Mitesh Dongre(2009). ***Studies on the mechanism of protease negative phenotype of vibrio cholerae non-01,non-0139 strain.*** Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.
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SUMMARY OF THE THESIS

Vibrio cholerae, a formidable human pathogen and principal vector for cholera, has quailed millions of people worldwide (Kaper *et al.*, 1995). *V. cholerae* was first cultured by Robert Koch, about 125 years ago in Kolkata (then Calcutta), India, and has since been the subject matter of several investigations directed towards understanding the biology of this pathogen. Most of these studies were carried out, rather rationally, in toxigenic strains of *V. cholerae* belonging to O1 and O139 serogroups. These studies revealed the presence of an intricate signal transduction system involving several overlapping regulatory circuits, which work in coalesce to bring about environmental survival, pathogenesis and evolution of this bacterium. In recent decades, many dedicated approaches delving into the exact nature of this signalling phenomenon, established the crucial role of cell density sensing or quorum sensing system which regulates several important cellular events in *V. cholerae* (Miller *et al.*, 2002).

Quorum sensing is a complex cell-to-cell communication process which coordinates differential gene expression in response to population density. For adaptation to different environmental conditions, *V. cholerae* has evolved multiple quorum sensing systems (Miller *et al.*, 2002; Lenz *et al.*, 2005; Lenz & Bassler, 2007), operating in a concerted fashion to administer the activity of LuxO, a critical regulator of quorum sensing cascade. So far, four different quorum sensing circuits have been identified in *V. cholerae*. The first two circuits are constituted by unique autoinducer response regulator pairs CAI 1/CqsS and AI2/LuxPQ, respectively, which integrate a shared histidine phosphotransfer protein, LuxU, to control the phosphorylation of LuxO and thus, its activity. Key element of the third system is a sensor kinase response regulator pair known as VarS/VarA phosphorelay system which induces expression of three redundant small RNAs known as CsrBCD sRNAs. These sRNAs govern expression of CsrA, the effector of third circuit which in turn inputs signalling information to LuxO independent of LuxU, however, through a hitherto unknown mechanism.

At low cell density these three quorum sensing systems of *V. cholerae* undergo a series of phosphorylation events culminating in the activation of LuxO (phospho-LuxO). Activated LuxO along with the sigma factor $\sigma 54$ activates expression of 4 sRNAs known as Qrr sRNAs. These sRNAs antagonizes the expression of master

regulator HapR, the downstream effector of quorum sensing. Recent advances in the understanding of *V. cholerae* quorum sensing system unveiled the fourth feeder of sensory information as Fis, a small nucleoid protein whose role as a global regulator of exponential and stationary phase genes in many Gram-negative bacteria is well established (Ishihama, 1999). In *V. cholerae* Fis work in parallel with LuxO at low cell density to stimulate the expression of *Qrr* 1-4 sRNAs, thus control the production of HapR in a growth dependent manner (Lenz & Bassler, 2007).

In contrast, at high cell density the scenario reverses when reciprocal flow of phosphate renders LuxO inactive. The *hapR* expression is thus derepressed leading to high cellular accumulation of HapR protein. In *V. cholerae*, HapR negatively regulates the expression of virulence cascade genes (Kovacikova & Skorupski, 2002) and other critical cellular processes. In essence, depending upon cell density, quorum sensing co-ordinately controls genes and operons controlling disparate cellular events such as, virulence, biofilm formation, natural competence and hemagglutinin protease production (Jobling & Holmes, 1997; Kovacikova & Skorupski, 2002; Hammer & Bassler, 2003; Lenz *et al.*, 2004; Meibom *et al.*, 2005).

V. cholerae is a natural inhabitant of aquatic environment including rivers, estuaries and ocean coasts, where it is predominantly found attached to submerged surfaces in the form of biofilms. Interestingly, the environmental population of *V. cholerae* is overtly dominated by strains belonging to serogroups other than O1 and O139, collectively known as non-O1, non-O139 strains (Colwell & Huq, 1994). These non-O1, non-O139 strains has been found to show greater genetic variability than their toxigenic O1 and O139 counterparts (Dziejman *et al.*, 2005). For historic reasons, non-O1, non-O139 strains are considered of negligible epidemiological relevance since they have only been associated with mild gastrointestinal illness and/or extraintestinal infections (Mukhopadhyay *et al.*, 1995; Ou *et al.*, 2003). However, recent years have seen an episode of upsurge in the incidence and abundance of drug resistant non-O1, non-O139 *V. cholerae* (Thungapathra *et al.*, 2002). Majority of these strains lacks genetic system responsible for production of cholera toxin and other known virulence factors; however, they can cause diarrhoea, clinically indistinguishable from that associated with cholera. Comparative genome sequence analysis of some non-O1, non-O139 strains revealed that these strains carry chromosomal islands that encodes a

putative exotoxins and type III secretory system (T3SS), predicted to be involved in virulence (Dziejman *et al.*, 2002; Chen *et al.*, 2007). Recent studies also identified a novel secretion system know as type 6 (T6SS) secretory system in a non-O1, non-O139 strain. The T6SS is shown to be responsible for translocating proteins directly into the eukaryotic host (Pukatzki *et al.*, 2006; Pukatzki *et al.*, 2007). Moreover, owing to the relatively higher rates of horizontal gene transfer events in *V. cholerae* (Faruque *et al.*, 2004*)* it seems likely that these T3SS and T6SS systems will be integrated with other virulence mechanisms and result in the emergence of new pathogenic clones of *V. cholerae*.

One aspect of cholera research deals with vigilant monitoring of the emergence of new genetic variants among the existing serogroups, as well as emergence of altogether new serogroups that can replace these old ones. This surveillance for new strains of *V. cholerae* seems to be a prudent undertaking which will likely help in the beforehand preparedness to counter the evolutionary adaptations of *V. cholerae* that may change the pattern of cholera epidemiology in future. Keeping this credo, we initiated a detailed analysis of several clinical isolates of non-O1, non-O139 *V. cholerae*. Since quorum sensing plays a crucial role in the environmental survival and pathogenesis of these bacteria, a major thrust of our research was streamed to investigate extent of the cell density sensing system in non-O1, non-O139 strains.

We proceeded with characterizing twelve randomly selected non-O1, non-O139 *V. cholerae* strains, for the integrity of their quorum sensing network. Since, most notable quorum sensing signatures includes secretion of HAP and regulated production of biofilms, selected non-O1, non-O139 strains were simultaneously analysed for these two phenotypes. Preliminary experimentation suggested existence of normal population density sensing mechanism in 8 out of twelve selected strains. On the other hand, four strains, namely PG95, PL91, SC117 and V2, turn out to be protease negative. Genetic analysis revealed that protease negative phenotype of PG95 and SC117 can be attributed to the absence of *hapA* (HAP structural gene). The remaining two strains namely PL91 (O110) and V2 (O37), which despite carrying functional *hapA* did not show any protease activity even at high cell density, were selected as putative quorum sensing mutants. Moreover, these strains formed robust biofilms with thick pellicle, further suggesting the abnormality of quorum sensing system in these strains.

Earlier it has been reported that mutants carrying constitutively active LuxO or non- functional HapR, exert similar phenotypes (Zhu *et al.*, 2002; Hammer & Bassler, 2003; Vance *et al.*, 2003). To investigate a similar possibility, we performed epistasis analysis of *hapR* and *luxO* genes in strains PL91 and V2. In the process we identified a variant LuxO molecule in PL91 (designated as $LuxO_{PL91}$) whose activity remained unperturbed by the LuxU (system 1 & 2) mediated phosphorylation-dephosphorylation events, thus conferring a protease negative phenotype as exhibited by this strain. Further, the constitutive activity of $LuxO_{PL91}$ turns out to be independent of CsrA (system 3) and Fis (system 4). Sequencing analysis of the *luxO*$_{PL91}$ revealed the deletion of a 12 amino acid stretch, precisely, I^{80}DTAVEAMRHGA^{91}. Through a series of mutagenesis and subsequent epistasis analysis, we identified that the constitutive activity of $LuxO_{PL91}$ is indeed contributed by the absence of two residues in the stretch namely, H89 and G90. We surmised that absence of these two residues might confer a conformational change in the three-dimensional structure of LuxO which mimics the active state of LuxO (phospho-LuxO) and thus results in its constitutiveness. Further, we observed that this constitutive activity of $LuxO_{PL91}$ is not strain specific, rather, can be generalized to other strains of *V. cholerae* as well as to other closely related *Vibrio* species like *V. mimicus*.

In a simultaneous effort to understand the protease negative phenotype of another short-listed non-O1, non-O139 strain V2 (O37), we applied a similar strategy to that used for PL91. In the pursuit, we identified a mutant HapR in V2, harboring a novel mutation (G39D) in the N-terminal DNA binding domain, which renders this quorum sensing master regulator catalytically dead. Restoration of glycine at the position 39 revived the normalcy this non functional HapR variant, thus restituted protease production and smooth morphotype in this strain. Interestingly, the Gly39 is situated in a glycine rich hinge region (G^{34}IGRGG^{39}) that links two putative DNA binding motifs $\alpha 1$ and $\alpha 2$ in the N-terminal domain of HapR. This stack of glycine residues (G^{34}IGRGG^{39}) in the hinge region of HapR has also been identified in its close

homologues from other *Vibrio* spp. Thus, in an effort to evaluate the role of the N-terminal glycine residues in functioning of HapR, we generated alanine and aspartate congeners of glycines, at the respective positions. Functional analysis of the resulting mutants showed a wide spectrum of protease and rugose phenotypes as well as differential DNA binding properties. Essentially, our results underscore the significance of glycine residues in the hinge region for maintaining the proper function of HapR as a quorum sensing master regulator.

Collectively, this study identified two novel quorum sensing mutants of *V. cholerae* non-O1, non-O139 serogroups, harboring unique variants of critical quorum sensing regulators, LuxO and HapR. Holding the epidemiological significance of the LuxO and HapR, it is interesting to observe how these mutations are going to attribute in pathogenesis of *V. cholerae*. Moreover, a surmised survival advantage, in the form of enhanced biofilm production by the strains carrying these mutations, would make them a suitable tool to maintain the infectious foci during and in between epidemics; however, this apriorism warrants further investigation. Additionally, these natural quorum sensing mutants would provide a tool for exploring and screening small molecule inhibitors of density sensing mechanism and biofilm formation, in *V. cholerae*.