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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.

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 asociation 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*).

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 EA, 2001*, *Shi Y 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 O et al., 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.*

MATERIALS AND METHODS

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.

Bacterial strains, plasmids used and culture conditions

In this study, streptomycin resistant *V. cholerae* N16961 (O1 El Tor Inaba) was used as a wild type strain.The suicide vector pCVD442 was maintained in *E. coli* strain DH5αλpir (*Philippe 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); 50mM 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.

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 (*chi*S 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 *chi*S 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 (*Philippe et al., 2004*). To harbour these deleted genes in *V. cholerae*, the resultant chimeric plasmid was transformed into *E. coli* SM10λpir (*Philippe 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.

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).

β-hexosaminidase assay

β-hexosaminidase activity was estimated by previously followed procedure (*Li and Roseman, 2004*) with PNP-GlcNAc (*p*-nitrophenyl-β, D-*N* acetylglucosaminide) 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 1M 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.

157 **Generation of *V. cholerae* growth curve**

158 Log phase cultures of wild type *V. cholerae* ChiS^{+}, ChiS^{-}, its constitutive mutant ChiS* and
159 ChiS^{c} were harvested by centrifugation, washed three times with PBS, cell number was adjusted
160 to 1×10^{8} c.f.u/ml and mixed in a ratio of 1:1000 either lactate or mucin supplemented minimal
161 medium. The cultures were maintained at 37°C under constant shaking at 180 rpm for 72 h
162 (*Mondal et al., 2014*). For analysis of viable counts cultures were diluted and plated on LB agar
163 supplemented with streptomycin (*Vercruysse et al., 2014*).

164

165 ***In vitro* growth assay in HT-29 cell line**

166 Mucin secreting human intestinal cell line HT-29 cells were maintained in Dulbecco's Modified
167 Eagle's Medium (DMEM, Sigma) , supplemented with 10% fetal bovine serum (FBS)
168 (HiMedia), 1% (vol/vol) non-essential amino acid and 1% (vol/vol) penicillin/streptomycin
169 (Sigma) mixture at 37°C under 5% CO2 . The survival of *V. cholerae* in the presence of mucin
170 secreting HT-29 cells were analysed by using previously described protocol (*Mondal et al*.,
171 2014). The 80% confluent, serum starved HT-29 cells in 12-well plate were infected with log
172 phase cultures of all *V. cholerae* strains at an infectious dose of 10^{7} c.f.u/ml. After 12 h of
173 incubation unbound cells were collected from the supernatant and cells were then treated with
174 0.1 % Triton X-100 for 2–3 min to detach the bound bacteria. Both the unbound and the bound
175 bacteria were collected, washed in PBS, serially diluted and plated on to LB agar to get viable
176 bacterial count.

177

178 **Motility Assay on semi solid agar**

Motility of all *V. cholerae* strains were examined on soft agar plates by a previously mentioned protocol (*Yeung et al., 2012*). The soft agar plates contained minimal media supplemented with 0.4% porcine mucin and 0.3% agar. All the strains were grown to log phase and 1 µl of each of the cultures were spotted on soft agar plates and incubated at 37°C for 15 h. Motility were analysed by measuring the diameter of the surface motility zone.

Mucin penetration assay

The assay was performed according to previously described protocol (*Liu et al., 2008*). In brief, 1% mucin columns were prepared in 1ml syringes. Log phase cultures were taken, washed and 0.1 ml of culture containing equal number bacteria (10^8 c.f.u/ml) were added from the top of 1% mucin columns. Columns were then kept at 37°C under static conditions. After 30 min of incubation 500 µl fractions were collected from the bottom of the columns, serially diluted and plated onto LB agar to measure the bacterial count.

HT-29 cell adhesion assay

For detection of bound bacteria in HT-29 cell, we followed a modified procedure from previously used protocol was followed (*Debnath et al., 2015*). 80% confluent HT-29 cells maintained in DMEM as mentioned before in 12 well plates and were serum starved overnight before treatment. These were then treated with log phase cultures of all three strains of *V. cholerae* at a dilution of 10^7 c.f.u/ml and incubated at 37 °C for 1 h in 5% CO2, cells were washed three times with PBS and detached using 0.1% Triton X-100. Adherent bacteria were counted after serial dilution by plating on LB agar plates.

For qualitative analysis of bacterial adhesion we used GFP labelled bacterial strains and followed a previously mentioned protocol (*Debnath et al., 2015*). HT-29 cells were cultured on glass coverslips in 12 well plates until (70-80) % confluent and infected with 10^7 c.f.u/ml of GFP labelled strains. After 1 h of incubation, bound bacteria were washed 3 times with PBS and mounted on glass slides with mounting medium. Glass slides were observed under fluorescence microscope (Olympus AX-70) to show the GFP labelled bacteria bound to HT-29 cells.

Suckling mouse colonization

Bacterial colonization in suckling mice intestine were assessed by *in vivo* competition assay in the procedure described before (*Ding et al., 2004*). Log phase cultures of of wild type *V. cholerae* ($LacZ^-$) strain was mixed at a ratio of 1:1 with each of the strains i.e $ChiS^+$, $ChiS^-$, $ChiS^*$ and complemented $ChiS^c$ strains ($LacZ^+$). The mixed cultures were orally inoculated at a concentration of approximately 5×10^7 c.f.u/ml into five day old infant mice and incubated for 18 h. Mice intestine were then harvested, homogenized, washed and serially diluted to plate on LB agar supplemented with streptomycin (100 μg/ml). Competitive index was calculated by the following equation:- ratio $out_{(mutant/wild\text{-}type)}$/ratio $in_{(mutant/wild\text{-}type)}$. The competitive Index (CI) value of $CI<1$ indicates a fitness defect and that of $CI>1$ indicates an increased fitness..

Fluid accumulation in ileal–ligated rabbit model and bacterial recovery from rabbit intestine.

New Zealand rabbits were used for the fluid accumulation assay. Rabbit weighing approximately 2.5 kg was used for the assay as described (*Mondal et al., 2014, Debnath et al., 2015*). Bacterial inoculums of each of the strains were adjusted to 10^9 c.f.u/ml and introduced in rabbit ileum.

Fluid accumulation was measured after 18 h infection in rabbit. Fluid accumulation was calculated as FA ratio= volume of fluid accumulation (ml) / intestinal length (cm). PBS was used as a negative control. Bacteria were counted by homogenizing the intestinal sections in 1 ml PBS. To determine the actual bacterial c.f.u at the time of intestinal harvest, bacteria were collected from the intestine, washed, serially diluted and plated on LB agar supplemented with streptomycin (100 μg/ml). β-hexosaminidase assay were also performed under *in vivo* conditions by collecting bacteria from intestinal samples of rabbit during ileal loop experiment.

RNA isolation and quantitative RT-PCR *in vitro* and *in vivo*

Bacteria were also harvested from rabbit intestinal loops (*in vivo*) after infecting with all the *V. cholerae* strains separately in each loop. Bacterial pellets were washed thrice in PBS and then used for RNA isolation. Total RNA was isolated using Trizol (Invitrogen) following the manufacterer's protocol. DNase treatment was performed using DNA free kit (Ambion) for elimination of contaminating genomic DNA followed by cDNA synthesis using reverse transcription kit (Promega) according to the manufacturer's protocol with 1 μg of total RNA for each of the 20 μl reactions. The mRNA transcript levels were quantified by quantitative PCR (qPCR) using 2×SYBR green PCR master mix (Applied Biosystems) and 0.2 μM of specific primers (*toxT, tcpA, ctxA*) designed using IDT for each transcripts **(Table S1)**. Data analysis was done using 7500 Real Time PCR detection system (Applied Biosystems, Foster City, California). The relative expression of the target transcripts were calculated according to Livak method (*Livak and Schmittgen., 2001*) using *recA* as an internal control.

GM1 ELISA for CT estimation *in vivo*

The ability of *V. cholerae* strains to express cholera toxin (CT) *in vivo* was assayed by GM1 enzyme-linked immunosorbent assay (ELISA) (*Holmgren., 1973*) using polyclonal anti-CT antibody (Sigma). CT was detected in the intestinal fluid accumulated in rabbit ligated ileal loop. The fluid collected was centrifuged and filtered using 0.45 μm membrane filter (Millipore). The amount of CT produced was determined using a standard curve obtained with purified CT and absorbance was measured at 492 nm. The average OD_{492} obtained from triplicate wells of each experimental sets were considered to estimate the amount of CT present in the samples using the standard curve (*Patra et al., 2012*).

Statistical analysis

The suckling mice colonization data were graphically plotted by using Graphpad Prism software and analysed by using one way ANOVA. Rest of the experiments were analysed by student's t test. Each of the experiments were done in triplicates and the results were represented as mean ± SEM. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Activation of ChiS in the presence of mucin

β-hexosaminidase activity is a measure of ChiS activation and its effect on the chitin utilization pathway (*Li and Roseman, 2004*). Here, we measured the total β-hexosaminidase activity in all the *V. cholerae* strains in presence or absence of mucin as a sole nutrient source. Total hexosaminidase activity in the $ChiS^+$ strain in the presence of mucin was 180.5 nmoles/min/mg compared to 24 nmoles/min/mg in the absence of mucin. So, in the presence of mucin, ChiS activation was induced 7.4 fold higher in $ChiS^+$ strain in the presence of mucin (**Fig:1**).

However, the ChiS⁻ strain showed negligible activity of the enzyme in presence or absence of mucin. On the other hand, the ChiS* strain showed constitutive activation of β-hexosaminidase without requiring any induction by mucin. The ChiSᶜ strain also showed similar activation to the ChiS⁺ strain in presence of mucin. Additionally, we also found RNA expression of ChiA2 was 5 fold less and total mucinase activity to be 9 fold less in ChiS⁻ strain than the ChiS⁺ strain in mucin supplemented media **(Fig:S1, S2)**. Therefore, this indicated that mucin induced the activation of ChiS which further turned on the chitin utilization pathway genes as well as the extracellular chitinase ChiA2 .

ChiS helps *V. cholerae* to utilize mucin

Next, we measured the growth rate of all the strains in minimal media supplemented with mucin (**Fig:2A**) or sodium lactate (**Fig:2B**). The growth rate of the ChiS⁺ strain in mucin supplemented minimal medium after 72 h was 6.1×10^8 c.f.u/ml compared to the ChiS⁻ strain with that of 3×10^7 c.f.u/ml . So, the growth rate of the ChiS⁻ strain was severely 20 fold diminished compared to the ChiS⁺ strain. The ChiS* and ChiS☒strains showed similar growth as of the ChiS⁺ strain in mucin supplemented medium. However, the growth rate of all the strains were similar in sodium lactate supplemented minimal medium indicating equal fitness of all the strains. This indicated ChiS is essential for utilizing mucin as a sole nutrient source.

Motility and mucin penetration depends on ChiS

We investigated the motility of different *V. Cholerae* strains in presence of mucin (**Fig:3A, 3B**) . In plate assay, we found all the strains except ChiS⁻ showed similar motility. However, we found that motility zone in case of the ChiS⁻ strain was 0.36±0.07 cm and that of the ChiS⁺ strain was

1.8±0.11 cm. Therefore, motility of the ChiS$^-$ strain was reduced to 5 fold compared to the ChiS$^+$ strain ($P< 0.05$). Taken together, this indicates ChiS is required to promote motility in *V. Cholerae* in the presence of mucin.

Next, we investigated the role of ChiS on mucin layer penetration *in vitro* (**Fig:3C**). Out of all the loaded bacterial cells 2.6×10^7 c.f.u/ml ChiS$^+$ viable cells penetrated through mucin layer, whereas, 2×10^6 c.f.u/ml ChiS$^-$ strain was detected following mucin penetration. . Therefore, our data showed 13 fold reduction in mucin penetration ability by the ChiS$^-$ mutant strain compared to the ChiS$^+$ wild type strain ($P< 0.05$). ChiS* and ChiS^c showed almost similar mucin penetration compared to ChiS$^+$ strain. This indicates that ChiS helps *V. cholerae* to penetrate the mucin layer *in vitro*.

Adhesion and survival of *V. cholerae* in the presence of HT-29 cells is dependent on ChiS.

After penetration through the mucin layer of the intestine *V. cholerae* needs to adhere to the epithelial cells in the intestine to initiate the infection. We studied the effect of ChiS on initial adherence of *V. cholerae* to HT-29 cells under fluorescence microscopy **(Fig:4A).** The GFP labelled ChiS$^-$ strain was less visible in adhered form with HT-29 cells compared to the ChiS$^+$ strain. We also studied the adhesion assay quantitatively **(Fig:4B)**. The bacterial count for ChiS$^+$ bound to HT-29 cells was 1.08×10^8 c.f.u/ml and that of ChiS$^-$ was 1.83×10^7 c.f.u/ml. Therefore, we found that the ChiS$^-$ strain to be 6 fold more defective to adhere to the HT-29 cells when compared to the ChiS$^+$ strain ($P< 0.05$). ChiS* and ChiS^c showed adherence almost similar to the ChiS$^+$ strain.

Here, the impact of ChiS on survival of *V. cholerae* was also analysed by infecting mucin secreting HT-29 cells (**Fig:4C**). After 12 h of infection, the viable counts for the ChiS$^+$ strain

was 7.7×10^{7} c.f.u/ml and that of the $ChiS^{-}$ strain was 5.9×10^{6} c.f.u/ml in the presence of HT-29 cells. Our result showed that the $ChiS^{-}$ strain was 13 fold less efficient to survive when compared to the $ChiS^{+}$ strain ($P< 0.05$). ChiS* and ChiS⊠strains showed survival similar to that of the $ChiS^{+}$ strain. This indicated that ChiS was important for *V. cholerae* survival in the presence of HT-29 cells.

ChiS affects suckling mice colonization in mice

Bacterial binding to intestinal epithelial cell facilitates bacterial colonization in the intestine. We have already showed the $ChiS^{-}$ strain to be defective in adhesion *in vitro*. Therefore, we next examined the role of ChiS in colonization of suckling mice by using competition assay **(Fig:5)** . The input ratio during bacterial infection was 1:1 of *V. cholerae*. After 18 hrs the output ratio of $ChiS^{-}lacZ^{+}/ChiS^{+}LacZ^{-}$ was ≈0.0001 indicating a high fitness defect for the $ChiS^{-}$ strain ($P< 0.05$). In contrast, $ChiS^{c}$ and ChiS* strains showed almost no competitive disadvantage. Additionally, we also determined the Competitive Index (CI) between $ChiS^{+}LacZ^{-}/ChiS^{+}LacZ^{+}$ and we found CI≈1 indicating no fitness defect of the $LacZ^{-}$ mutant over $LacZ^{+}$. Taken together, this indicated that the $ChiS^{+}$ strain outcompeted $ChiS^{-}$ strain in the infant mice colonization. Therefore, we concluded that *V. cholerae* ChiS contributes in intestinal colonization.

ChiS depletion in *V. cholerae* results in reduced pathogenesis in rabbit intestine.

Till now, we have shown that ChiS affects *V. cholerae* colonization efficiency. In this experiment, we have qualitatively shown and measured the intestinal fluid accumulation in rabbit ileal ligated model by evaluating FA ratio **(Fig:6A, 6B).** In rabbit intestine, infection with the $ChiS^{-}$ strain showed 6 fold reduction in fluid accumulation compared to the wild type *V.*

cholerae ChiS^{+} after 18 h of infection ($P< 0.05$). Infection with ChiS* and ChiS^{c} strain showed fluid accumulation similar to the ChiS^{+} strain. We also measured the c.f.u recovered from the rabbit intestine **(Fig:6C)**. In case of the ChiS^{+} strain, bacteria recovered was 1.03×10^{7} c.f.u/gm of intestine and that of the ChiS^{-} strain was 7×10^{5} c.f.u/gm of intestine. Therefore, we found upto 15 fold less recovery in case of the ChiS^{-} strain ($P< 0.05$).This indicated that ChiS is involved in colonization of *V. cholerae* and fluid accumulation in the host intestine, which is one of the critical aspects of its pathogenesis.

Activation of ChiS in the host intestine.

We also analysed total β-hexosaminidase activity to evaluate ChiS induction in each strains *in vivo* from fluid accumulated samples in the rabbit intestine **(Fig:7)**. β-hexosaminidase activity in ChiS^{+} was 102 nmoles/min/mg whereas the ChiS^{-} strain showed activity of 23 nmoles/min/mg. The ChiS^{+} strain therefore, showed 4.4 fold higher β-hexosaminidase activity compared to the the ChiS^{+} strain ($P< 0.05$). Induction of β-hexosaminidase activity in ChiS* and ChiS^{c} strains were similar to ChiS^{+} strain. Therefore, this indicated that ChiS is activated in the host intestine and thus affects pathogenesis of *V. cholerae.*

ChiS contributes in virulence gene expression and cholera toxin (CT) production in *V. cholerae*

Since we found differential colonization and less fluid accumulation in rabbit intestine, we analyzed the virulence gene expression (*ctxA, toxT,* and *tcpA*) in *V. cholerae* strains harvested from rabbit ileal loop samples **(Fig:8A)**. We found *ctxA, toxT,* and *tcpA* RNA levels to be significantly reduced by 3 fold, 4.5 fold and 4 folds less, respectively, in the ChiS^{-} strain when

362 compared to the ChiS^{+} wild type (P< 0.05). ChiS* and ChiS^{c} showed *ctxA, toxT,* and *tcpA* RNA
363 levels similar to the ChiS^{+} strain**.** We also measured cholera toxin production of all the strains of
364 *V. cholerae* in the intestinal fluid samples from the rabbit ileal loop after 18 h of infection
365 **(Fig:8B)**. We found fluid from the ChiS^{-} infected ileal loop sample to contain less cholera toxin
366 (210 ng/ml) with a difference of 6.5 fold compared to the ChiS^{+} (1220 ng/ml) (P< 0.05).
367 Additionally, in AKI media ChiS^{-} strain showed significant decrease in the RNA levels of these
368 virulence genes (*ctxA, toxT,* and *tcpA*) compared to ChiS^{+} strain **(Fig:S4)**
369
370 **DISCUSSION**
371 It has been previously reported that there are many TCS in pathogenic bacteria that contributes to
372 virulence. ChiS is a component of TCS in *V. cholerae*. Although ChiS is the regulator of *V.*
373 *cholerae* extracellular chitinases like ChiA2 (*Meibom et al., 2004*)**,** its function in pathogenesis is
374 still unknown. Therefore, in this study we have aimed to understand its role in pathogenesis.
375 It is known that, *V. cholerae* ChiS is activated in the presence of GlcNAc oligosaccharides
376 of chitin in the aquatic environment (*Li and Roseman., 2004*). The activation of ChiS promotes
377 the expression of downstream chitin utilization pathway components like periplasmic-β-N-
378 acetylglucosminidase, etc (*Meibom et al., 2004*). Our results showed that ChiS is also activated
379 in the presence of intestinal mucin. Most probably the GlcNAc oligosaccharide residues of
380 mucin activates ChiS in the same way as it does in the aquatic environment. This leads to the
381 activation of the chitin utilization pathway in a similar manner as mentioned before and results
382 into the expression of extracellular chitinases like ChiA2.
383 The activation of ChiS is governed by chitin oligosaccharide binding protein (CBP) that
384 binds to keep ChiS in a deactivated mode in the absence of GlcNAc residues. Once CBP when

385 binds to GlcNAc residues, it is released from ChiS leaving the sensor kinase in activated mode
386 (*Li and Roseman., 2004*). We also observed that activation and deactivation cycle of ChiS takes
387 place in presence of intestinal mucin. In absence of CBP, ChiS remains constitutively active even
388 in the absence of GlcNAc oligosaccharides (*Li and Roseman., 2004*). In our case also, the
389 induction by mucin was not required in the *cbp* mutant strain (ChiS*). Therefore, we confirmed
390 that *V. cholerae* ChiS is induced in the presence of mucin.

391 *V. cholerae* can utilize mucin as a sole nutrient source (*Mondal et al, 2014*). Our results here
392 showed that mucin utilization by *V. cholerae* depends upon ChiS. In absence of ChiS, *V.*
393 *cholerae* showed poor growth even in presence of mucin in minimal media as well as in the
394 mucin secreting intestinal cells. This suggests that ChiS contributes in utilization of mucin by *V.*
395 *cholerae* which helps the bacteria to survive in mammalian host intestine. There are many
396 intestinal microbes that utilize mucin as an energy source (*Chen et al., 2002, Deplancke et al.,*
397 *2002, Derrien et al., 2010*). *Clostridium perfringens*, an opportunistic intestinal pathogen was
398 able to grow on medium with mucin as a substrate (*Deplancke et al., 2002*) and $(GlcNAc)_2$ (*Chen*
399 *et al., 2002*). Other intestinal microbes like *Bacteroides fragilis* could utilize
400 GlcNAc; *Escherichia coli, Lactococcus lactis* and *Proteus vulgaris* could utilize $(GlcNAc)_{1–6}$
401 (*Chen et al., 2002*). *Bifidobacterium adolescentis* and *Eubacterium limosum* could use
402 $(GlcNAc)_{1–6}$ to some extent as their main carbon source (*Chen et al., 2002*).

403 Earlier, it has been shown that *V. cholerae* utilizes mucin by the help of an extracellular
404 chitinase ChiA2 (*Mondal et al, 2014*). ChiA2 cleaves the oligosaccharide moieties of mucin
405 (*Mondal et al., 2014*). These residues then help to switch on the chitin utilization pathway that
406 results in catabolism of GlcNAc residues of mucin. ChiS contributes in the utilization of mucin
407 as nutrient source by inducing the extracellular chitinases like ChiA2. Additionally, here we also

found significant differences in RNA expression of ChiA2 and chitinase activity assay between ChiS^{+} and ChiS^{-} strain in mucin supplemented media **(Fig:S2, Fig:S3).** .

When *V. cholerae* reaches the small intestine, the mucosal layer acts as a barrier. Thus trespassing this mucosal barrier is one of its aspects of virulence. Motility is important for *V. cholerae* in order to carryout mucin penetration (*Liu et al., 2015*). In this study, we found ChiS is important for *V. cholerae* motility in mucin and its penetration. This can be explained by the fact that when ChiS is activated by mucin ChiA2 is induced along with other chitinases to remove the sugar residues from mucin. This weakens the integrity of mucin. This provides easy access for the proteases to degrade mucin (*Sanders et al., 2007*). This leads *V. cholerae* to swim faster as well as penetrate into mucin layer to reach the intestinal epithelium for successful colonization. Our result suggested that the ChiS^{-} strain showed reduced adherence to intestinal cells, leading to defective colonization. Therefore, ChiS, a component of TCS, is found to be important for intestinal colonization by *V. cholerae*. A previous study with VprAB which is also a *V. cholerae* TCS has been found to contribute to its intestinal colonization (*Herrera et al., 2014*).

The ChiS^{-} strain in rabbit intestine showed reduced fluid accumulation, which is due to the reduced cholera toxin production. This was in accordance with our result, where we found reduced expression of *ctxA*. Decreased expression of *ctxA* along with *tcpA* was due to reduced expression of *toxT*. It is well established that lower *toxT* expression is linked to reduced *ctxA* and *tcpA* (*DiRita et al., 1991*). This indicates that ToxR regulon might be affected in the ChiS^{-} strain. The unability to utilize mucin by *V. cholerae* in the intestine decreases GlcNAc residues in the ChiS^{-} strain which might activate cyclic AMP (cAMP) receptor protein (CRP) (*Kovacikova et al., 2004*). This negatively regulates the ToxR regulon via cAMP-CRP pathway (*Skorupski and Taylor., 1997*). *In vitro*, we have also observed decreased production of virulence genes in AKI

media **(Fig:S4)**. However, further experiments are needed to establish the link between ChiS and ToxR regulon.

Additionally, delivery of the cholera toxin requires successful *V. cholerae* colonization in the small intestine (*Taylor et al., 1987, Ritchie et al., 2010*). Reduced colonization by ChiS$^-$ leads to decreased cholera toxin production as well as less fluid accumulation.

Taken together, our data indicate that *V. cholerae* ChiS gets activated in the host intestine by mucin. It contributes to mucin utilization by the bacteria which helps *V. cholerae* to survive in the intestine. On the other hand, ChiS plays a role in *V. cholerae* pathogenesis, probably through nutrient acquisition from mucin in the intestine during infection. However, further studies are needed for a complete understanding of the function of ChiS in this event.

CONFLICT OF INTEREST

The authors have no conflict of interest.

ACKNOWLEDGEMENTS

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562
563 **FIGURE LEGENDS**
564 **Fig.1: Activation of ChiS is promoted in the presence of mucin**: Bacteria were grown
565 in minimal medium supplemented with or without porcine mucin. 0.5% of sodium lactate
566 was added in each medium to obtain similar bacterial growth. Log phase cultures were
567 taken to measure the total hexosaminidase (ChiS regulated periplasmic enzyme) activity
568 in ChiS^{+} (*V. cholerae* N16961 wild type), ChiS^{-} (isogenic *ChiS* mutant), ChiS* (*cbp*

mutant expressing ChiS constitutively) and ChiS^{c} (complement of ChiS^{-}) strains in presence (■) or absence (■) of 2% mucin. †, $P < 0.05$. Error bars represent standard errors from three biological replicates (n=3).

Fig.2: ChiS contributes in utilization of mucin as a sole nutrient source. . ChiS^{+} (*V. cholerae* N16961 wild type), ChiS^{-} (isogenic *ChiS* mutant), ChiS* (*cbp* mutant expressing ChiS constitutively) and ChiS^{c} (complement of ChiS^{-}) strains were inoculated separately in **(A)** minimal media supplemented with 2% (w/v) porcine mucin and **(B)** 0.5% sodium lactate as the only carbon source . The viable bacterial counts were detected by plate count method and represented graphically. Each of the experiment was repeated three times (n = 3) and the data were expressed as mean ± SEM.

Fig.3: Motility and mucin penetration is promoted by ChiS in *V. cholerae*: ChiS^{+} (*V. cholerae* N16961 wild type), ChiS^{-} (isogenic *ChiS* mutant), ChiS* (*cbp* mutant expressing ChiS constitutively) and ChiS^{c} (complement of ChiS^{-}) strains were separately grown in LB till log phase. **(A)** Soft agar plates showing differences in motility between ChiS^{-} strain and all other strains. 1 µl of each of the cultures were spotted on plates containing minimal media supplemented with 0.4% porcine mucin and 0.3% agar. Plates were incubated for 15 h at 37°C. **(B)** Diameter of the surface motility zones are graphically represented. Motility were analysed by measuring the diameter of the surface motility zone. †, $P < 0.05$. The result shown is a mean of ±SEM of three biological replicates (n = 3). **(C)** 10^{7} c.f.u/ml of ChiS^{+} (*V. cholerae* N16961 wild type), ChiS^{-} (isogenic *ChiS* mutant), ChiS* (*cbp* mutant expressing ChiS constitutively) and ChiS^{c}

591 (complement of ChiS⁻) were loaded on top of 1ml mucin (1%) columns and were
592 allowed to penetrate. Bacteria were collected from the bottom of the columns, serially
593 diluted and plated on LB agar to obtain the bacterial number by plate count method. †, P
594 < 0.05. The result shown is a mean of ±SEM of three biological replicates (n = 3).
595

596 **Fig.4: ChiS is important for bacterial adhesion and survival in presence of HT-29**
597 **cells.** ChiS⁺ (*V. cholerae* N16961 wild type), ChiS⁻ (isogenic *ChiS* mutant), ChiS* (*cbp*
598 mutant expressing ChiS constitutively) and ChiS^c (complement of ChiS⁻) strains were
599 grown to log phase and adjusted to 1 O.D. 80 % confluent HT-29 cells were then infected
600 with 10^7 c.f.u/ml of each strain and incubated at 37°C in 5 % humidified CO_2 incubator.
601 **(A)** Fluorescent Images of GFP labeled bacteria bound to HT-29 cells seen under Phase
602 contrast. i) HT-29 cells infected with ChiS⁺ strain, ii) HT-29 cells infected with ChiS⁻
603 strain, iii) HT-29 cells infected with ChiS*, iv) HT-29 cells infected with ChiS^c and v)
604 Non-infected HT-29 cells. **(B)** Adhesion assay: HT-29 epithelial cells were infected with
605 *V. cholerae* strains for 1 h. Bound bacteria were collected and plated. †, P < 0.05. Each of
606 the experiment was repeated three times (n = 3) and the data were expressed as mean ±
607 SEM. **(C)** Both bound and unbound bacteria were collected after 12 h incubation with
608 HT-29 cells. Samples were washed and serially diluted to plate on LB agar. Number of
609 bacteria were enumerated by plate count method. †, P < 0.05. The result shown is mean
610 of ±SEM of three biological replicates (n = 3).
611

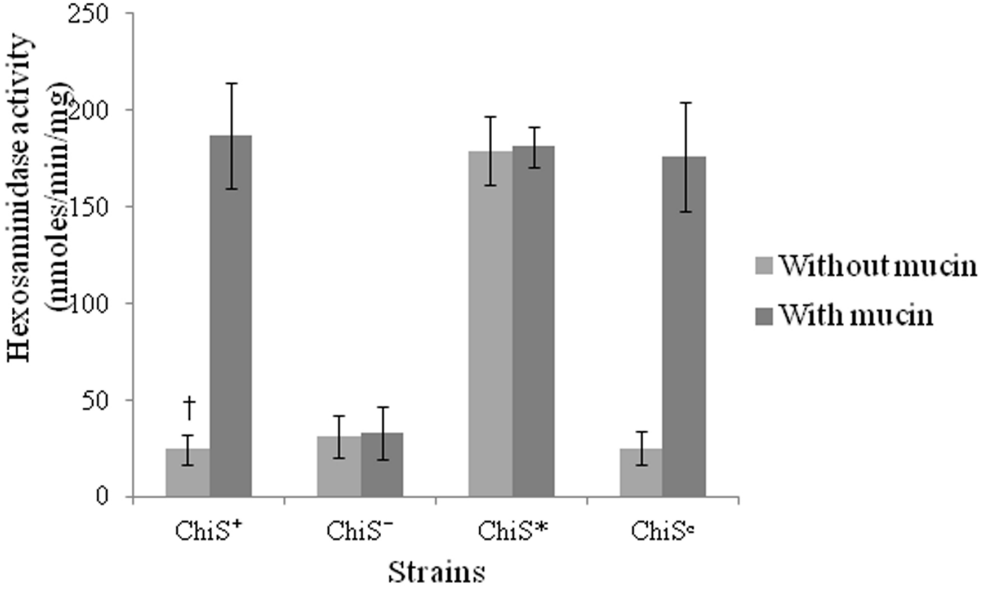
Fig.5: ChiS helps in *invivo* colonization of *V. cholerae*: Comparative study of colonization of ChiS^{+} (*V. cholerae* N16961 wild type), ChiS^{-} (isogenic *ChiS* mutant), ChiS* (*cbp* mutant expressing ChiS constitutively) and ChiS^{c} (complement of ChiS^{-}) strains in 5 days old suckling mice is presented here. Mice were orally inoculated with 5×10^{7} c.f.u/ml of wild type *V. cholerae* (LacZ^{-}) strain mixed at a ratio of 1:1 with each of the strains i.e ChiS^{+} (LacZ^{+}), ChiS^{-} (LacZ^{+}), ChiS* (LacZ^{+}) and complemented ChiS^{c} (LacZ^{+}) strains and incubated for 18 h. Mice intestine were harvested, homogenized, washed, serially diluted and plated onto LB agar. Competitive index (CI)=ratio $\text{out}_{(\text{mutant/wild-type})}$/ratio $\text{in}_{(\text{mutant/wild-type})}$. The competitive Index (CI) value of CI<1 indicates the a fitness defect, CI>1 indicates an increased fitness and CI≈1 indicates no fitness defect. $P < 0.05$. Each of the experiment was repeated three times (n=3) and the data were expressed as mean ± SEM.

Fig 6: *V. cholerae* ChiS contributes in fluid accumulation as well as colonization in rabbit intestine: Log phase cultures of ChiS^{+} (*V. cholerae* N16961 wild type), ChiS^{-} (isogenic *ChiS* mutant), ChiS* (*cbp* mutant expressing ChiS constitutively) and ChiS^{c} (complement of ChiS^{-}) strains were adjusted to 1 O.D and 10^{9} c.f.u./ml were inoculated into the intestinal ligated loops of a rabbit. **(A)** A representative rabbit intestine is presented here. Effects of *V. cholerae* strains in fluid accumulation are shown. PBS is used as a negative control. **(B)** Fluid accumulation ratio in rabbit ligated ileal loop were determined and represented graphically. †, $P < 0.05$. The result shown is a mean ±SEM of three biological replicates. **(C)** Rabbit intestinal samples were also harvested, homogenized, washed, serially diluted and plated onto LB agar to enumerate the intestinal colonization and the recovered c.f.u of each strain are graphically represented.

†, $P < 0.05$. Each of the experiment was repeated three times (n = 3) and the data were expressed as mean ± SEM.

Fig 7: *V. cholerae* ChiS is activated in the intestine: Log phase cultures of ChiS$^+$ (*V. cholerae* N16961 wild type), ChiS$^-$ (isogenic *ChiS* mutant), ChiS* (*cbp* mutant expressing ChiS constitutively) and ChiS^c (complement of ChiS$^-$) strains were adjusted to 1 O.D and 10^9 c.f.u./ml were inoculated into the rabbit intestinal ligated loops. *In vivo* hexosaminidase assay was performed by the samples collected from fluid accumulated in the intestinal loops. †, $P < 0.05$. Each of the experiment was repeated three times (n = 3) and the data were expressed as mean ± SEM.

Fig.8: *V. cholerae* ChiS affects cholera toxin production and virulence gene expression in the intestine: Log phase cultures of ChiS$^+$ (*V. cholerae* N16961 wild type), ChiS$^-$ (isogenic *ChiS* mutant), ChiS* (*cbp* mutant expressing ChiS constitutively) and ChiS^c (complement of ChiS$^-$) strains were adjusted to 1 O.D and 10^9 c.f.u./ml were inoculated into the intestinal loops. **(A)** *In vivo* cholera toxin production was analyzed from the accumulated fluid samples of ligated ileal loop assay. †, $P < 0.05$. Each of the experiment was repeated three times (n = 3) and the data expressed as means ± SEM. **(B)** Bacteria were also harvested from rabbit intestinal ligated loops after infection for 18 h, RNA was isolated and virulence gene expression was measured by qRT-PCR. †, $P < 0.05$. Each of the experiment was repeated three times (n = 3) and the data expressed as means ± SEM.



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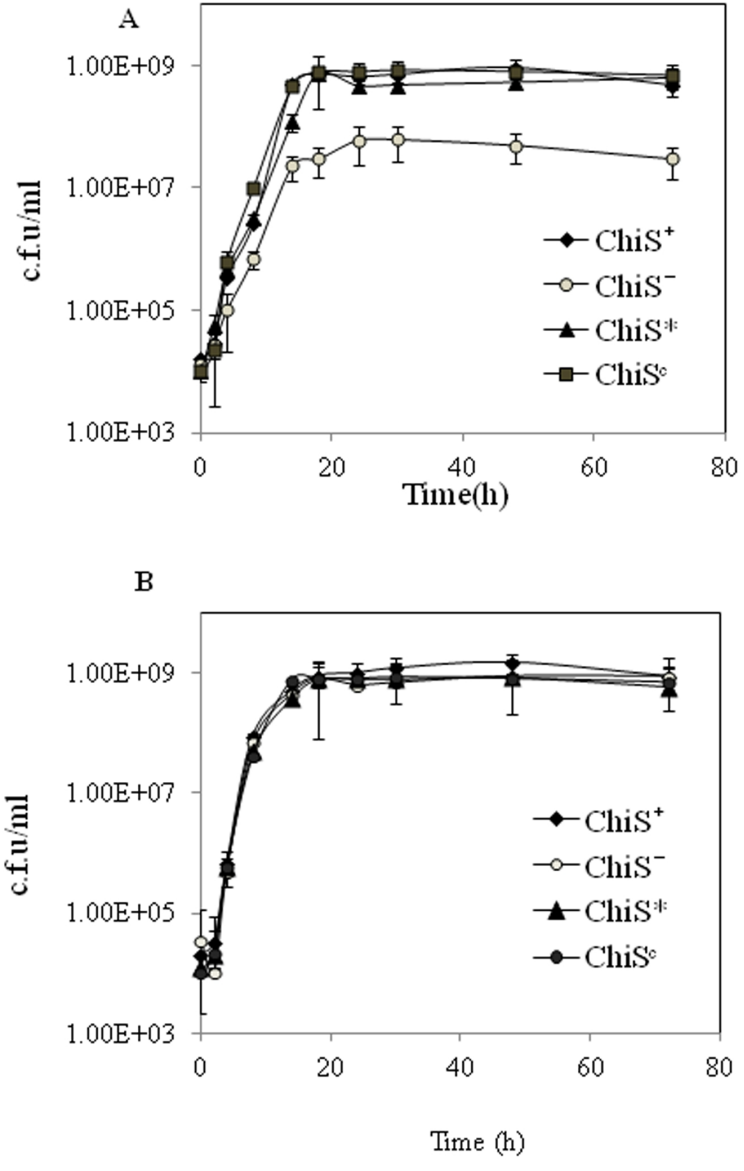

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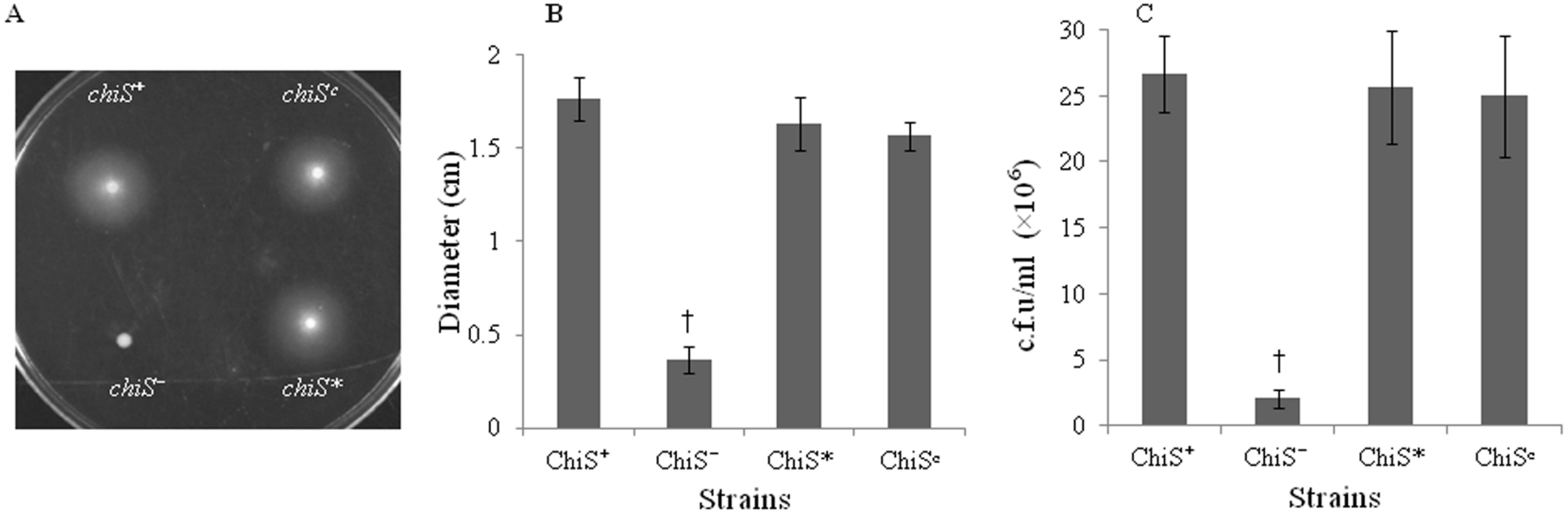

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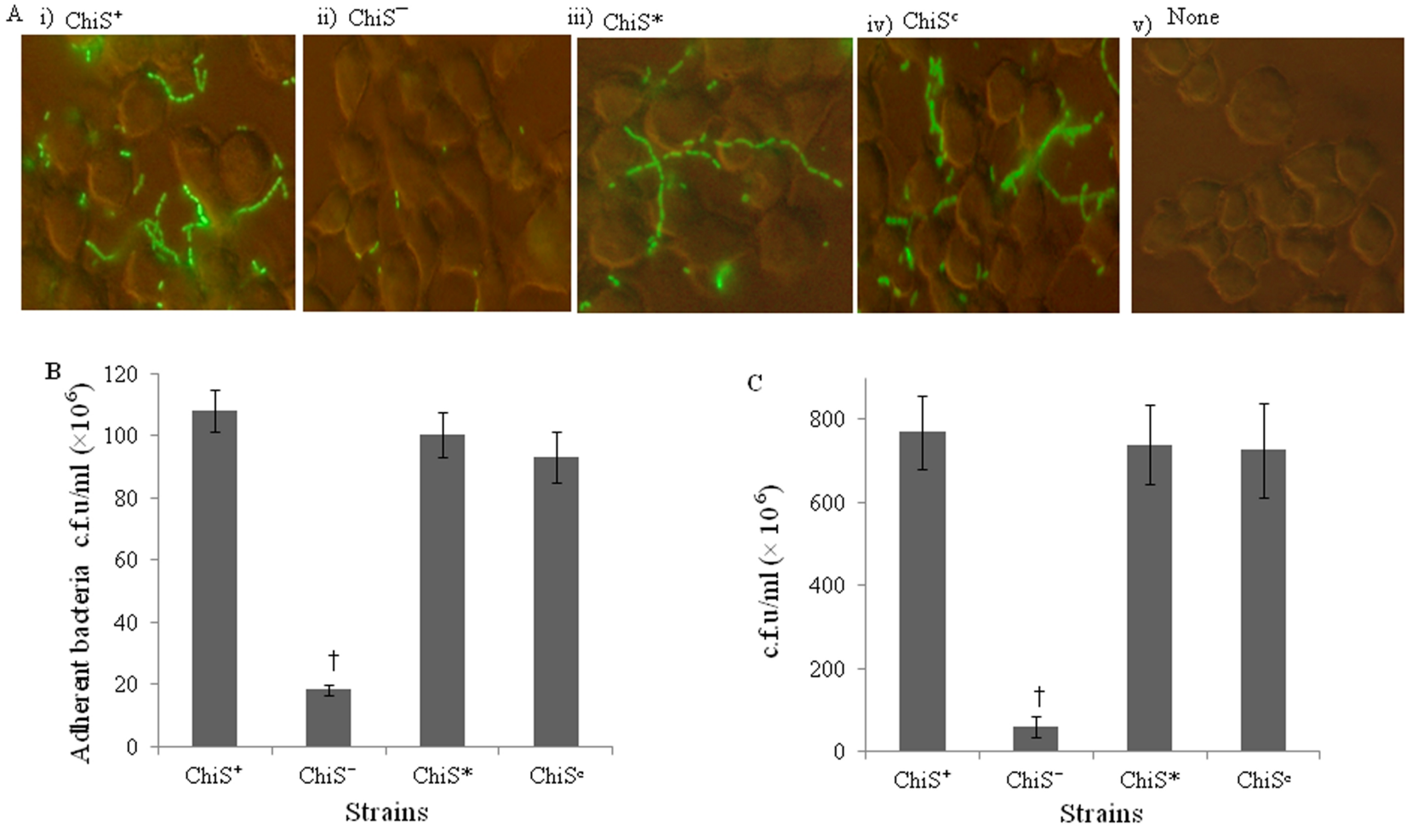

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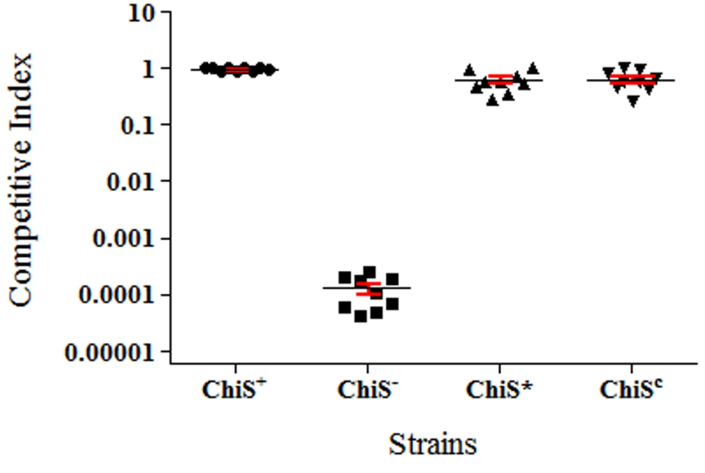

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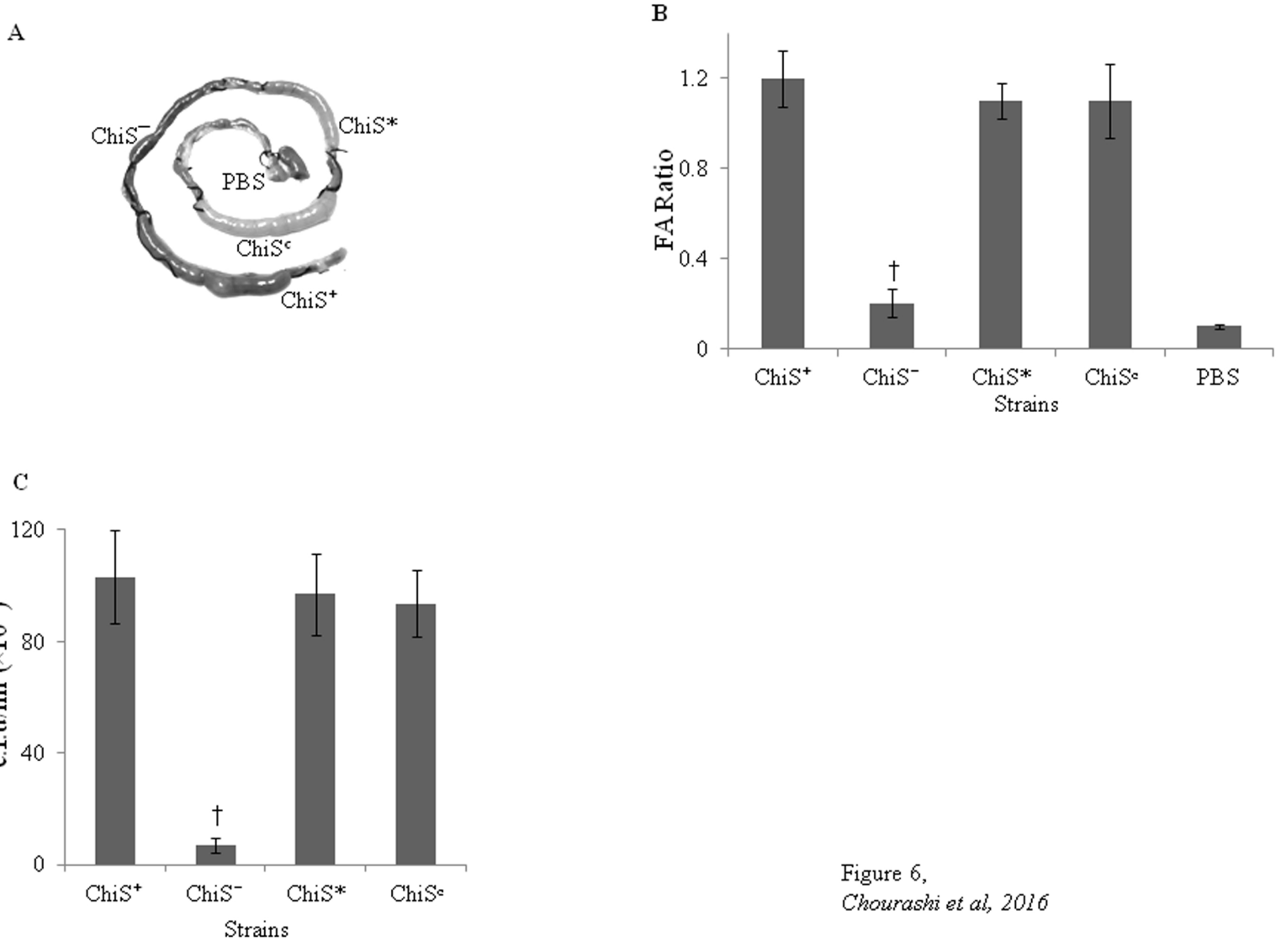

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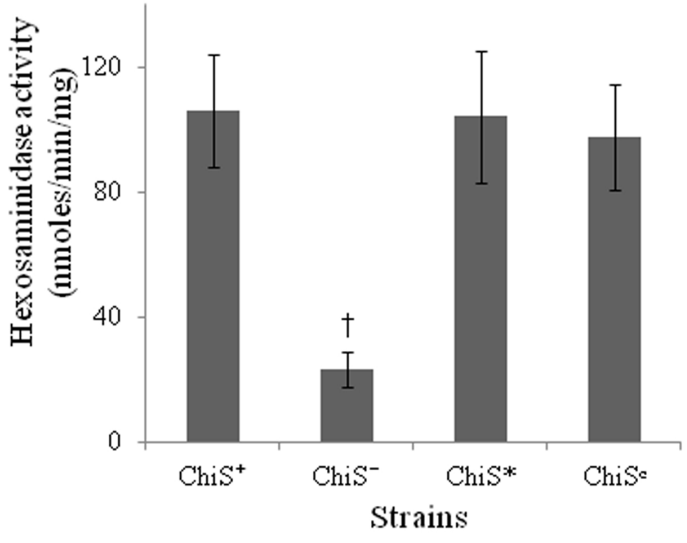

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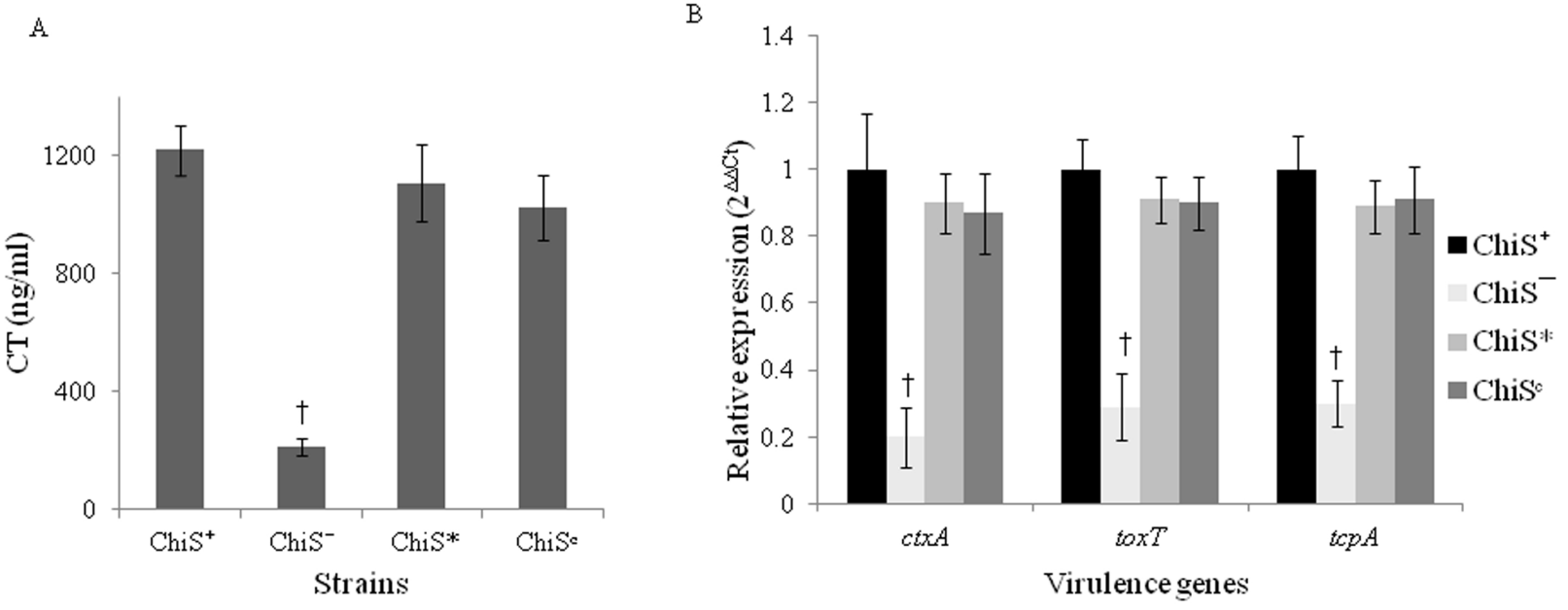

Figure 8,
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Supplementary material

Elaborated methodology

Bacterial strains and growth conditions

All strains used were streptomycin resistant *V. cholerae* N16961 (O1 ElTor Inaba) , streptomycin resistant strain was used as a wild type strain. . In each case bacteria were grown overnight in LB media (BD Difco) and then inoculated into suitable media for experiments. For showing depletion of ChiA2 expression analysis in ChiS¯ strain, bacteria were grown in minimal–lactate media containing M9 minimal medium (BD Difco), 50mM HEPES pH-7.5 (Sigma), filter sterile 0.2% $MgSO_4$ (Merck) and 0.01% $CaCl_2$ (SRL) with or without porcine mucin (sigma) as a sole nutrient source. Sodium lactate (Sigma) was also added to support equal growth of wild type and mutant strains during RNA analysis. For virulence gene expression study, bacteria were also cultured in AKI media containing (0.5% NaCl,0.3% $NaHCO_3$ purchased from Merck, 0.4% yeast extract and 1.5% peptone purchased from BD Difco) pH-7.2 at 37°C under static condition (*Abuaita et al., 2009*).

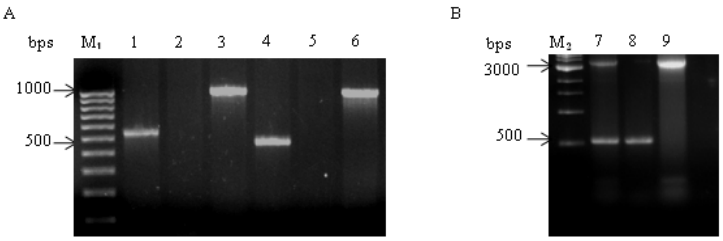
Chitinase activity assay

The N-acetylglucosamine concentration in the reaction mixture and the chitinase activity were determined by previously followed di-nitrosalicylic acid (DNS) method (*Mondal et al., 2014*). This method tests the free carbonyl groups in the reducing sugars. Chitinase activity was assayed here by estimating reducing sugars. Equal no bacteria were inoculated in minimal medium supplemented with mucin (pH-7.5). 0.5% sodium lactate (Sigma) was also added to support equal growth of both the strains. Log phase cultures were taken, bacteria were pelleted by

centrifugation and the crude supernatant from each of the bacterial culture were used as samples for the enzymatic assay. The samples were used to incubate with 0.5 mg/ml porcine mucin (Sigma) for 1 h at 37°C. In each case the control was done by using heat inactivated samples. The reaction was stopped by adding DNS solution. The mixture was boiled at 100°C for 10 min and cooled by keeping it in ice immediately after boiling. The amount of reducing sugar was estimated by measuring the OD at 540 nm.

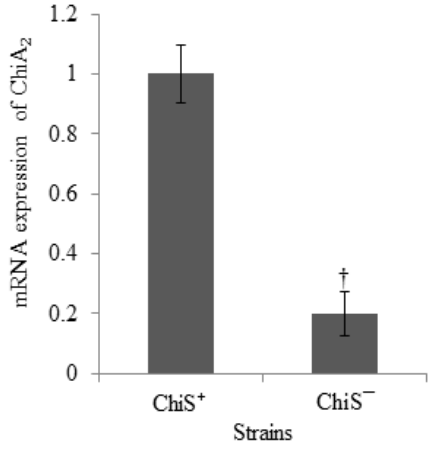
The amount of reducing sugar was calculated from a previously prepared standard curve. Total enzymatic activity were analyzed after measuring total protein by lowry method and then calculated by measuring the amount of GlcNAc produced in nmoles /mg of protein/ min.

RESULTS

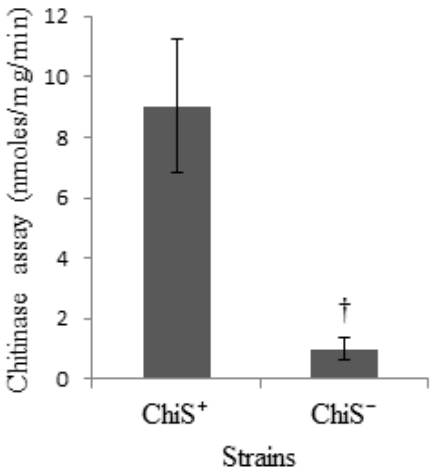


Supplementary Fig S1: Conformation of the in-frame deletion/insertion mutation of *chi*S gene (ChiS^{-}) and *cbp* gene (ChiS*) in *V. cholera* N16961 and complementation of ChiS^{-} mutant to obtain ChiS☒**.(A)** (M_1) 100 bp ladder, (1) Internal amplicon (540 bps) of *chiS* gene in ChiS^{+} or WT and (2) ChiS^{-} strain, (3) amplicon of inserted unmarked *chiS* fusion construct of 960 bps in ChiS^{-} strain, (4) Internal amplicon (450 bps) of *cbp* gene in WT and (5) ChiS* or Δ*cbp* strain and (6) amplicon of inserted unmarked *cbp* fusion construct of 945 bps. (B) (M_2)

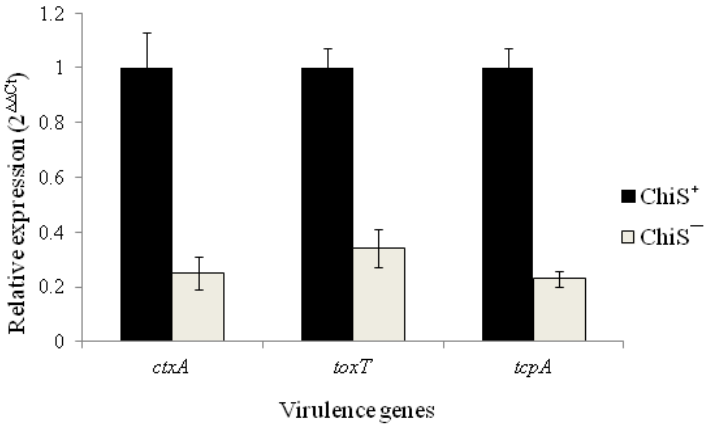
1Kb ladder, (7) complemented ChiS⁻ mutant strain with cloned 3.5 kb full length amplicon of *chiS* and amplicon of 538 bps from the deleted gene of *chiS,* (8) amplicon of 538 bps from the deleted gene of *chiS* in ChiS⁻ strain, (9) 3.5 kb full length amplicon of *chiS* from ChiS⁺ strain



Supplementary Fig S2: **ChiS knockout strain shows ChiA2 depletion in mucin supplemented media**. Bacteria were grown in minimal medium supplemented with 2% mucin as a nutrient source. 0.5% of sodium lactate was added in each medium to obtain similar bacterial growth. Log phase cultures were taken in every case. RNA expression of *ChiA2* in *V. cholerae* wt ChiS⁺ and the mutant strain ChiS⁻ were analyzed by qRT PCR and graphically represented. The transcript levels were normalized to *rec*A mRNA. †, $P < 0.05$. Each experment were repeated three times (n = 3) and the data were expressed as mean ± SEM.



Supplementary Fig S3: **ChiS knockout strain shows reduced mucinase activity**. $ChiS^+$ and $ChiS^-$ strains were grown in minimal medium supplemented with 2% mucin as a nutrient source. 0.5% of sodium lactate were added in each medium to obtain similar bacterial growth. Log phase cultures were taken in each case. Bacteria were pelleted and the culture supernatant were used for mucinase activity assay. †, $P < 0.05$. Each experment were repeated three times (n = 3) and the data were expressed as mean ± SEM.



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73 **Supplementary Fig.S4: ChiS affects virulence gene expression under *in vitro* conditions**: **(A)**

74 Log phase cultures of ChiS$^+$ (*V. cholerae* N16961 wild type) and ChiS$^-$ (isogenic *ChiS* mutant),

75 strains were inoculated (1:1000) in AKI media and grown at 37^{o}C for 5 h statically at 37°C.

76 RNA was isolated and virulence gene expression was measured by qRT PCR**.** †, $P < 0.05$. Each

77 of the experiment was repeated three times (n = 3) and the data were expressed as mean ± SEM.

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Table S1. List of primers used in this study

Names	Sequence 5'-3'	Purpose
ChiSFP(A) XbaI	CAGCTCTAGACCGGGCATCACTACAACTAT	deleted *chiS* construct
ChiS RP (B) Fus	CCCATCCACTATAAACTAACAAACGCAGCCATCAAGGTATT	deleted *chi*S construct
ChiS FP (C) Fus	TGTTAGTTTATAGTGGATGGGATTTGATGCGTGCCGTGTTA	deleted *chiS* construct
ChiS RP (D) SacI	GAATCGAGCTCATTCAGTTGTTGCCTAGCGG	deleted *chiS* construct
ChiS internal F	GAACAACTGGAGCACATCTT	Screen Δ*chiS* mutants
ChiS internal R	CGTCAGCATAATAATAGGCA	Screen Δ*chiS* mutants
CBP FP(A) XbaI	CAGCTCTAGAACTCAGGCAAAGAGCCAT	deleted *cbp* construct
CBP RP (B) Fus	CCCATCCACTATAAACTAACACGCTGGAGCTGAAACACT	deleted *cbp* construct
CBP FP (C) Fus	TGTTAGTTTATAGTGGATGGGGAAGAAAATCCGAAGGGC	deleted *cbp* construct
CBP RP (D) SacI	GAATCGAGCTCTGATTGAAGTGCCCAGCT	deleted *cbp* construct
CBP internal F	CCTACAATGGTACGTAACTTC	Screen Δ*cbp* mutants
CBP internal R	AAGGACCAGTACCTACTGGAT	Screen Δ*cbp* mutants
ChiS comp F	ATGTTTAGGTTCTATCGAAA	Cloning into pBad for complementation of deleted ChiS strain
ChjS comp R	TTATTCACTGGTCAGGAGTT	
ctxA RT FP	CTCAGACGGGATTTGTTAGGCACG	RNA levels of *ctxA*
ctxA RT RP	TCTATCTCTGTAGCCCCTATTACG	RNA levels of *ctxA*
toxT RT FP	CAGCGATTTTCTTTGACTTC	RNA levels of *toxT*
toxT RT RP	CTCTGAAACCATTTACCACTTC	RNA levels of *toxT*
tcpA RT FP	GCTACCGCAAACGCAAATG	RNA levels of *tcpA*
tcpA RT RP	CCCATAGCTGTACCAGTGAAAG	RNA levels of *tcpA*
ChiA2 RT FP	CTACCGCCCAGTTTACTTATCC	RNA levels of *ChiA2*
ChiA2 RT RP	AACCATCGGTATCCGCAATAG	RNA levels of *ChiA2*
RecA RT FP	GCAATCAAAGAAGGCGAAGAAG	Internal control for RT
RecA RT RP	GGCCATACATGATCTGAGTGTT	Internal control for RT

FP: Forward Primer; RP: Reverse Primer: Fus: Fusion; RT: Real Time; *chiS*-Locus No: VC0622; *cbp*-VC0620; *ctxA*-VC1457; *tcpA*-VC0828; *toxT*-VC0838; *ChiA2*-VCA0027; *recA*-VC0543.

Table S2. List of strains and plasmids used in this study

Strains orPlasmids	Description	Source
Strains		
E. coli		
SM10 λpir	*E. coli*, λpir host for R6K origin plasmids and mobilizing strain, Kan^R	(1)
V.cholerae		
N16961 $ChiS^+$	Wt*Vibrio cholerae* O1 El Tor, Str^R	(2)
N16961 $ChiS^-$	Δ*vc0622,* Str^R	This study
N16961 $ChiS^c$	Δ*vc0622,* Str^R +*chiS* in pBad (complemented strain)	This study
N16961 ChiS*	Δ*vc0620,* Str^R, constitutive expression of *chi*S	This study
Plasmids		
pGEM-T easy	TA-cloning vector, Amp^R	Promega
PCVD442	Suicidal conjugation vector carrying *sacB*, Amp^R	(3)
pBad-Topo	TA-cloning vector, Amp^R	Invitrogen

Resistance to Amp^R: Ampicillin, Str^R: Streptomycin

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4. Skorupski, K., Taylor, R.K., 1996. Positive selection of vectors for allelic exchange. Gene 169, 47–52.