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Role of a sensor histidine kinase ChiS of *Vibrio cholerae* in pathogenesis

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ABSTRACT

Vibrio cholera survival in an aquatic environment depends on chitin utilization pathway that requires two factors, chitin binding protein and chitinases. The chitinases and the chitin utilization pathway are regulated by a two-component sensor histidine kinase ChiS in *V. cholerae*. In recent studies these two factors are also shown to be involved in *V. cholerae* pathogenesis. However, the role played by their upstream regulator ChiS in pathogenesis is yet to be known. In this study, we investigated the activation of ChiS in presence of mucin and its functional role in pathogenesis. We found ChiS is activated in mucin supplemented media. The isogenic *chiS* mutant (ChiS$^-$) showed less growth compared to the wild type strain (ChiS$^+$) in the presence of mucin supplemented media. The ChiS$^-$ strain also showed highly retarded motility as well as mucin layer penetration *in vitro*. Our result also showed that ChiS was important for adherence and survival in HT-29 cell. These observations indicate that ChiS is activated in presence of intestinal mucin and subsequently switch on the chitin utilization pathway. In animal models, our results also supported the *in vitro* observation. We found reduced fluid accumulation and colonization during infection with ChiS$^-$ strain. We also found ChiS$^-$ mutant with reduced expression of *ctxA, toxT* and *tcpA*. The cumulative effect of these events made *V. cholerae* ChiS$^-$ strain hypovirulent. Hence, we propose that ChiS plays a vital role in *V. cholerae* pathogenesis.

1. Introduction

Vibrio cholerae causes the fatal diarrheal disease cholera. *V. cholerae* normally resides in the aquatic environment, where it colonizes on the chitinous surface of crustaceans (Huq et al., 1983) and utilize chitin as nutrient source. Chitin is an un-branched long chain polymer of β-1, 4 linked *N*-acetylglucosamine residues (GlcNAc). In *V. cholerae,* a two-component sensor histidine kinase, ChiS (VC0622) located in the inner membrane controls the expression of genes involved in chitin degradation. These include $(GlcNAc)_2$ catabolic operon (*chb*), two extracellular chitinase genes *chiA1* and *chiA2*, and an outer membrane chitoporin gene *chiP* (Meibom et al., 2004). ChiA1 and ChiA2 hydrolyze the β-1, 4 linkages between the GlcNAc residues in chitin, yielding soluble $GlcNAc_n$ oligosaccharides, where n = 2–6 (Svitil et al., 1997; Meibom et al., 2004; Orikoshi et al., 2005) which enter through chitoporin and are utilized sequentially via a downstream cascade of catabolic operon (*chb*) (Hunt et al., 2008). It has been recently known that ChiS also regulate chitin induced natural competence through the involvement of another transmembrane regulator TfoS (Yamamoto et al., 2014).

ChiS is a 133 kDa sensor histidine kinase which belongs to the 'Two component system' (TCS). It has a short N-terminal peptide chain in the cytoplasm, a membrane domain, a periplasmic domain, a second membrane domain, and finally a long polypeptide chain extending into the cytoplasm (Li and Roseman., 2004). ChiS remains inactive by a periplasmic chitin oligosaccharide binding protein, CBP through the ChiS-CBP complex formation. The presence of GlcNAc oligosaccharides as an environmental signal leads to the dissociation of ChiS-CBP complex by mediating association of CBP with GlcNAc, thereby activating ChiS. Like other TCS, a conserved histidine residue in the cytoplasmic domain of the active ChiS is autophosphorylated followed by the transfer of the phosphoryl group to a conserved aspartate residue of the cytoplasmic response regulator which is not yet characterized for ChiS. This regulator finally interacts with the genes under ChiS regulation. This typically activates an output domain which includes chitinolytic genes of chitin utilization pathway (Li and Roseman, 2004).

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TCS in various other pathogenic bacteria are reported to control virulence. VieSAB, a TCS of *V. cholerae* is reported to contribute to its motility and biofilm regulation (Hector et al., 2008). Another *V. cholerae* TCS, VprA-VprB is found to be involved in virulence through its endotoxin modification in host intestine (Herrera et al., 2014). Similarly, TCS PhoP-PhoQ in *Salmonella enteric* is involved in LPS modification and resistance to antimicrobial peptides (Groisman, 2001; Shi et al., 2004). CpxR-CpxA in *Shigella sonnei* is found to be involved in the activation of the master virulence gene regulator virF (Gal-Mor and Segal, 2003).

Several reports indicate that *V. cholerae* chitinase and chitin binding protein are also important for pathogenesis apart from their role in chitin utilization program (Bhowmick et al., 2008; Mondal et al., 2014). GbpA, a chitin binding protein, helps in adherence of *V. cholerae* to the intestinal epithelial cells through a coordinated interaction with mucin (Bhowmick et al., 2008). A recent study shows that ChiS dependent chitinase, ChiA2 is important for survival and pathogenesis of *V. cholerae* within the host intestine (Mondal et al., 2014). Since TCS are found to be involved in virulence, it is important to explore the role of ChiS in *V. cholerae* pathogenesis. In this study, we determined the effect of intestinal mucin on ChiS activation. Further, in order to define the role of ChiS in *V. cholerae* pathogenesis, we explore the impact of *chiS* deletion. We found that isogenic *chiS* mutant (ChiS$^-$) showed repression in mucin utilization. We also demonstrated that disruption of *chiS* gene has marked effects on survival, motility, mucin penetration and utilization, expression of virulence in *V. cholerae.*

2. Materials and methods

2.1. Ethics statement

All the animal experiments were done according to the guidelines provided by Committee for the Purpose of Supervision and Control Experiments on Animals (CPCSEA), Government of India. The protocols followed for the animal experiments were approved by the Institutional Animal Ethics Committee of National Institute of Cholera and Enteric Diseases (Registration no: PRO/106/May, 2014–September 2017). Four to five days old infant Swiss mice were used for intestinal colonization studies. New Zealand white rabbits were used for fluid accumulation assay. Animals were euthanized in CO_2 chamber assuring minimum pain to the animals during the intestinal harvest.

2.2. Bacterial strains, plasmids used and culture conditions

In this study, streptomycin resistant *V. cholerae* N16961 (O1El Tor Inaba) was used as a wild type strain.The suicide vector pCVD442 was maintained in *E. coli* strain DH5αlpir (Philippen et al., 2004). For TA cloning, we used pGEMT Easy vector (Promega) was used and maintained in *E. coli* JM109 (Table S1). Strains were grown in LB medium (BD, Difco) at 37 °C with appropriate antibiotics. For β-hexosaminidase assay, bacteria were grown in minimal–lactate media containing M9 minimal medium (BD Difco); 0.5% sodium lactate (Sigma); 50 mM HEPES, pH 7.5 (Sigma), filter sterile 0.2% $MgSO_4$ (Merck) and 0.01% $CaCl_2$ (SRL) with or without mucin (Sigma) as a sole source of carbon. Sodium lactate was added to support equal growth of wild type and mutant strains. To study the expression of virulence genes, bacteria were cultured in AKI media containing 0.5% NaCl, 0.3% $NaHCO_3$ (Merck), 0.4% yeast extract and 1.5% peptone (BD Difco) pH 7.2 at 37 °C under static condition.

2.3. Construction of deletion mutants of ChiS and CBP

Construction of isogenic mutants were done following earlier mentioned procedure (Skorupski and Taylor, 1996). In brief, *V. cholerae* N16961 was used for genomic DNA isolation. Almost 500 bps of flanking sequences of both the genes (*chiS* and *cbp*) were amplified by PCR using primers (Table S1). The flanking sequences were then annealed by fusion PCR using primers (Table S2) to get in-frame 3017 base pairs and 1509 base pairs deleted constructs for *chiS* and *cbp* mutants respectively. These unmarked fusion products were amplified and subcloned into pGEM-T Easy vector (Promega). The DNA fragments containing the unmarked deleted gene were digested with Xba1 and Sac1 restriction enzymes and ligated into the counter selectable *sacB*-based suicidal plasmid pCVD442 (Philippen et al., 2004). To harbour these deleted genes in *V. cholerae*, the resultant chimeric plasmid was transformed into *E. coli* SM10λpir (Philippen et al., 2004) and were conjugally transferred to N16961. The transconjugants were selected in ampicillin-streptomycin double antibiotic Luria Bertani (LB) agar plates. The unmarked gene replacements were done by double-crossover recombination mutation using the sucrose plates (Liu et al., 2015). Isogenic deletions and insertions of the unmarked gene were confirmed by using PCR based assay (Fig. S1) from the genomic DNA of the respective mutants using primers mentioned (Table S1) (Herrera et al., 2014).

V. cholerae strains were denoted as wild type (ChiS$^+$) and *chiS* isogenic mutant strain (ChiS$^-$). A constitutive mutant of *chiS* was constructed by deleting the *cbp* gene (chitin oligosaccharide binding protein) from *V. cholerae* and was denoted as ChiS* in all the experiments.

2.4. Complementation of chiS mutant

For complementation of *chiS* mutant, the open reading frame of *chiS* was PCR amplified by using Taq polymerase and Pfu polymerase (Promega) at a ratio of 2:1 and primers mentioned in Table S1 and cloned into pBAD-TOPO TA expression vector as previously mentioned protocol (Mondal et al., 2014). The cloned vector was transformed into *chiS* mutant strain (ChiS$^-$) and the complemented strain was denoted as ChiS^c. The complemented strain was induced by 0.2% arabinose (Sigma).

2.5. β-hexosaminidase assay

β-hexosaminidase activity was estimated by previously followed procedure (Li and Roseman, 2004) with PNP-GlcNAc (*p*-nitrophenyl-, *N*-acetyl-β-D-glucosa) purchased from Sigma. To analyse its activity wild type *V. cholerae* (ChiS$^+$), ChiS$^-$, its constitutive mutant ChiS* and ChiS^c were grown up to log phase in minimal–lactate media with or without mucin as mentioned previously. In case of *in vivo* hexosaminidase assay bacteria were collected from intestinal samples. Equal amount of bacteria (1×10^8 c.f.u/ml) were taken from each sample, washed and treated with toluene at a ratio of 10 μl/ml of culture. The mixture was shaken vigorously and kept at RT for 20 min. 0.1 ml of each of these treated bacteria was mixed with 0.1 ml of 1 mM substrate i.e PNP-GlcNAc in 20 mM Tris-HCl (pH 7.5). The reaction mixture was incubated at 37 °C for 60 min. 0.8 ml of 1 M Tris-base was added to stop the reaction. The reaction mixture was centrifuged to separate the cell debris and optical density of the supernatant was measured at 400 nm. Total enzymatic activity was analyzed after measuring total protein by Lowry method and then calculated as *p*-nitrophenol produced per minute per mg of total protein.

2.6. Generation of V. cholerae growth curve

Log phase cultures of wild type *V. cholerae* ChiS$^+$, ChiS$^-$, its constitutive mutant ChiS* and ChiS^c were harvested by centrifugation, washed three times with PBS, cell number was adjusted to 1×10^8 c.f.u/ml and mixed in a ratio of 1:1000 either lactate or