analysis indicates a conserved aspartate residue (Asp-47) that is critical for its signalling function (Freeman & Bassler, 1999). Signal transduction is proposed to occur via a series of phosphorylation and dephosphorylation reactions. Inactivation of LuxO function occurs by dephosphorylation at Asp-47. Thus far, a number of variant LuxO molecules have been characterized from various strains of V. cholerae. One category of LuxO molecules harbour 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 mutations 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 Vibrio harveyi. In contrast, a mutant protein mimicking phospho-LuxO (D47E, F94W) resulted in a constitutively active form, which repressed luminescence in V. harveyi. Because of the constitutive nature of its activity, the mutant form D47E is referred to as constitutively active LuxO (Con-LuxO) (Freeman & Bassler, 1999). Similarly, a LuxOL104Q variant that was isolated from V. cholerae El Tor strain E7946 also acts constitutively, and thus represses protease production and enhances biofilm formation by this strain (Vance et al., 2003). It is noteworthy to mention that these variant LuxO molecules are not functionally equivalent. For example, the repressor phenotype of LuxO D47E is more pronounced than that of LuxO F94W. Similarly, AKR LuxO protein has a stronger repressor phenotype than does the LuxO D47E protein (Freeman & Bassler, 1999).

Previously, we identified a variant LuxO molecule from a non-O1, non-O139 V. cholerae PL91 (henceforth designated LuxOPL91) whose function remains unaffected in the absence of LuxU, thus conferring the protease-negative phenotype exhibited by this strain (Raychaudhuri et al., 2006). In this work, we wanted to evaluate whether LuxO functioning at high cell density in PL91 is due to a gain of function mutation or whether there exists a continuous input from CsrA and Fis to quorum sensing system in PL91, which in turn maintain the activity of LuxOPL91 at high cell density and thus render this strain protease negative.

In pursuit of our interest, csrA and fis single mutants of V. cholerae strain PL91 were generated by disrupting the chromosomal copy with suicidal constructs pSM and pSRF, respectively. For the construction of plasmid pSM, a 147 bp internal fragment of the csrA gene was amplified with oligonucleotides designated CsrA XbaI and CsrA EcoRI (Table 1). The fragment was cloned into similarly digested suicide vector pGP704 at XbaI and EcoRI and verified by sequencing. pSM was maintained in E. coli SM10 :: pir as a host. In a similar fashion to the pSM construction, a 174 bp internal fragment of fis gene was amplified with oligonucleotides Fis XbaI and Fis EcoRI (Table 1) and cloned into similarly digested suicide vector pGP704. The resulting recombinant plasmid was designated pSRF and maintained in E. coli SM10 :: pir as a host. Subsequently, pSM and pSRF were transferred in V. cholerae PL91 by conjugation. csrA and fis mutants of PL91 were screened by streaking onto Luria–Bertani agar plates containing ampicillin (100 µg ml⁻¹) and streptomycin (10 µg ml⁻¹). The csrA and fis mutants of PL91 were designated PL91-SM and PL91-SRF, respectively (Table 1). To further examine the integration of pSM, a PCR was carried out using a long PCR kit (Fermentas) following the manufacturer’s protocol with the primer pair 100 bp UP CsrA and 100 bp DN CsrA (Table 2). An expected 4.2 kb band was obtained from the disrupted strain, which was purified and sequenced using an ABI PRISM dye terminator cycle sequencing kit (Perkin Elmer). Further analysis confirmed the disruption of csrA, which occurred at amino acid position 59 (CsrA is constituted of 65 aa) in PL91-SM. A similar approach was used to confirm the integration of pSRF. Towards this end, the genomic DNA of PL91-SRF was amplified by using Phusion polymerase (Finnzyme) with the primer pair Smal FIS and PstI FIS (Table 2). An expected 4.2 kb band was obtained and sequenced using an ABI PRISM dye terminator cycle sequencing kit. Further analysis confirmed the disruption of fis, which occurred at amino acid position 72 (Fis is constituted of 98 aa) in PL91-SRF. As protease production in the stationary growth phase is one of the parameters used to evaluate the functional perturbation of wild-type LuxO, we wanted to evaluate the performance of LuxO in PL91-SM (PL91 ΔcsrA) and PL91-SRF (PL91 Δfis) by measuring the protease activity in culture supernatants of PL91-SM and PL91-SRF, along with PL91-C (PL91 carrying vector pKK177-3R1) and PL91-SVM (PL91ΔluxO). All these strains were grown in tryptic soy broth without dextrose (glucose) (TSB-D) containing appropriate antibiotics, with agitation, at 37 °C to stationary phase. The cell-free supernatants harvested from stationary phase cultures were tested for protease production using an azocasein assay as described elsewhere (Benitez et al., 2001; Raychaudhuri et al., 2006). We found that PL91-C, PL91-SM and PL91-SRF remain protease negative in comparison to PL91-SVM (Fig. 1a). These data clearly indicate that the absence of CsrA and Fis could not incapacitate the performance of LuxOPL91, which suppresses protease production in the corresponding mutants of PL91 at high cell density. In essence, our data clearly suggested that LuxOPL91 is a gain of function variant that is capable of maintaining its activity at high cell density in csrA and fis mutants of PL91, thus conferring a protease-negative phenotype in wild-type PL91 and isogenic csrA and fis mutants of PL91. Next, we wanted to examine whether the function of LuxOPL91 is a strain-dependent phenomenon or not. To resolve this issue, overexpression constructs of luxOPL91 and luxO N16961 were used to transform into non-O1, non-O139 protease-positive strain of V. cholerae SC134 (a kind gift of Dr Ranjan K. Nandy, National Institute of Cholera and Enteric Diseases, Beliaghata, Kolkata, India). The only difference between LuxOPL91 and LuxO N16961 is the absence of 12 aa in
LuxO PL91 as described previously (Raychaudhuri et al., 2006). The recombinant strains SC134-PL (carrying luxO PL91 overexpression plasmid pPL) and SC134-NL (carrying luxO N16961 overexpression plasmid pNL) were tested for protease production at high cell density under aerated conditions. Our data showed that SC134-NL remains protease positive, whereas SC134-PL becomes protease negative, which further suggested that the function of LuxO PL91 is not restricted to PL91, and can maintain its constitutive activity in strains other than PL91 (Fig. 1b). Taken together, our data confirmed that LuxO PL91 is gain of function variant, and the absence of CsrA and Fis does not perturb its activity. It can also exert its constitutive activity in strains other than PL91.

Thus far, it appears that the quorum sensing system of V. cholerae requires the involvement of two global regulators, CsrA and Fis. The exact mechanism by which CsrA promotes LuxO function is not known. It has been proposed to mediate its action through an unidentified factor that in turn increases the activity of phospho-LuxO (Lenz et al., 2005). It has been demonstrated that Fis binds to the qrr promoters. It is believed that the binding

### Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Source/reference</th>
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<tbody>
<tr>
<td><strong>V. cholerae strains</strong></td>
<td></td>
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</tr>
<tr>
<td>PL91</td>
<td>Non-O1, non-O139, serogroup O110, Sm' (10 μg ml⁻¹)</td>
<td>Thungapathra et al. (2002)</td>
</tr>
<tr>
<td>PL91-C</td>
<td>Non-O1, non-O139, serogroup O110, carrying pKK177-3R1, Sm' (10 μg ml⁻¹), Ap' (100 μg ml⁻¹)</td>
<td>Raychaudhuri et al. (2006)</td>
</tr>
<tr>
<td>PL91-SVM</td>
<td>Non-O1, non-O139, serogroup O110, Sm' (10 μg ml⁻¹), luxO::pSVM, Ap' (100 μg ml⁻¹)</td>
<td>Raychaudhuri et al. (2006)</td>
</tr>
<tr>
<td>PL91-SM</td>
<td>Non-O1, non-O139, serogroup O110, Sm' (10 μg ml⁻¹), csrA::pSM, Ap' (100 μg ml⁻¹)</td>
<td>This study</td>
</tr>
<tr>
<td>PL91-SRF</td>
<td>Non-O1, non-O139, serogroup O110, Sm' (10 μg ml⁻¹), fis:: pSRF, Ap' (100 μg ml⁻¹)</td>
<td>This study</td>
</tr>
<tr>
<td>SC134</td>
<td>Non-O1, non-O139, serogroup O125</td>
<td>Ranjan K. Nandy, National Institute of Cholera and Enteric Diseases, India</td>
</tr>
<tr>
<td>SC134-C</td>
<td>Non-O1, non-O139, serogroup O125, carrying pKK177-3R1, Ap' (100 μg ml⁻¹)</td>
<td>This study</td>
</tr>
<tr>
<td>SC134-PL</td>
<td>Non-O1, non-O139, serogroup O125, carrying pPL, Ap' (100 μg ml⁻¹)</td>
<td>This study</td>
</tr>
<tr>
<td>SC134-NL</td>
<td>Non-O1, non-O139, serogroup O125, harbouring pNL, Ap' (100 μg ml⁻¹)</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
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<tr>
<td>SM10 : apr</td>
<td>thi thr leu tonA lacY supE recA:: RP4-2-Tc:: Mu, λ-pir R6K</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pKK177-3R1</td>
<td>Ap'</td>
<td>Gisela Storz, National Institute of Health, Bethesda, MD, USA</td>
</tr>
<tr>
<td>pSM</td>
<td>147 bp EcoRI-Xbal fragment from PCR amplification of genomic DNA with Xbal-CsrA and EcoRI-CsrA, cloned into pGP704 digested with same restriction enzymes.</td>
<td>This study</td>
</tr>
<tr>
<td>pSRF</td>
<td>174 bp EcoRI-Xbal fragment from PCR amplification of genomic DNA with Xbal-Fis and EcoRI-Fis, cloned into pGP704 digested with same restriction enzymes</td>
<td>This study</td>
</tr>
<tr>
<td>pPL</td>
<td>1.332 kb luxO (ORF) of PL91 was amplified with Smal-luxO and HindIII-luxO and cloned into pKK177-3RI</td>
<td>This study</td>
</tr>
<tr>
<td>pNL</td>
<td>1.368 kb luxO (ORF) of N16961 was amplified with Smal-luxO and HindIII-luxO and cloned into pKK177-3RI</td>
<td>This study</td>
</tr>
</tbody>
</table>

### Table 2. Oligonucleotides

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>100 bp UP CsrA</td>
<td>5'-TTAGGTGTAAAACGCGGACCCATTTGAG-3'</td>
</tr>
<tr>
<td>100 bp DN CsrA</td>
<td>5'-GCAAATTAACCAAAGCGCTGAG-3'</td>
</tr>
<tr>
<td>Xbal CsrA</td>
<td>5'-CTAGTCTAGGTTGCGAAACCCCTGTGAG-3'</td>
</tr>
<tr>
<td>EcoRI CsrA</td>
<td>5'-CCGGATCTACCTACCTTCTACGTTGAG-3'</td>
</tr>
<tr>
<td>Xbal FIS</td>
<td>5'-CTAGTCTAGAACCCTAATCTGCAAGAC-3'</td>
</tr>
<tr>
<td>EcoRI FIS</td>
<td>5'-ATAGAATTCACGCCGAGTATGATGCTGAG-3'</td>
</tr>
<tr>
<td>Smal FIS</td>
<td>5'-TCCCCGGGATGTTGCAAGAATAATGCTGAG-3'</td>
</tr>
<tr>
<td>PstI FIS</td>
<td>5'-AAAACTGCAATGGTATGCGTTCATTCGTTG-3'</td>
</tr>
<tr>
<td>LuxO Smal</td>
<td>5'-TCCCCGGGATGTTGATAGAAGACAC-3'</td>
</tr>
<tr>
<td>LuxO HindIII</td>
<td>5'-CCCAAGCTTACCCGCTTCCTTTCAG-3'</td>
</tr>
</tbody>
</table>
of Fis introduces a bend between phospho-LuxO and the σ^{34}/RNA polymerase binding site that facilitates σ^{34}-dependent activation of qrT sequences (Lenz & Bassler, 2007). As suppression of protease production continues even at high cell density under aerated condition in PL91-C, PL91-SRF and PL91-SM, it is attractive to hypothesize that the binding of LuxOPL91 in the absence of Fis to qrT promoter sequences is sufficient enough to foster σ^{34}-dependent activation of qrT expression, thus repressing production of HA/protease in wild-type as well as csrA and fis mutants of PL91. This warrants further investigation.

As optimum functioning of LuxO depends on the integrated activity of multiple factors, we wanted to explore the effect of such factors on the performance of LuxOPL91. Among various LuxO variants, LuxO D47E has been shown to exert control over density-dependent gene expression at high cell density in csrA and fis mutants, thus locking the mutants in a low cell density phenotype (Lenz et al., 2005; Lenz & Bassler, 2007). In other words, the LuxO D47E variant acquires a functional independency over the activity of CarA and Fis, which prompted us to examine the performance of LuxOPL91 in csrA and fis mutants of PL91. Previously, we showed that the function of LuxOPL91 does not depend on LuxU (Raychaudhuri et al., 2006). In the current study, we provide evidence of its functional independency over the activity of CarA and Fis. In essence, we have identified another gain of function variant of LuxO that bears a functional resemblance to LuxOD47E despite harbouring a different pattern of mutation. There remains a possibility of an alteration in the binding of various gain of function variants of LuxO to the promoter regions of the genes encoding qrT small RNAs, thus affecting the transcription of these small RNAs in the respective wild-type as well as csrA and fis mutants of V. cholerae strains. In this regard, LuxOPL91, in addition to other LuxO derivatives, might serve as a molecular tool for understanding the structural basis of LuxO function as a transcriptional activator. Additional studies are necessary to address this issue.

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