Identification of the Role of Quorum Sensing Signaling Molecule Cholera Autoinducer-1 (CAI-1) in Flagellum Dependent Cell Signaling Pathway and Regulation of Virulence Gene Expression in *Vibrio cholerae*

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Synopsis

Background

In *Vibrio cholerae* O139 Bengal strain MO10 that has high epidemiological importance, a flagellum-dependent cell signaling pathway has been proposed that controls exopolysaccharide (EPS) expression, biofilm formation and virulence gene expression (Lauriano et al., 2004). This pathway has been found to involve a sodium–driven flagellar motor and phosphorylation of VpsR, an important regulator for biofilm formation. HapR, a common quorum-sensing regulator of *Vibrio cholerae* was found not to be involved in EPS expression in MO10.

Aims and objectives

In the present study an attempt was made to identify whether the flagellum-dependent signal transduction pathway controlling EPS expression, biofilm formation as well as virulence gene expression is under the control of quorum sensing signaling autoinducer molecule like cholera autoinducer-1 (CAI-1, *cqsA* gene product) that may regulate the above activities either directly or indirectly involving other intermediary genes in quorum sensing pathway but not involving *hapR*. The present study also intends to identify the involvement of CAI-1 on virulence gene expression like cholera toxin, activity of the *toxR-tcpP* virulence regulatory genes and to reveal the role of CAI-1 in inducing intestinal colonization.

Materials and methods

MO10 Δ*cqsA* was developed by introducing deletion mutation in *cqsA*. For better understanding of the proper signaling mechanism and related regulators for EPS expression and biofilm formation, deletion mutations were further developed in *flaA* (major structural component of flagellar filament), *luxO* (σ^{54}-dependent quorum sensing regulator), *motX* (essential structural unit of flagellar motor), *vpsR* (a response regulator protein homologous to σ^{54}-

dependent activators and stimulator of transcription of *vps* gene cluster). For supporting the present study MO10 Δ*luxS* construct (the gene responsible for the synthesis of autoinducer-2 i.e., AI-2), available in the laboratory, was used also for mutant strains development.

Plasmid constructs containing deletions in the above mentioned functional genes were prepared by inserting the PCR generated gene deletions in a suicide vector pKEK229 using specific restriction endonucleases following the plasmid restriction map and the constructs were electroporated in *E. coli* SM10λ *pir* cells.

The mutations (deletions) of the respective above mentioned genes have been introduced in *Vibrio cholerae* O139 Bengal MO10 Δ*lacZ* chromosome via homologous recombination method. Thus *Vibrio cholerae* mutant strains MO10 Δ*cqsA* (CG103), Δ*cqsA* Δ*luxS* (CG175), Δ*flaA* (CG100), Δ*cqsA* Δ*flaA* (CG109), Δ*cqsA* Δ*luxS* Δ*flaA* (CG177), Δ*luxO* (CG170), Δ*cqsA* Δ*luxO* (CG172), Δ*cqsA* Δ*luxS* Δ*luxO* (CG174), Δ*flaA* Δ*luxO* (CG171), Δ*motX* (CG166), Δ*cqsA* Δ*motX* (CG168), Δ*cqsA* Δ*luxS* Δ*motX* (CG169), Δ*flaA* Δ*motX* (CG163), Δ*vpsR* (CG111), Δ*cqsA* Δ*vpsR* (CG113), Δ*cqsA* Δ*luxS* Δ*vpsR* (CG179), and Δ*flaA* Δ*vpsR* (CG112) were generated.

Phenotypes like EPS expression (in the form of *vpsL* transcription level (*vpsL* being one of the important member of *vps* gene cluster causing EPS expression), colony morphology, biofilm formation abilities were studied in the mutant strains and comparative analyses were done with wild type strain as well as among the mutants itself.

Autoinducer cross-feeding experiment was performed to establish whether CAI-1-deficiency is responsible for excess EPS expression as well as colony rugosity in MO10 Δ*cqsA* strain. To make better understanding of the EPS signaling phenomenon in MO10, transcription of *flaA*, *vpsR* and *luxS* were studied by preparing promoter-*lacZ* transcriptional reporter construct and subsequent electroporation into different mutant backgrounds of MO10 along with wild type strain and by β-galactosidase assay. In vitro cholera toxin production abilities were measured in those single mutated and double mutated strains. β-galactosidase activity of *toxR* (cytoplasmic transcriptional activator of

cholera toxin and pilus production), *tcpP* (membrane-localized transcription factor) and *toxT* (membrane-localized transcription factor) promoter genes were conducted for determine the influence of CAI-1 on *toxT-tcpP-toxR* mediated virulence factor expression. In vivo colonization ability were also studied to reveal the role of CAI-1 in inducing intestinal colonization.

Result and discussion

Results reveal that CAI-1-deficient condition leads to significantly ($p < 0.05$) elevated EPS expression, rugose colony formation and significantly ($p < 0.05$) elevated biofilm formation in comparison to wild type strain. Moreover, deficiency of both autoinducers also produced similar results as observed in case of CAI-1 deficiency alone.

Mutant causing deficiency of flagellum, led to significantly ($p < 0.05$) elevated EPS expression, rugose colony formation and significantly ($p < 0.05$) elevated biofilm formation in comparison to wild type strain as was reported earlier. Most interestingly, mutants causing deficiency in CAI-1 and flagellum both as well as deficiency in CAI-1, AI-2 and flagellum together, demonstrated significant ($p < 0.05$) defect in EPS expression, smooth colony formation and significant ($p < 0.05$) defect in biofilm formation.

Further, autoinducer cross-feeding to the respective mutant reveals that CAI-1-deficiency is indeed responsible for elevated EPS expression under Δ*cqsA* state of MO10. Moreover, CAI-1 is mainly responsible for the observed elevated EPS expression in Δ*flaA* state of MO10.

Gene expression analysis of *cqsA*, *luxS* and *flaA* under wild type and mutant backgrounds were studied by producing promoter-*lacZ* transcriptional fusion reporter constructs and subsequent β-galactosidase assay. The results reveal that gene expression of any of the above mentioned factors is not dependent on others. On the other hand, motility was not changed upon introduction of mutation in *cqsA* gene and the strain remains motile. But introduction of *flaA* mutation resulted a non-motile strain as expected and also reported earlier.

Moreover, results of the present study also reveal that excess EPS production, colony rugosity and biofilm formation under CAI-1 and flagellum-deficient conditions are independent of the regulatory action of LuxO.

Further analyses of the CAI-1 mutants reveal that elevated EPS expression and biofilm formation under CAI-1 deficient condition are dependent on sodium-driven flagellar motor and VpsR similar as observed earlier in case of flagellum–deficient condition. Gene expression analysis reveals that *vpsR* expression is not dependent on CAI-1.

Analysis of cholera toxin production ability in the mutants generated reveal that CAI-1-deficiency in Δ*cqsA* rugose strain renders the strain defective in cholera toxin production. Whereas other mutants like Δ*flaA* (CG100) which was also rugose in phenotype was found to be defective in cholera toxin production. On the other hand the mutants like Δ*cqsA* Δ*flaA* (CG109), Δ*vpsR* (CG111), Δ*cqsA* Δ*vpsR* (CG113) and Δ*flaA* Δ*vpsR* (CG112) those are smooth in phenotype were able to produce substantial level of cholera toxin as wild type strain. But there were no change in cholera toxin production in *luxO* mutants i.e., MO10 Δ*luxO* (CG170), Δ*cqsA* Δ*luxO* (CG172) and Δ*flaA* Δ*luxO* (CG171) from their corresponding mother strains i.e., wild type, Δ*cqsA* and Δ*flaA* strains of MO10, respectively.

Gene expression analysis of *toxR*, *tcpP* and *toxT*, the important regulators for *ctxAB* expression, under CAI-1 and flagellum-deficient mutant backgrounds of MO10 i.e., Δ*cqsA* and Δ*flaA* respectively, reveal significantly ($p < 0.05$) lower expression of *toxR*, *tcpP* and *toxT* and this lower expression also correlate with the deficiency ($p < 0.05$) in cholera toxin production by the respective mutants mentioned above. In case of other mutants i.e., MO10 Δ*cqsA* Δ*flaA*, Δ*vpsR*, Δ*cqsA* Δ*vpsR* and Δ*flaA* Δ*vpsR* those demonstrated wild type level of cholera toxin production also demonstrated wild type level of *toxR*, *tcpP* and *toxT* expression.

Intestinal colonization assay reveals that MO10 Δ*cqsA* and Δ*flaA* to be defective for intestinal colonization. But intestinal colonization was found to increase in MO10 Δ*cqsA* Δ*flaA* than MO10 Δ*cqsA* and Δ*flaA* mutant strains, respectively.

Conclusion

Quorum sensing cholera autoinducer-1 (CAI-1) (as an independent and essential member of “autoinducers” as an intra-genus signaling unit) and flagellar filamentous structure are two predominant signaling units for regulating EPS expression and biofilm formation, such that one plays leading role in absence of other. CAI-1 signal for EPS expression either singly or along with AI-2 together through independent circuits to the pathway driven by flagellar structure. On the other hand, CAI-1 alone also has characteristics pattern of influence on EPS expression. The proposed EPS signaling units i.e., CAI-1 and flagellum are mutually inhibitory towards each others’ action for EPS expression and they finally converge as both of them involve sodium-driven flagellar motor and VpsR, the positive regulator for EPS biosynthesis. Most importantly, the biofilm regulatory process under study is independent of the regulatotry action of quorum sensing central regulator LuxO. CAI-1 also influence cholera toxin expression. CAI-1 and flagellum, both have great influence on the expression of regulators like, ToxR, TcpP and ToxT those are well known for influencing cholera toxin production under environmental signals and cholera autoinducer-1 also influences intestinal colonization.

Significance

It is evident from this investigation that the *Vibrio cholerae* strain under study has evolved an alternative mechanism of EPS expression where the organisms involve different flagellar components like flagellar filamentous structure and sodium-driven flagellar motor in the EPS signaling process in addition to quorum sensing autoinducers. Moreover, the cholera autoinducer-1 (CAI-1) -driven EPS signaling demonstrates an alternative signaling circuit involving sodium-driven flagellar-motor and VpsR rather than previously described LuxO-HapR regulatory action (Zhu & Mekalanos, 2003). The proposed EPS signaling process is probably responsible for rendering the adaptive feature more selective and advantageous for the organism to survive in distinct environment and that is reflected in the efficient and rapid biofilm forming ability of O139 strain MO10 on solid surfaces over O1 El Tor strains studied (Watnick et al., 2001). Bacterial biofilm is suggested to represent a complex habitat where

differentiation on the basis of selective advantage takes place (Hammer & Bassler, 2003). Taken together, it can be suggest that complex molecular interplay is involved in the EPS signaling mechanism in these organisms.