Role of Quorum Sensing Signaling Molecule Cholera Autoinducer-1 (CAI-1) on Exopolysaccharide Expression

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ABSTRACT

The Gram-negative bacterium *Vibrio cholerae*, is the causative agent causing lethal diarrheal disease cholera. This organism gains adaptive advantage for survival in different environmental reservoirs by expressing different molecular signaling loop. The organism also found to express an exopolysaccharide (EPS) molecule which is essential for their survival in the environment. Quorum sensing mechanism control many responses including EPS expression by modulating gene expressions through involvement of important regulatory protein LuxO. On the other hand, in a subset of epidemic-causing *Vibrio cholerae*, absence of flagellum but not motility was identified to induce elevated EPS expression. Therefore, the role of quorum sensing cholera autoinducer-1 on EPS expression was tested on different mutated backgrounds. Mutation was introduced in vital genes like *cqsA* (cholera autoinducer-1), *flaA* (flagellin protein), *luxO* (response regulator of quorum sensing pathway) and *vpsR* (response regulator of flagellum mediated signal transduction pathway) in the model strain MO10. Subsequent phenotypic alterations in terms of colony morphology were analyzed. It was found that EPS signaling cas cade is independent of LuxO contribution and essentially involve VpsR.*flaA* function here as epistatic gene for controlling of colony rugosity. Finally, it may be inferred that a quorum sensing regulatory loop is functional in those subset of organism, independent from LuxO response and finally converge on VpsR, a well-known component of flagellum mediated signaling loop. These two signaling unit also mutually inhibitory to each other in different cell populations. Therefore, *Vibrio cholerae* enhancing their survival potential in the environment even in the inter epidemic period.

Introduction

Vibrio cholerae is a human pathogen as well as natural inhabitant of aquatic ecosystems. These organisms face a multitude of environmental stresses in different phases of life cycle that create selective pressure on them. To combat those stress and enhance survival fitness in the stressful environment,

these organisms accommodate some responses those typically involve signal transduction systems resulting in distinct patterns of gene expression. Ability to form biofilm on solid surfaces (Watnick *et al.,* 19, 20, 21)is believed to be such an adaptive feature of *Vibrio cholerae* that enables the pathogenic strains to persist long in the aquatic reservoirs. Biofilm formation of *Vibrio cholerae* and many other bacterial species is regulated by a phenomenon of cell-cell communication, called quorum sensing (23,24,6).

Quorum sensing mechanism control many responses including exopolysaccharide (EPS) expression at different cell densities by modulating target gene expression through involvement of two important regulatory proteins, LuxO and HapR (1, 2, 3, 4, 10, 15, 24, 12, 13). Quorum sensing mechanism for EPS expression in O139 strain MO10 (isolated during Calcutta outbreak in 1992) was unknown before starting this work although, it was reported that MO10 can produce the quorum sensing autoinducer (9). Some reports clearly indicate that in the strain MO10, EPS expression was found not to be mediated through HapR although, the organism possesses a functional HapR (10, 11).

However, different *Vibrio cholerae* biotypes utilize distinct signaling systems for EPS expression (16, 7). In a subset of *Vibrio cholerae,*it was reported (21, 11) that lack of flagellar structure induces EPS expression. Studies also revealed that phosphorylated VpsR play significant roles in high level *vps* gene transcription as well as signal transduction for enhanced EPS expression (11). Suggestion is there that at least three distinct types of EPS signaling pathways are functional in *Vibrio cholerae* i.e., a HapR–dependent pathway, a flagellum–dependent pathway, and both HapR- and flagellum-independent pathway (11). Depending on this background knowledge an attempt was made to identify the role of quorum sensing signaling molecule cholera autoinducer-1 (CAI-1) on EPS expression through important intracellular messengers like FlaA (flagellum dependent pathway), LuxO (classical quorum sensing dependent pathway) and VpsR (*vps* gene transcription and EPS expression) in this subset.

Materials and Methods

Model strains and plasmids used to carry out the present work

Vibrio cholerae MO10

The model *Vibrio cholerae* strain for the proposed study was *Vibrio cholerae* MO10 (18). *Vibrio cholerae* MO10 is a member of the O139 serogroup and was isolated during the cholera outbreak in India and Bangladesh in 1992. This strain gifted by Prof. Karl E. Klose, Professor at the University of Texas at San Antonio.

E. coli SM10 *λpir*

E. coli SM10 *λpir* strain was used here for cloning and propagation of plasmid (14). Antibiotic marker used for selection here is kanamycin (50 mg/ml).

Suicide plasmid vector pKEK229

Suicide (as it is unable to replicate in the target host) plasmid vector pKEK229 was used here to engineer mutations in host *Vibrio cholera*e strains.

Culture media and growth conditions

Strains were grown in Luria-Bertani (LB) broth. The LB used here was also supplemented with the following concentrations of antibiotics, when appropriate: 2 μg of chloramphenicol per ml for smooth strains, 20 μg of chloramphenicol per ml for rugose strains, 25 μg of kanamycin per ml, 100 μg of streptomycin per ml, and 50 μg of ampicillin per ml (Lauriano *et al.,* 2004).

Preparation of *Vibrio cholerae* MO10 deletion mutant strains

The *Vibrio cholerae* MO10 *ΔcqsA*, *ΔflaA*, *ΔluxO*and *ΔvpsR* mutant strains and subsequent double mutants were prepared to fulfill the aims of the study. The mutant strains were developed through the experiments described in the following sections.

Preparation of deletion construct in suicide plasmid vector

Vibrio cholerae MO10 *ΔcqsA*,*ΔflaA*, *ΔluxO* and *ΔvpsR,* the deletion fragments of *cqsA*, *flaA*, *luxO* and *vpsR* were cloned in suicide plasmid pKEK 229 (Correa *et al.,* 2000) to get the suicide plasmid-deletion fragment constructs. Detailed procedure is described in the following sections.

Plasmid vector DNA isolation

E. coli SM10*λ pir* which harbored suicide plasmid pKEK229 was isolated by standard protocol using kit from HiMedia, India.

Isolation of chromosomal DNA of *Vibrio cholerae*

Vibrio cholerae O139 Bengal strain MO10 chromosomal DNA was isolated following methods of Sambroke and Russel 2001.

PCR Primer designing

Whole genome sequences of *Vibrio cholerae* was downloaded and specific primers were designed for the genes (*cqsA, flaA, luxO, vpsR*) to be mutated. The primers were designed such a way that the 5' fragment to the deletion was generated using primer pairs A and B and the fragment 3' to the proposed deletion was generated using primer pair C and D for the gene.

Table 1. Oligonucleotide primer sequences for gene deletion prepared in our Lab.

Name of gene deleted and location in chromosome	Name of designed primer	Position of primer in respective gene	Sequences of primer (5'-3')
cqsA (VCA0523)	*cqsA* P-A	-6 to +12	GCTCTAGAAGATCTATGCATTTAACGAAAATA
	cqsA P-B	+188 to +208	GCGGATCCAACAGGAGATGAACGAAATAC
	cqsA P-C	+959 to +979	GCGGATCCTGTTGTTCTTCCAGTAATGAC
	cqsA P-D	+1151 to +1170	GCGGTCGACGATATCATGAACAAGCCTCAACTT CC
flaA (VC2188)	*flaA* P-A	+1 to +21	GCTCTAGACTACTGCAATAACGAGATTGC
	flaA P-B	+167 to +188	GCGGATCCGTCACAGCATCAGTAACCTGC
	flaA P-C	+954 to +974	GCGGATCCCATCCAAACCACGAGATTGCG
	flaA P-D	+1120 to +1139	GCGGTCGACATGACCATTAACGTAAATAC
luxO (VC1021)	*luxO* P-A	+1 to +17	GCTCTAGAATGGTAGAAGACACGGC
	luxO P-B	+168 to +184	GCGGATCCCTGCATAGAGTACGTCC
	luxO P-C	+1185 to +1201	GCGGATCCAGTATCCGTGCATGAGA
	luxO P-D	+1353 to +1368	GCGGTCGACTTACCGTTCCTTCTCT
vpsR (VC0665)	*vpsR* P-A	-197 to -177	GCTCTAGAAGAAATAATCGTGCCAAGTCG
	vpsR P-B	+265 to +285	GCGGATCCGGTATCTGAACTGAGCTGCGC
	vpsR P-C	+859 to +879	GCGGATCCCTGATCGATGGTGATTTTAAC
	vpsR P-D	+1321 to +1340	GCGTCGACAAAACTTAGAAGTTTTCATC

Development of deletion (mutated) fragments of the desired genes through polymerase chain reaction (PCR)

PCR amplification of the desired portion of genes

Thermal cycler (Eppendorf, Germany) was used here for amplification of DNA.

Purification of PCR generated deleted gene fragments

In this method PCR amplified products were purified and were stored at -20 °C.

Restriction enzyme digestion of gene deletion fragment

PCR generated fragment 5' to the gene deletion was digested with Xba I and BamHI, generating fragment-1. The deletion fragment 3' to the gene deletion was digested with BamHI and Sal I, generating fragment-2. Purification was done using gel-extraction kit (GeNei, India)

Restriction enzyme digestion of suicide vector pKEK229

pKEK229 having restriction site Xba I and Sal I was digested in two stages. At first stage, the vector was digested with single restriction enzyme followed by purification using gel-extraction kit (GeNei, India). At the next step second enzyme digestion was done followed by second purification step using gel-extraction kit.

Ligation of the deletion fragment with suicide vector

PCR generated DNA fragment 5' to the deletion (digested with Xba I and Bam HI restriction endonucleases) and PCR generated DNA fragment 3' to the deletion (digested with Bam HI and Sal I restriction endonucleases) for each gene were ligated into suicide vector, pKEK229 (digested with Xba Iand Sal I restriction endonucleases) using the ligation enzyme T4 DNA ligase to produce plasmid constructs containing respective gene deletion.

Preparation of mutant strains using the prepared deletion constructs

The deletion plasmid constructs prepared as described in previous section were electroporated in *E. coli* SM10*λpir*.

Preparation of competent cells for electroporation

Bacterial cells of *Escherichia coli* SM10*λpir* were inoculated into 5 ml LB broth containing kanamycin (25 µg/ml) and allowed to grow in shaking condition for 2 - 4 h until OD_{600}=0.2 - 0.4. 1.5 ml culture was taken and kept in ice for 20 minutes. It was then centrifuged for 1 minute at 4°C. The palate was collected and kept in ice and the supernatant was poured off. The cell palate was washed with ice cold milli Q water and resuspended in 1 ml 10% glycerol and kept

in ice for 5 minutes. Centrifugation was done at 5000 rpm for 1 minute at 4°C and the supernatant was carefully poured off. The palate was again resuspended in 1 ml 10% glycerol and stored in ice.

Electroporation into *E. coli* cells

In this procedure plasmid construct was mixed with ice cold competent *E. coli* SM10 *λpir* cells and the mixture was placed in electroporator (Eppendorf, Germany). Immediately after completion of electroporation the mixture was then spreaded on LB agar plate containing 50µl/ml ampicillin and was incubated at 37°C overnight. Colonies of *E. coli* SM10*λpir* cells grown on LB agar plate in presence of 50 µl/ml ampicillin was considered primarily to contain deletion plasmid construct and further confirmed by PCR

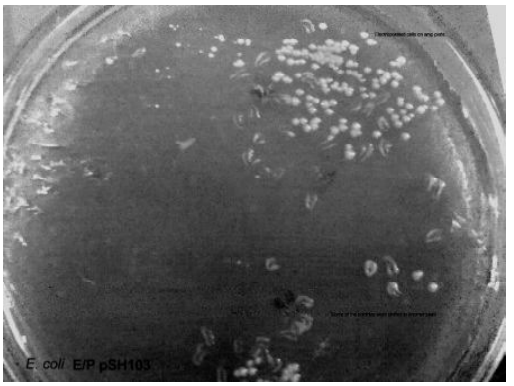


Fig. 2. Electroporated pSH103 (*cqsA* deletion plasmid construct) in *E. coli* SM10ë*pir* on ampicillin-kanamycin LB agar plate.

Screening of the desired clone through PCR

10 colonies of each transformed *Escherichia coli* SM10ë*pir* were randomly selected for plasmid DNA isolation for the search of the transformed colony containing desired fragment size of the gene deletion mentioned earlier through PCR.

Conjugation

Mating of *Vibrio cholerae* and *Escherichia coli* SM10 *λpir* containing deletion plasmid construct and colonies of *Vibrio cholerae* transconjugants are shown in Fig. 3A and 3B.

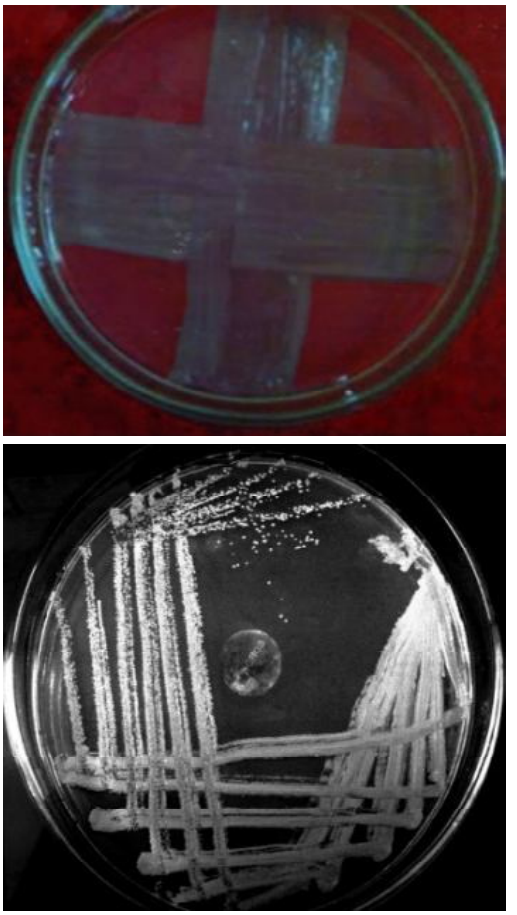
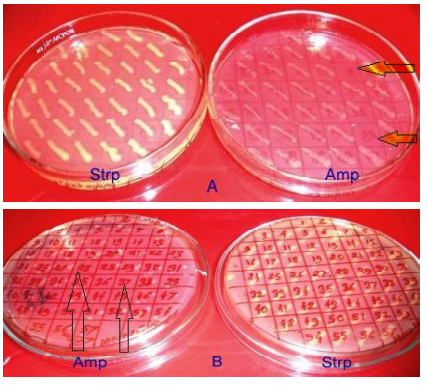


Fig. 3. (A) Conjugation of *Vibrio cholerae* with *Escherichia coli* SM10 *λpir* containing

pSH104 on LB agar plate. ***Vibrio cholerae*** **strain was streaked horizontally whereas *E. coli* cell was streaked vertically. After 24 hours' growth a cell patch was found in that overlap zone. (B) Colonies of *Vibrio cholerae*transconjugant on streptomycin-ampicillin LB agar plate for the selection of mutants.**

Recombination

Four colonies from strp-amp plate were collected and cultured in streptomycin (100 µg/ml) containing 1 ml LB broth for a time period of 8-12 hours at 37 °C. Finally, 50 µl culture was transferred to 10% sucrose LB agar plate and incubated at 30 °C for overnight. Then colonies were patched on specific antibiotic plate (Fig. 4) for selection through positive and negative marker. The colony which could grow on streptomycin LB agar plate (LB agar containing streptomycin) only, but did not grow on ampicillin LB agar plate (LB agar containing ampicillin) was considered as the final recombinant colony.



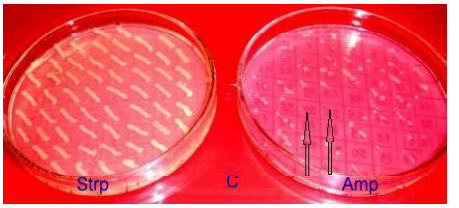


Fig. 4. Final selection of mutated strains. Here A, B and C showing three parallel sets of *cqsA* mutant strains selection from streptomycin (Strp, 100 µg/ml) and ampicillin (Amp, 50 µg/ml)) containing plates. The arrows indicate recombinant mutants those were streptomycin resistant and ampicillin sensitive.

Confirmation of strains containing desired gene deletion through PCR and gel documentation

Chromosomal DNA of each of the mutant strains were isolated and used as template DNA to carry out PCR. PCRs were carried out using primer A and primer D of the corresponding gene and the amplified product were subjected for gel documentation. Size reduction to a desired and predicted level from mother gene to mutated gene was considered the strain to be correct mutated form.

Identification of colony morphology

Vibrio cholerae mutant colonies were grown on LB agar plates containing streptomycin and incubated at 30 °C for a time period of 24 to 48 hours. Morphologies of the colonies were studied by careful observation of the upper surface of the colonies grown on LB agar plates. Appearance of wrinkles on the upper

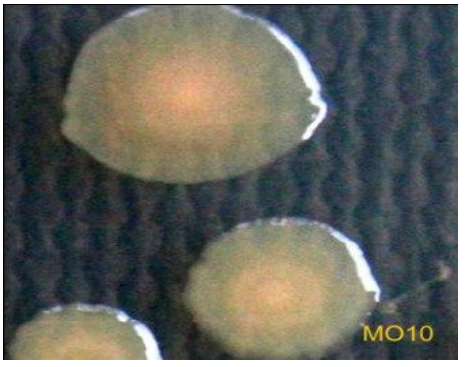
surface of the single colony was considered the colony to be rugose (11). Colonies with absence of wrinkles on the upper surface were considered the colonies to be smooth. Colony morphologies were photographed by Canon EOS 350D digital camera.

Table 2. Plasmid constructs prepared with gene deletions

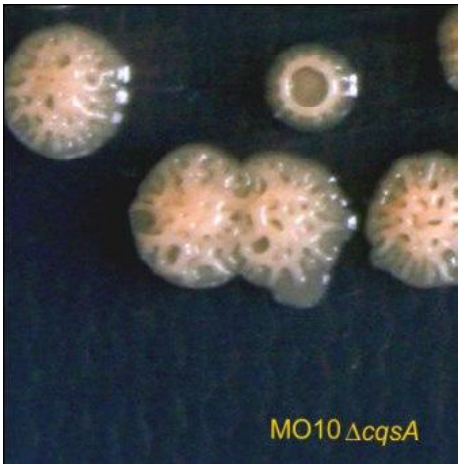
Plasmid	Description	Source
pSH103	*ΔcqsA* in pKEK229	This study
pSH102	*ΔflaA* in pKEK229	This study
pSH115	*ΔluxO* in pKEK229	This study
pSH105	*ΔvpsR* in pKEK229	This study

Table 3. Mutant strains generated in this study

Strain	Description	Source
CG103	MO10 *ΔcqsA*	This study
CG100	MO10 *ΔflaA*	This study
CG109	MO10 *ΔcqsAΔflaA*	This study
CG170	MO10 *ΔluxO*	This study
CG172	MO10 *ΔcqsA ΔluxO*	This study
CG111	MO10 *ΔvpsR*	This study
CG113	MO10 *ΔcqsA ΔvpsR*	This study



Wild (smooth)



ΔcqsA (rugose)

Fig. 5. Autoinducer influence expression as evident by colony morphologies of relevant mutant. Colony morphology was studied by carefully observing upper surface of bacterial colonies. *Vibrio cholerae* strains with rugose or wrinkle-surfaced colonies reflect enhanced EPS expression over strains demonstrating smooth-surfaced colonies. The figure demonstrates colony morphology of MO10 (wild type) smooth, CG103 (*ΔcqsA*) rugose.

Results and Discussion

To identify the role of CAI-1 in EPS expression, mutant strain MO10 *Δ"cqsA* (CG103) was developed by introducing deletion in the gene responsible for biosynthesis of CAI-1 through preparation of MO10 *ΔcqsA* deletion construct (pSH103) in suicide plasmid pKEK229 and subsequent homologous recombination.

ΔflaA (rugose)

ΔcqsA ΔflaA (smooth)

Fig. 6. Autoinducer and flagellum influence EPS as evident by colony morphologies of relevant mutants. Colony morphology was studied by careful observation of the upper surface of bacterial colonies. *Vibrio cholerae* strains with rugose or wrinkle-surfaced colonies reflect enhanced EPS expression over strains demonstrating smooth-surfaced colonies. The figure **demonstrates colony morphology of CG100 (*ΔflaA*), CG109 (*ΔcqsA ΔflaA*).**

MO10 *ΔcqsA* strain produced rugose colonies (Fig. 5) whereas, wild type strains of MO10 produce smooth colonies. Therefore, it is clear that deletion of quorum sensing *cqsA* gene resulted in diminished production of quorum sensing cholera autoinducer-1, are producing excess amount of EPS that turns the colony morphology into rugose phenotype. This diminished concentration of CAI-1 signifies the low cell density condition of *vibrio* species that increases their protective function in the environment in low cell density condition by producing excess EPS and consequently increases their survival potential in the inter-epidemic period.

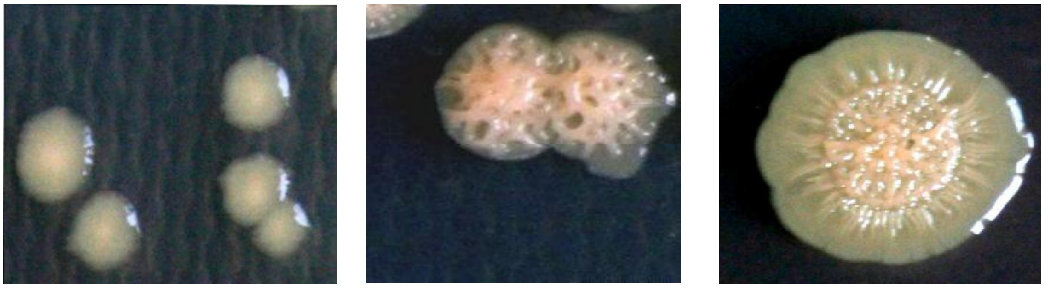
Most interestingly, when MO10 *ΔcqsA ΔflaA* strain (CG109) was further prepared by introducing deletion in *flaA* (major structural component of flagellar filament), they were found to produce smooth colonies (Fig. 6). Deletion of single *flaA* produce rugose colony morphology (Fig. 6) produces significant EPS than wild type strain.

Beside this, when MO10 *ΔcqsA ΔflaA* (CAI-1$^-$, Flagellum$^-$) recipient strain was grown in presence of the cell-free culture fluids from autoinducer donor, colony morphology of recipient strain was found to be shifted from smooth to rugose type. This observation suggests that CAI-1 deficiency (*ΔcqsA*) may be the underlying reason for

the defective EPS expression in MO10 *ΔcqsA ΔflaA* strain (Fig 6), as supplementation of CAI-1 through the cell-free culture fluid increased EPS expression significantly to the level of MO10 *ΔflaA* state and also shifted the colony morphology of the recipient from smooth to rugose type. Hence, these results also reveal that the quorum sensing molecule CAI-1 is responsible for observed excess EPS expression and colony rugosity in MO10 *ΔflaA* strain.

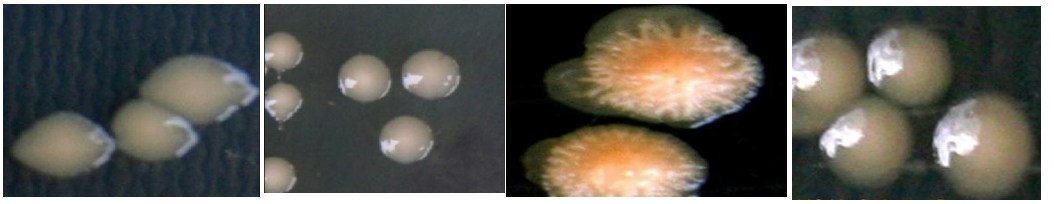
All the results presented here suggest that quorum sensing autoinducer and flagellar filament are two major signaling units driving independently two discrete signaling circuits for EPS expression as it was observed that absence of both the signaling units in MO10*ΔcqsA ΔflaA* strains resulted in disruption of EPS expression. On the other hand, absence of single unit i.e., either autoinducer as observed in MO10*ΔcqsA* (Fig. 5) or flagellum in MO10*ΔflaA* strain (Fig. 6) leads to excess EPS production. It clearly indicates that in absence of one signaling unit [either autoinducer or flagellum], the other plays predominant role for elevated EPS biosynthesis because further mutation in the second unit renders the cell defective for EPS production. More specifically, it appears that, under autoinducer deficit conditions, the observed excess EPS production is flagellum-dependent, whereas the excess EPS observed during absence of flagellar filament is autoinducer dependent. Moreover, defective EPS production during absence of autoinducer and flagellar filament both reveal that these two components are independent and major signaling units responsible for *vps* transcription in this study strain as absence of both leads to EPS disruption.

LuxO was first characterized in *Vibrio harveyi* to play crucial role in cell density-dependent target gene expression through HapR, which controls behavioral responses in the process of quorum sensing (3). In the strain MO10, EPS expression and biofilm formation was found not to be mediated through HapR although, the organism possesses a functional *hapR* with R12L substitution in comparison to the active *hapR* from El Tor strain 3083-2 (10,11). On the basis of this information, goal of this study was to understand the role of LuxO in EPS expression in MO10. For that purpose, mutant strains MO10 *ΔluxO*, MO10 *ΔcqsA ΔluxO* were developed by introducing deletion in *luxO* in wild type and MO10 *ΔcqsA* strains respectively. Results reveal that *luxO* deletion causes no significant change and no shift in colony morphology (Fig.7). These findings suggest that, in this study strain, LuxO has no influence on EPS expression under the conditions of absence of CAI-1. Hence, it appears that the CAI-1 signal transduction pathways those control EPS expression in *Vibrio cholerae* strain



ΔluxO (smooth) *ΔcqsA (rugose)* *ΔcqsA ΔluxO (rugose)*

Fig.7. Autoinducer influence EPS as evident by colony morphologies of relevant mutants. Colony morphology was studied by careful observation of the upper surface of bacterial colonies. *Vibrio cholerae* strains with rugose or wrinkle-surfaced colonies reflect enhanced EPS expression over strains demonstrating smooth-surfaced colonies. The figure **demonstrates colony morphology of CG170 (*ΔluxO*), CG103 (*ΔcqsA*), CG172 (*ΔcqsA ΔluxO*).**



***ΔvpsR* (smooth)** ***ΔcqsA ΔvpsR* (smooth)** ***ΔflaA* (rugose)** ***ΔflaA ΔvpsR* (smooth)**

Fig. 8. Colony morphology of *vpsR*-mutants and respective mother strains. Colony morphology was studied by careful observation of the upper surface of bacterial colonies. *Vibrio cholerae* strains with rugose or wrinkle-surfaced colonies reflect enhanced EPS expression over strains demonstrating smooth-surfaced colonies. The figure demonstrates colony morphology of CG111 (*ΔvpsR*) (smmoth), CG113 (*ΔcqsA ΔvpsR*) (smmoth), CG100 (*ΔflaA*) (rugose), CG112 (*ΔflaA ΔvpsR*) (smmoth).

MO10 do not involve LuxO-HapR regulatory circuit.

VpsR, a response regulator protein homologous to $\acute{o}^{54}$-dependent activators have been identified as a stimulator of transcription of *vps* gene clusters in *Vibrio cholerae* (22). To determine the involvement of VpsR on EPS expression under quorum sensing signaling molecule CAI-1-deficient condition in *Vibrio cholerae* strain MO10, *ΔvpsR* (CG111), *ΔcqsA ΔvpsR* (CG113) mutant strains of MO10 were developed through the construction of *ΔvpsR* construct of MO10 (pSH105) in suicide plasmid pKEK229 and subsequent homologous recombination. The single *vpsR* mutant and CAI-1-*vpsR*-double

mutant demonstrated reduced EPS expression and shift in colony morphology from rugose to smooth type was observed in the MO10 *ΔcqsA ΔvpsR* strain (Fig.8). The flagellum mediated signaling circuit also channeled through VpsR as flagellum-*vpsR* double mutant also produced less EPS and changed the colony morphology from rugose to smooth.

Here, the results indicate that mutation in *vpsR* is epistatic to the mutations in *cqsA* and to the mutation of *flaA* (as also observed in Lauriano *et al.,* 2004) as the autoinducer (or flagellum)-*vpsR* double mutant and *vpsR* single mutant produced identical phenotypes in respect of EPS expression. These observations suggest that VpsR is an important positive regulator for transcription of *vps* genes causing high level EPS expression under CAI-1 or flagellum-deficient state. Lauriano *et al.,* in 2004 also identified phospho-VpsR as the activated form of VpsR for stimulating *vps* transcription and to be essential for EPS expression in wild type as well as *ΔflaA* strains of MO10. Taken together, it is suggesting that the proposed EPS signaling unit i.e., the CAI-1 probably influence the activation of VpsR through stimulating its phosphorylation but not its expression, and in this manner include it in the EPS signaling cascades. The exact mechanism of phosphorylation remains unidentified. In relation to this, it is also tempting to speculate that the defective EPS expression in *ΔcqsA ΔflaA* mutant strains of MO10 probably takes place due to lack of phosphorylation of VpsR because of the absence of CAI-1 and flagellum both, the two major EPS signaling units.

Conclusion

The Calcutta strain MO10 used in this study, modulate their genetic responses for long persistence in nature and subsequent expression of their virulence factors. Quorum sensing autoinducer is one of them which is involve in expression of EPS but not in classical quorum sensing pathway as there is no involvement of LuxO rather, it is converging on VpsR which is the component of flagellum mediated signal transduction pathway of EPS expression. On the other hand, both the signaling units are mutually inhibitory to each other.

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