Studies on the Biofilm Formation by V. Cholerae non-01, non-0139 Strains.

The dreaded disease 'cholera' that afflicts millions every year all over the world, is caused by *Vibrio cholerae* strains belonging to O1 and O139 serogroups (Kaper *et al.*, 1995). Strains belonging to serogroup other than O1 and O139 collectively known as non-O1, non-O139 and were considered to cause localized outbreaks and extra-intestinal infections in man. However, in recent past, an unusual upsurge in the isolation of the non-O1, non-O139 strains as compared to O1 and O139 strains were reported (Sharma *et al.*, 1998). In fact, the relative preponderance of *V. cholerae* non-O1, non-O139 strains as compared to epidemic causing counterparts of O1 and O139 strains from diarrhoeal patients admitted to Infectious Diseases Hospital, Kolkata ranged between 33 to 36% in the last three years. (Unpublished data by RK Nandy *et al.*).

The genome of *V. cholerae* is consisted of two chromosomes and complete nucleotide sequences of both the chromosomes are available for O1 El Tor strain N16961. Available data suggest that genome of *V. cholerae* is very dynamic in nature in regards to acquisition of potential virulence factors through horizontal transfer and to generate of new clones (Heidelberg *et al.*, 2000). Although much attention was attributed towards the biology and pathogenesis of O1 and O139 strains, very little or no information is available on the nature of pathogenesis of non-O1, non-O139 strains which cause diarrhea clinically indistinguishable from cholera. Interestingly, recent genome sequencing and comparative genome analysis of non-O1, non- O139 strains revealed that these strains harbor genes of putative exotoxins and type III secretory system (Chen *et al.*, 2007; Dziejman *et al.*, 2002). These studies suggested the possibility of distinct pathogenic mechanism by these strains.

V. cholerae is a versatile bacterium that flourishes in diverse environments, including the human intestine, rivers, lakes, estuaries, and the ocean. Studies have shown that non-O1, non-O139 *V. cholerae* are abundantly present in environment (Montilla *et al.*, 1996). These strains shows better survival in adverse conditions and resistance to several drugs, detergents and chelating agents (Montilla *et al.*, 1996; Thungapathra *et al.*, 2002). The appearance of these drug resistant non-O1, non-O139 strain pose a serious problem not only because of the incidences of "cholera like disease" caused by such strains in hospitalized patients are increasing but also they can act as reservoirs for drug resistant genes and emerged as new threat. It is important to understand the mechanism of pathogenicity and environmental survival of these strains.

Quorum sensing plays a crucial role in the pathogenesis and biofilm development of *V. cholerae* strains of O1 and O139 origin (Zhu and Mekalanos, 2003). Biofilm is a dynamic

multicellular structures where microbes are communicating among themselves, thus coordinating their behavior through series of molecular events, involving adhesions, aggregation and signal exchange as well as community expansion (O'Toole *et al.*, 2000). Biofilm development plays a crucial role in the survival and sustenance of the organism in its natural environment. Another hallmark of biofilm-associated cells is their remarkable ability to confer resistance against conventional antibiotics and biocides compared to their planktonic counterparts (Donlan and Costerton, 2002). Ability to form biofilm on the various biotic and abiotic surfaces in different environmental conditions can be the possible reason of innocuous existence of non-O1, non-O139 strains in nature.

In the present study, we analyzed the biofilm development and effect of various conditions on the ability to form biofilm by non-O1, non-O139 *V. cholerae*. Nine strains of eight different serogroups were included in the study. These nine strains were earlier reported to be diverse in their antibiotic resistance pattern and several genetic markers like *toxR, toxT, hlyA, hlyU* and *SXT* elements (Thungapathra *et al.*, 2002). Great variation in biofilm development of these strains was observed in LB medium. Strains PG9, PG92 and PL61 were unable to form biofilm while strain PG95 and PL91 showed the robust biofilm in the form of pellicle. Nature of surface play important role in surface adherence and biofilm formation (Cerca *et al.*, 2005), thus we examined the biofilm development of these strains on acrylic surfaces i.e., polystyrene and polypropylene substratum. It was observed that some of the non-O1, non-O139 *V. cholerae* can form enhanced biofilm on hydrophobic surfaces in comparison to hydrophilic glass surface. The strains which showed defect in forming biofilm on borosilicate glass tubes were also unable to form biofilm on acrylic surfaces. It suggests that possibly the mechanism of biofilm formation and surface adherence is independent of the nature of surface. Enhance biofilm formation by some strains on the hydrophobic surfaces might be due to more initial adherence of bacteria through non-covalent interaction between substrata and bacteria (Cerca *et al.*, 2005).

In recent years, increase numbers of infections by non-O1, non-O139 *V. cholerae* were found either near sea areas or due to ingestion of sea food (Lukinmaa *et al.*, 2006). It is possible that ability of these strains to form biofilm is the reason of their persistence in marine water. We analyzed the biofilm development of these strain in artificial sea water medium to mimic sea conditions and found that all the nine strains were able to form good biofilm in sea water. Interestingly, strains such as PG9, PG92 and PL61 which show defect in biofilm formation in LB medium, form good biofilm in sea water medium. Biofilm

development of *V. cholerae* O139 in artificial sea water is calcium dependent (Kierek and Watnick, 2003a). Role of calcium can be through interaction with negatively charged groups of O-antigen chain of lipopolysaccharides (Kierek and Watnick, 2003a). We checked the effect of calcium ions on biofilm development of these strains in sea water medium. Our data suggests that biofilm development in non-O1, non-O139 *V. cholerae* strains is calcium dependent as well and possibly same pathway of biofilm formation is involved in these strains as observed in *V. cholerae* O139. Presence of smooth type of LPS with O-antigen chain in these strains further supports this hypothesis.

Biofilm development in *V. cholerae* O1, O139 strains take place through two different pathways. In medium rich in monosaccharide like LB or fresh water biofilm formation is dependent upon the *vps* (*Vibrio* Polysaccharide Synthesis) genes mediated pathway. These genes regulate the secretion of exopolysaccharide matrix of biofilm. In sea water medium, biofilm formation is *vps*-independent and Calcium dependent (Kierek and Watnick, 2003a, b). It might be speculated that the strains which show defect in biofilm formation in LB are having problem in *vps*- dependent pathway. It is also possible that these strains may have problem in their initial adherence with the substratum in particular conditions. Further studies are needed to find out the possible reason of such behavior of these strains.

It was found that in EZ RDM medium, iron also play crucial role in biofilm development of these non-O1, non-O139 strains. Mode of action of iron in this development is yet not clear. Iron was found to be important for rugose switching and biofilm formation in O1 *V. cholerae* (Mey *et al.*, 2005). It is possible that similar pathway of iron dependent biofilm development is present in these strains also.

To determine the architecture of biofilm and spatial distribution on surface, confocal microscopy was done with strain PL91. We also observed that a strain which is defective in forming biofilm in particular condition can be trapped in the biofilm matrix of another strain. It is possible that two strains with different phenotypic behavior can help each other in environmental survival sharing same ecological niche. Studies revealed that rugose strains of *V. cholerae* acquire high resistance to environmental stress due to high EPS secretion and biofilm formation.(Wai *et al.*, 1998; Wai *et al.*, 1999).

Quorum sensing is found to play important role in life cycle of *V. cholerae*. Presence of quorum sensing pathways was observed in several *Vibrio* species (Milton, 2006). Quorum sensing regulates biofilm formation and hemagglutination protease (Hap) secretion in *V. cholerae*. Hap protease secretion of these nine strains was analyzed using azocasein substrate

assay and hemagglutination assay. Great heterogeneity was observed in the ability of strains to secrete Hap, some strains secreted high protease, while three strains were found to be defective in Hap secretion. Earlier, dysfunction and defective quorum sensing circuits were observed in several clinical and environmental isolates of non-O1, non-O139 as well as O1, O139 *V. cholerae* (Joelsson *et al.*, 2006). There are several possible explanations for defect in Hap protease secretion by some strains like (i) defect in quorum sensing genes (ii) mutation in gene which encodes Hap (iii) problem in protease secretory machinery of the strains.

On the basis of these studies it can be speculated that possibly biofilm formation and quorum sensing pathways of non-O1 and non-O139 are identical with O1, O139 *V. cholerae*. The reason for variation in phenotypes of these strains might be because of mutation in genes involved in these pathways due to environmental selective pressures. To further confirm, whether genes required for quorum sensing and biofilm development of O1, O139 are present or not in these strains, we performed the molecular analysis of five important genes in all nine strains (Chapter 4). It was found that *luxO, luxU* and *csrA* were present in eight strains out of nine. These genes are involved in quorum sensing circuits of *V. cholerae*. In addition to these two important genes related to exopolysaccharide secretion and biofilm formation i.e., *vpsR* and *vpsL* were also present in eight strains.

In strain PG61 out of these five genes only *csrA* was present. Sequence analysis of 16S rRNA of this strain suggested that it belongs to *V. fluvialis* species not with *V. cholerae*. This strain was previously categorized as *V. cholerae* might be due to identical biochemical characteristics with *V. cholerae*. It is the first report where *csrA* gene is identified in *V. fluvialis*. PL61 showed good biofilm in defined sea water. Biofilm development in this strain was found to be calcium dependent. *V. fluvialis* cause 'chlolera" like disease and show prolonged survival in seawater under starvation (Allton *et al.*, 2006; Amel *et al.*, 2006). It can be speculated that ability to form biofilm can be the reason of its prolonged survival in coastal water. Further studies need to be done to find out the pathways involved in the biofilm development of this bacterium.

Two strains PG95 and PL91 showed Hap negative phenotype and developed the robust biofilm in the form of pellicle. Earlier it has been reported that mutants carrying constitutively active LuxO or non-functional HapR show similar phenotypes (Hammer and Bassler, 2003; Vance *et al.*, 2003; Zhu *et al.*, 2002). Disruption of *luxO* gene does not affect the biofilm forming ability of strain PG95 and maintain protease negative phenotype

(Unpublished data by Raychaudhuri *et al.*,). Further studies are necessary to reveal the reason behind such phenotypic behavior of this bacterium.

Overexpression of *hapR* gene of PL 91 in trans restores the protease secretion in both PL91 and *V. cholerae* O1 El tor strain N16961. N16961 carry a natural frame shift or point mutation in *hapR*, thus defective in protease secretion. This data in combination with sequencing data suggested that there is no functional mutation in *hapR* gene. These results also ruled out the possibility of any problem in secretory pathway of protease secretion.

DGGE profiling and sequence analysis of *luxO* gene of this strain revealed that a stretch of 12 amino acid is missing in the LuxO of this strain. Disruption of *luxO* gene restores the protease secretion and negatively affects the biofilm development. This data suggest that the phenotypes observed in this strain are due to defective quorum sensing pathways. There was no affect of *luxU* or *CsrA* disruption on the function of LuxO, suggesting that functioning of $LuxO_{PL91}$ is independent of sensory signal from both LuxU and CsrA mediated quorum sensing circuits. It can be speculated that $LuxO_{PL91}$ is structurally mimicking the phosphorylated active LuxO and thus remain active even at high cell density and degrading the *hapR* mRNA. Further studies are necessary to find out the importance of missing amino acids. These studies may provide an avenue of understanding towards the functionality of this con-$LuxO_{PL91}$ molecule. It will be interesting to find out the importance of constitutively active LuxO variant in regulation of other genes of metabolic pathways.

LuxO controls the expression of number of genes in *V. cholerae* O1 strains but its role in the *in vivo* survival of the organisms remains to be elucidated. Recent work from various groups have demonstrated a possible interaction and intracellular survival of *V. cholerae* in free living as well as parasitic protozoa (Abd *et al.*, 2005; Jain *et al.*, 2006). It could be possible that quorum sensing regulators might be involved in such interactions. Recently, it was found that quorum sensing enhances oxidative stress tolerance in *V. cholerae* (Joelsson *et al.*, 2007). Further studies are necessary to reveal that whether constitutively active form of LuxO helps bacteria in survival during interaction with other organisms or not.