Canadian Journal of Microbiology
Revue canadienne de microbiologie

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Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2015-0556.R1
Manuscript Type:	Article
Date Submitted by the Author:	30-Oct-2015
Complete List of Authors:	Mondal, Moumita; National Institute of Cholera and Enteric Diseases, Biochemistry Chatterjee, Nabendu; National Institute of Cholera and Enteric Diseases, Biochemistry
Keyword:	<i>V. cholerae</i> chitinase, ChiA2, chitin, horizontal gene transfer, tfoX

1 **Manuscript ID: cjm-2015-0556**

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3 **Role of a *Vibrio cholerae* exo-chitinase ChiA2 in Horizontal Gene Transfer**

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Abstract

Vibrio cholerae exo-chitinase ChiA2 play a key role in acquisition of nutrients by chitin hydrolysis in the natural environment as well as in pathogenesis in the intestinal milieu. In this study we demonstrate the importance of ChiA2 in horizontal gene transfer in the natural environment. We found the expression of ChiA2 and TfoX, the central regulator of *V. cholerae* horizontal gene transfer, varied with changes in environmental conditions. The activity of ChiA2 was also dependent on these conditions. In three different environmental conditions tested here, we observed that the supporting environmental condition for the maximum expression and activity of ChiA2 was at 20 °C, pH 5.5 and 100 mM salinity in presence of chitin. The same condition also induced TfoX expression and was favorable for horizontal gene transfer in *V. cholerae*. HPLC analysis showed ChiA2 released significant amount of (GlcNAc)₂ from chitin hydrolysis under the favorable condition. We hypothesized that under the favorable environmental condition, ChiA2 was up-regulated and maximally active to produce significant amount of (GlcNAc)₂ from chitin. The same environmental condition also induced *tfoX* expression followed by its translational activation by (GlcNAc)₂ produced leading to efficient horizontal gene transfer.

Key words: *V. cholerae* chitinase; ChiA2; chitin; horizontal gene transfer, *tfoX*

49 Introduction

50 Cholera, caused by the facultative pathogen *Vibrio cholerae* is far from being extinct. The
51 current Haiti out- break clearly shows that it is a re-emerging disease (Morens *et al.* 2004).

52 The causative organism *V. cholerae*, apart from being a human pathogen also share an
53 ecological relationship with the host chitinous planktonic copepods during its reside in the
54 aquatic environment (Huq *et al.* 1983). During their growth on chitin *V. cholerae* can acquire
55 new genetic material from the environment by natural transformation, a mode of Horizontal
56 Gene Transfer (HGT) (Meibom *et al.* 2005). HGT has been reported as an important
57 phenomenon that contributes in the development of high level of genomic diversity among
58 the *Vibrio Spp.* (Thompson *et al.* 2005; Purdy *et al.* 2005). The transforming foreign DNA
59 acquired by HGT is either used in the repair of damaged genes and therefore helps in genome
60 maintenance or to the acquisition of new alleles or genes leading to the genetic diversity or
61 evolution (Blokesch *et al.* 2007). The sero-conversion of O1 to O139 by means of natural
62 transformation indicates that HGT may lead to the emergence of new and more virulent
63 strains of *V. cholerae* (Blokesch *et al.* 2007; Faruque *et al.* 2003).

64 The major component of the exoskeleton of copepods are chitin, a polymer of β - 1, 4-linked
65 N-acetylglucosamine (GlcNAc), has been reported to induce natural competence and
66 transformation in *Vibrio Spp.* (Meibom *et al.* 2005). Chitin induces the genes that encode
67 type IV pilus assembly complex. Type IV pilus assembly complex is known to be potentially
68 involved in the DNA uptake process (Meibom *et al.* 2004; Suckow *et al.* 2011). The
69 importance of type IV pilus assembly complex in natural competence was determined by
70 disrupting following genes in *V. cholerae* strain A1552: *pilA* (VC2423), the gene predicted to
71 encode a type IV pilin; *pilQ* (VC2630) which encode a homolog of the secretin protein
72 family; and *pilB* (VC2424) encoding a traffic nucleotide triphosphatase (NTPase), speculated
73 to energize assembly of the pilus filament (Meibom *et al.* 2004). When the mutant strains

74 were tested for natural competence, no transformants were detected compared to the wild
75 type strain.

76 The initiation of competence and transformation occurs via a chitin dependent activation of
77 TfoX, the main regulator of natural transformation (Meibom *et al.* 2005; Meibom *et al.*
78 2004). A small RNA (sRNA) *tfoR* which is transcriptionally induced in presence of
79 (GlcNAc)₂ activates the translation of *tfoX* mRNA (Yamamoto *et al.* 2011). TfoX regulates
80 the expression of diverse genes including those encoding the proteins that functions in chitin
81 degradation and chitin-induced competence. The three pilus assembly genes pilA, pilB and
82 pilQ, genes encoding four chitinases including ChiA1 and ChiA2, and a chitoporin gene are
83 reported to be induced by TfoX (Meibom *et al.* 2005).

84 ChiA2, the extracellular chitinase of *V. cholerae* along with the endo-chitinase ChiA1 plays
85 the key role in acquisition of nutrients from the natural environment and helps *V. cholerae* in
86 initialization of chitin utilization pathway (Hunt *et al.* 2008). The identification of chitin and
87 its oligomers as the inducer of *tfoX* expression and horizontal gene transfer creates an obvious
88 interest about the role of chitinases in HGT which has not been mentioned or studied by any
89 researcher till date.

90 We show that the environmental condition that supports maximum expression and activity of
91 ChiA2 is also optimum for *tfoX* expression and HGT. We, by HPLC analyses provide
92 evidence that under the favorable condition (20 °C, pH 5.5 and 100 mM salt concentration)
93 ChiA2 produces significant amount of (GlcNAc)₂. We hypothesized that under the optimum
94 condition ChiA2 was up-regulated and maximally active to produce significant amount of
95 (GlcNAc)₂ from chitin. The same environmental condition also induced *tfoX* expression
96 followed by its translational activation by (GlcNAc)₂ produced leading to efficient horizontal
97 gene transfer.

98

99 **Materials and Methods**

100

101 **Bacterial culture and growth conditions**

102 *V. cholerae* O1 El Tor strain C6709 was used for the studies. A *chiA2* mutant strain of *V.*
103 *cholerae* C6709 was constructed as described previously (Mondal *et al.* 2014). Wild type *V.*
104 *cholerae* C6709 and the Δ *chiA2* C6709 was cultured and maintained in tryptic soy broth.

105 The expression studies of *chiA2* and *tfoX* was done under three different environmental
106 conditions (Table 1). *V. cholerae* was grown overnight in tryptic soy broth (TSB) medium
107 (BD Difco, Sparks, MD), centrifuged and washed in Phosphate buffered saline (PBS) and $1 \times$
108 10^5 cells were inoculated in seawater media (400 mM NaCl; 10 mM KCl; 25 mM $MgCl_2$; 28
109 mM $MgSO_4$) supplemented with chitin or (GlcNAc)₂. The inoculated culture then grown
110 separately under the mentioned three different environmental conditions for 24 h.

111

112 **Isolation of Total RNA**

113 The total RNA from *V. cholerae* was isolated by using TRIzol reagent (Invitrogen, Carlsbad,
114 CA). This reagent maintained the integrity of RNA during the homogenization of the cells
115 and at the same time it dissolved cellular components of the disrupted cells. Briefly, the cells
116 were homogenized thoroughly with TRIzol and incubated at room temperature for at least 5
117 mins for complete dissociation of the nucleoprotein complexes. Chloroform (1/5th volume of
118 TRIzol) was added to the homogenate and shaken vigorously for 15 s, incubated 2-3 mins at
119 room temperature; centrifuged at 12000 $\times g$ for 15 mins at 4 °C. The aqueous phase was
120 transferred to a fresh tube and isopropanol (1/2nd volume of TRIzol) was added, incubated at
121 room temperature for 10 mins and centrifuged at 12000 $\times g$ for 10 mins at 4° C. the pellet was
122 washed with 75% ethanol, air dried and dissolved in desired volume of nuclease free water.
123 The isolated RNA was stored at -70 °C.

124

125 **Quantitative PCR**

126 The expression of *tfoX* and *chiA2* was studied quantitatively by Quantitative PCR (qPCR)
127 using Power SYBR Green PCR master Mix (Applied Biosystems, Foster City, California)
128 and a set of primers (Table 2) in a 7500 Real Time PCR detection system (Applied
129 Biosystems, Foster City, California). *V. cholerae* or Δ *chiA2* *V. cholerae* was grown for 24 h
130 in sea-water media supplemented with chitin separately under the three different
131 environmental conditions as mentioned in Table 1. The total RNA isolated from *V. cholerae*
132 was converted to cDNA using Reverse Transcription System (Promega, Madison, WI) and
133 was used as template. The *recA* gene was used as internal control. Comparative RNA
134 expression was studied by comparing the $\Delta\Delta$ Ct values determined from the qPCR (Livak *et*
135 *al.*, 2001). To study the effect of (GlcNAc)₂ in *tfoX* expression in Δ *chiA2* mutants the
136 mentioned experiment was performed in a similar manner in sea-water media supplemented
137 with 6 mM (GlcNAc)₂ and the expression of *tfoX* was measured by qPCR. For the qPCR
138 reactions *recA* was used as internal control.

139

140 **Chitinase activity assay**

141 The N-acetylglucosamine concentration in the reaction mixture and the chitinase activity
142 were determined by previously established Di-nitrosalicylic acid (DNS) method (Miller *et al.*
143 1959). The experiment was performed as described in Mondal *et al.* 2014. The amount of
144 reducing sugar was then estimated spectrophotometrically. The specific activity of the
145 enzyme was calculated by measuring the amount of GlcNAc produced in μ mole/ mg of
146 protein/ min. The velocity of each reaction was calculated by measuring the amount of
147 GlcNAc produced in μ mole/ ml of reaction mixture/min. The Michaelis Menten constant K_m

and the maximum velocity $V_{\max}$ were calculated from a Lineweaver Burk plot. The catalytic constant K_{cat} and the catalytic efficiency (K_m/K_{cat}) were also determined.

Generation of *V. cholerae* growth curve

The variation in *V. cholerae* growth under the three different environmental conditions (Table 1) was studied by growth curve analyses. Overnight cultures of the wild type *V. cholerae* and ΔchiA2 *V. cholerae* were diluted separately in sea-water media (pH 8.0, salinity 300 mM) supplemented with of 2% chitin. The cultures were grown for 72 h with shaking. The viable cells were enumerated after 4h, 8h, 10h, 12h, 24h, 30h, 48h and 72 h time interval by plating the cultures on thiosulfate-citrate-bile-salts-sucrose (TCBS) agar plate.

Effect of environmental conditions on *V. cholerae* HGT

The *V. cholerae* HGT was performed by following a previously described method (Meibom *et al.* 2005) with a little modification. In brief, 1×10^9 CFU of overnight cultures of *V. cholerae* and ΔchiA2 *V. cholerae* were inoculated with sea-water media supplemented with chitin and the transformation reaction was performed under three different environmental conditions (Table 1) with 3 $\mu\text{g/ml}$ transforming ampicillin resistant GFP plasmid. The cultures were then incubated for 18 h in static condition. After 18 h the cultures were plated onto ampicillin containing LB plates.

HPLC analyses of the end products of ChiA2 treated mucin

HPLC analyses were done to estimate oligosaccharides produced as an end product of chitin after ChiA2 treatment. The oligosaccharides were eluted from the column by using a gradient of 60-80% acetonitrile and water. The retention time of each oligosaccharide was compared with commercially available standards. In detail, HPLC analyses were done using

a 4.6 nm × 250, 5 µm Zorbax Carbohydrate Analysis Column (Agilent Technologies, Santa Clara, CA) connected to a Shimadzu Prominence 20A, HPLC system. LC solutions (Shimadzu, Kyoto, Japan) program was used to control, calibrate and analyze the HPLC data. In 20 mM phosphate buffer pH 7.4, 1mg/ ml of crab shell chitin (Sigma, St. Louis, MO) was incubated with 50 µg/ ml purified ChiA2 at 30 °C for 3 h. The reaction was terminated by 10% trichloroacetic acid (HIMEDIA, Mumbai, India) (v/v). Eighty microliter of the terminated reaction mixtures were injected to the HPLC Column operated at 40 °C. The oligosaccharides were eluted from the column by using a gradient of 60-80% acetonitrile and water. The eluted fraction was passed immediately by LC PDA detection system to detect the components of the eluted fraction. The retention time of each oligosaccharide was compared with commercially available standards.

Statistical analysis

All the experiments were repeated at least three times. Data are expressed as means ± standard error (SE). One way ANOVA was used to analyze the data. A *P* value of <0.05 was considered statistically significant.

Results

Effect of environmental conditions on *chiA2* and *tfoX* expression

The expression of *chiA2* was analyzed in C6709 strains, under three different conditions mentioned in Table 1. The conditions were selected depending on the variation of growth of *V. cholerae* N16961 and the expression of *chiA2* over a range of temperature, pH and salt concentration (supplementary material Fig S1 to S3).

The maximum *chiA2* expression was observed under condition I (Fig 1A). The quantitative analysis of the expression showed, under condition I the expression of *chiA2* was 3.6-fold

higher compared to the expression under condition II. The expression of *chiA2* under condition III was lowest and was 5.9- fold less compared to the expression under condition I. This result indicated that the most favorable condition for the *V. cholerae* C6709 *chiA2* expression was condition I that is 20 °C, pH 5.5 and 100 mM salt concentration. This observation also showed that the optimum condition for *V. cholerae chiA2* expression varies with *V. cholerae* strain.

Analyses of the *tfoX* expression of *V. cholerae* strain C6709 under the three mentioned conditions showed condition I as the most favorable condition for *tfoX* expression (Fig 1B). Under condition I the *tfoX* expression was 4.5-fold higher compared to the expression under condition II. Under condition III *tfoX* expression was the lowest.

These results indicated that condition I is the most favorable condition for *chiA2* expression as well as *tfoX* expression.

Effect of environmental factors on activity of ChiA2

Activity of pure ChiA2 was studied under the mentioned three environmental conditions (Table I). The variations in ChiA2 activity under the different environmental conditions were studied by analyzing ChiA2 specific activity, using chitin as assay substrate. Condition I was found as the most favorable condition for *V. cholerae* C6709 ChiA2 activity compared to the rest two conditions (Fig 2). Under condition II also ChiA2 showed significant activity. Comparison of the specific activities of ChiA2 showed that it was 4.1-fold more active under condition I compared to condition III. Under condition II ChiA2 showed 3.3-fold more activity compared to condition III. Condition III was found as the most unfavorable condition for C6709 ChiA2 activity. This data showed that environmental condition I was most favorable for *V. cholerae* C6709 ChiA2 activity.

***V. cholerae* requires an active ChiA2 as well as optimum environmental condition for a successful growth**

The growth of *V. cholerae* was studied under the previously mentioned three different conditions (Table1). The $\Delta chiA2$ *V. cholerae* strain showed poor growth under the all three conditions. At 48 h the wild type *V. cholerae* showed ~100-fold higher growth under condition I compared to the $\Delta chiA2$ *V. cholerae* (Fig 3A). The growth of wild type *V. cholerae* was ~10-fold decreased under condition II compared to condition I (Fig 3B). Under condition III both wild type and $\Delta chiA2$ *V. cholerae* did not show significant growth and it was almost 1000-fold less compared to condition I (Fig 3C). These observations indicated that condition I was the most favorable condition for *V. cholerae* C6709 growth and ChiA2 is essential for the growth. In absence of *chiA2*, *V. cholerae* growth was compromised even under the favorable conditions. On the other hand it is also observed that even if *chiA2* is present *V. cholerae* shows poor growth under the unfavorable conditions.

We observed $\Delta chiA2$ *V. cholerae* was able to retrieve the wild type growth when the growth media was supplemented with (GlcNAc)₂. We observed that under condition I, $\Delta chiA2$ *V. cholerae* showed similar growth with respect to wild type, in (GlcNAc)₂ supplemented growth media (Fig 3A). Under condition I the $\Delta chiA2$ *V. cholerae* growth increased by 100-fold in presence of (GlcNAc)₂ compared to the non-(GlcNAc)₂ supplemented growth media. Under condition II, the similar change in $\Delta chiA2$ *V. cholerae* growth was observed (Fig 3B). Condition III was not favorable for the growth of *V. cholerae* (Fig 3C).

Effect of environmental condition on *V. cholerae* HGT

In the previous studies we found condition I among the three mentioned conditions (Table 1), as the most favorable for *chiA2* and *tfoX* expression as well as ChiA2 activity. *V. cholerae* HGT under the defined three environmental conditions showed that condition I was the most

favorable condition for *V. cholerae* HGT (Fig 4). Under condition I the number of transformants calculated was ~6.7 to 8.5- fold higher for wild type *V. cholerae* compared to the other two conditions. However, the $\Delta chiA2$ *V. cholerae* showed reduced level of natural competence and less number of transformants compared to the wild type strain irrespective of the condition selected. Under condition I, the number of transformants of $\Delta chiA2$ *V. cholerae* increased up to 13.5- fold when (GlcNAc)₂ was supplemented compared to the number of transformants of $\Delta chiA2$ *V. cholerae* without (GlcNAc)₂ supplement in the growth media. This observation indicated that in presence of (GlcNAc)₂ the $\Delta chiA2$ *V. cholerae* was capable of HGT and among the three environmental conditions condition I is the most favorable for *V. cholerae* HGT.

ChiA2 releases significant amount of GlcNAc dimer from Chitin

The end products of ChiA2-treated chitin were analyzed by HPLC and the components were identified with the corresponding standards. In Fig 5A, the chromatogram showed four distinct peaks. Peak 1 with retention time 5.5 min corresponded to the peak of GlcNAc (Fig 5B), the main product released from chitin on ChiA2 treatment. It occupied 35% of the total peak area. Peak 2A with retention time 6.2 min and peak 2B with retention time 6.5 min eluted in close proximity. Peak 2B with retention time 6.5 min (Fig 5A) corresponded to α -(GlcNAc)₂ (Fig 2B). Peak 2A did not have any standards and we presumed it with all possibilities as the peak of β -(GlcNAc)₂. Peak 2A occupied 22.2% of the total peak area and peak 2B occupied 18.2% of the total peak area (Table 3). Peak III with retention time 7.42 (Fig 5A) corresponded to the peak of (GlcNAc)₃ (Fig 5B) and it occupied only 9.6% of the total peak area (Table 3). So this data evidenced that chitin on treatment with ChiA2 releases significant amount of both the enantiomers of (GlcNAc)₂ besides the release of its main

product GlcNAc. The presence of small peak of (GlcNAc)₃ in the chromatogram was might be due to the un-degraded (GlcNAc)₃.

Discussion

In this study we demonstrated the importance of the extracellular chitinase ChiA2 in *V. cholerae* natural transformation, a mode of HGT. ChiA2 is important for initialization of *V. cholerae* chitin utilization pathway. It helps to degrade the insoluble chitinous exo-skeleton of copepods into its soluble monomers and oligomers which then be readily utilized by *V. cholerae* (Hunt *et al.* 2008). Chitin acts as an inducer of *V. cholerae* natural transformation and facilitates the uptake of new genetic material from the environment. Cholera is a climate related infectious disease (Lipp *et al.* 2002). Abiotic environmental factors regulate the survival and the association of *V. cholerae* with the zooplanktons via the regulation of expression and activity of chitinase genes (Bhowmick *et al.* 2007; Lipp *et al.* 2002). The most favorable conditions for ChiA2 expression and activity of *V. cholerae* O1 El Tor strain C6709 was observed at temperature 20 °C, pH 5.5 and 100 mM salt concentration. *V. cholerae* N16961 ChiA2 expression and activity works favorably under condition II (Supplementary material, Fig S1 to S5). However, this strain is a natural mutant of *hapR* and did not undergo natural transformation. So we used the *hapR* positive *V. cholerae* O1 El Tor strain C6709 for our study. Different environmental conditions were found as favorable for *chiA2* expression of two different strains of *V. cholerae* O1 El Tor.

As reported earlier, the chitinase genes of *V. cholerae* O1 El Tor mostly shows high level of expression and activity at low temperature (Bhowmick *et al.* 2007). Temperature around 25 °C has been reported to increase *V. cholerae* counts in aquatic reservoirs and increased chitinolytic activities (Louis *et al.* 2003). We found *V. cholerae* C6709 ChiA2 was maximally active under 20 °C. It is also reported that most of the bacterial chitinases were active at

acidic pH (Annamalai *et al.*, 2010; Dahiya *et al.*, 2005; Murao *et al.*, 1992; Ohtakara *et al.* 1979). *V. cholerae* C6709 ChiA2 was also observed to be active at pH 5.5 having a similarity with the chitinase of *Enterobacter spp.* NRG4 (Dahiya *et al.* 2005).

We observed that the expression of *tfoX*, the central regulator of *V. cholerae* HGT, was maximum under the same environmental conditions which were found optimum for ChiA2 expression and activity indicating regulation by the same environmental conditions.

The most favorable condition for *V. cholerae* C6709 growth was exactly the same that supports the expression of *chiA2* and *tfoX*. However, the $\Delta chiA2$ *V. cholerae* did not show significant growth under this condition indicating that ChiA2 is essential for *V. cholerae* C6709 survival and growth in aquatic environment during its reside on the chitinous surface of copepods. This data corroborated with the data of Meibom *et al.* (2004) in which it has been reported that a $\Delta chiA2$ *V. cholerae* of another O1 El Tor strain N16961 also exhibited reduced growth compared to the wild type (Meibom *et al.* 2004). This indicated that ChiA2 is important for survival of *V. cholerae* irrespective of the strains. However, we found that the $\Delta chiA2$ *V. cholerae* C6709 strain could retrieve wild type growth phenotype when the growth media was supplemented with (GlcNAc)₂. This observation suggested that (GlcNAc)₂ might be an important nutrient required for growth of *V. cholerae* C6709. Uchiyama *et al.* in year 2003 reported that in *Serratia marcescens* the expression of chitinases and chitin binding proteins are induced by (GlcNAc)₂ (Uchiyama *et al.* 2003). The *S. marcescens* mutants that are defective in (GlcNAc)₂ transport are unable to express the chitinases (Uchiyama *et al.* 2003). These reports suggest that (GlcNAc)₂ has an important role in induction of chitinase expression that are essential for nutrient supply to the bacteria via the chitin degradation pathway in the aquatic environment. Further detailed explanation of this phenomenon was given by a study that reported (GlcNAc)₂ activates translation of *tfoX* (Yamamoto *et al.*

2011), that regulates the expression of the genes those involved in chitin degradation and natural competence (Meibom *et al.* 2005).

Our results on the effect of environmental conditions on *V. cholerae* HGT demonstrated that conditions favoring the expression of *chiA2* and *tfoX* and the activity of ChiA2, was also optimum for *V. cholerae* HGT. However, dysregulation of ChiA2 under the optimum condition showed reduced natural competence compared to the wild type strain. Addition of exogenous (GlcNAc)₂ supported the role of ChiA2 in HGT. This result clearly states that ChiA2 acts on chitin to release (GlcNAc)₂ that supports *V. cholerae* HGT. Reported that, in presence of (GlcNAc)₂ translational activation of *tfoX* occurs via the transcriptional activation of *tfoR* small RNA. Activated *tfoX*, the regulator of HGT then up-regulates the expression of genes involved in natural competence (Yamamoto *et al.* 2011).

Taken together we observed that the maximum expression of *chiA2* and *tfoX* is optimum under the same environmental conditions that also favor the chitinolytic activity, *V. cholerae* growth as well as *V. cholerae* HGT. We speculated that, under the favorable environmental conditions, ChiA2 was up-regulated and maximally active to produce significant amount of (GlcNAc)₂ from chitin. Presence of significant amount of (GlcNAc)₂ in the end product of ChiA2 treated chitin provides a strong evidence about this phenomenon. The *tfoR* small RNA regulator is transcriptionally induced in presence of (GlcNAc)₂ and activates the translation of *tfoX* mRNA (Yamamoto *et al.*, 2011). Activated TfoX then regulates the expression of genes involved in chitin induced competence (Meibom *et al.* 2005). The same environmental conditions also induced *tfoX* expression followed by its translational activation by (GlcNAc)₂ produced leading to efficient HGT.

Acknowledgement

The authors are grateful to the Indian Council of Medical Research, internal to our organization for the financial support. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Moumita Mondal has been supported by the funds from University Grants Commission [UGC sanction No. F.2.77/98 (SA-I)].

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Table 1: the definition of the environmental conditions selected for the study

Conditions	Temperature	pH	Salinity (mM sodium chloride)	Media used
Condition I	20°C	5.5	100	Sea-water media supplemented with 0.8% chitin
Condition II	30°C	8.0	300	Sea-water media supplemented with 0.8% chitin
Condition III	42°C	9.0	600	Sea-water media supplemented with 0.8% chitin

Table 2: List of primers

<i>Primers</i>	<i>Sequence</i>
chiA2 real time primer F	5' ACAACGACACCGCCAGTAAAC 3'
chiA2 real time primer R	5' TGCCCCAGTCAAGGTCACAGC
chiA2XbaI A F	5'-CAGCTCTAGACTCTGATACTGACGTTTCGAG-3'
chiA2 B R	5'-CCCATCCACTATAAACTAACACAATGGCTTGAGGTGTGAAC-3'
chiA2 C F	5'-TGTTAGTTTATAGTGGATGGGGGAACAGGTGGTGATGCTT-3'
chiA2 SACI D	5'-GAATCGAGCTCGATTGCATTCCCAATGAG-3'

Table 3: HPLC analysis of the end products of ChiA2 treated chitin

			Standards		ChiA2-chitin reaction	
<i>Peak number</i>	<i>Sub peak</i>	<i>Peak Identity</i>	<i>Ret. Time (mins)</i>	<i>% Area</i>	<i>Ret. Time (mins)</i>	<i>% Area</i>
1.		(GlcNAc)	5.5 ± 0.05	35.8	5.56±0.05	35.09
2	A	(GlcNAc) ₂ β	NO*	19.7	6.20±0.03	22.23
	B	(GlcNAc) ₂ α	6.6 ± 0.03		6.57±0.03	18.21
3.		(GlcNAc) ₃	7.5 ± 0.02	16.1	7.42±0.02	9.66
4.		(GlcNAc) ₆	11.15 ± 0.03	6.5	NO*	-

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Figure legends

Figure 1. A. Graphical representation of the effect of environmental conditions on *chiA2*

expression. Effect of environmental conditions on *chiA2* expression was studied under the three mentioned conditions (Table 1). *V. cholerae* cells were cultured in sea water media supplemented with chitin separately under the conditions mentioned in Table 1. After 24 h growth the total RNA was isolated and *chiA2* expression was analyzed by quantitative PCR. Maximum expression was observed under condition I which was 3.6-fold higher compared to the expression under condition II. Lowest expression was observed under condition III. **B.**

Graphical representation of effect of environmental factors on *tfoX* expression. Under varying environmental conditions the expression of *tfoX* was studied following the similar method used to study the *chiA2* expression. Quantitative analyses of *tfoX* expression by qPCR showed condition I is the most favorable for *tfoX* expression and condition III least favorable for *tfoX* expression. Under condition I the *tfoX* expression was 4.5- fold higher compared to the expression under condition II. All the experiment was carried out thrice and the data represented as mean $\pm$ SEM.

Figure 2. Graphical representation of the effect of environmental conditions on ChiA2

activity. Activity of *V. cholerae* C6709 ChiA2 was studied under the varying environmental conditions. Activity assay of ChiA2 was performed separately under the mentioned environmental conditions (Table I) using 2% chitin as assay substrate. Under condition I *V. cholerae* C6709 ChiA2 showed maximum activity and under condition III the activity was minimum while, under condition II ChiA2 showed moderate activity. Under condition I the activity of ChiA2 was 4.3-fold higher compared to the activity under condition III. Under condition II ChiA2 showed significant activity but it was 1.2- fold less compared to the

activity under condition I. The experiment was performed thrice and the data presented here as mean $\pm$ SEM.

Figure 3. Graphical representation of the effect of environmental conditions on *V. cholerae* C6709 growth. Effect of environmental conditions on *V. cholerae* C6709 growth was studied in sea water media supplemented with 2% chitin separately under the mentioned three environmental conditions (Table 1). The growth curves were generated by plotting the number of viable cells (CFU/ml) in Y-axis against the time interval plotted in X-axis. The wild type *V. cholerae* C6709 was cultured in sea water media supplemented with 2% chitin separately under the three mentioned environmental conditions (Table 1). The number of viable cells after the time intervals (0 h, 2 h, 4 h, 8 h, 10 h, 12 h, 24 h, 30 h, 48 h and 72 h) was enumerated by plate count method using TCBS plates. The $\Delta chiA2$ *V. cholerae* was cultured in sea water media supplemented with 2% chitin or 6 mM (GlcNAc)₂ and was grown separately under the three mentioned environmental conditions (Table 1). The number of viable cells after the time intervals (0 h, 2 h, 4 h, 8 h, 10 h, 12 h, 30 h, 48 h and 72 h) was enumerated by plate count method using TCBS plates. Figure presents the growth curve of *V. cholerae* C6709 wild type and the $\Delta chiA2$ strains under **A.** condition I, **B.** condition II, **C.** condition III.

Figure 4. Graphical representation of the effect of environmental conditions on *V. cholerae* Horizontal Gene transfer (HGT). Effect of environmental factors on *V. cholerae* HGT was studied in sea-water media supplemented with chitin or (GlcNAc)₂. 1×10^9 CFU of overnight cultures of *V. cholerae* and $\Delta chiA2$ *V. cholerae* were inoculated with sea-water media and the transformation reaction was performed under three different environmental conditions (Table 1) with 3 μ g/ml transforming ampicillin resistant GFP plasmid. The graph

shows that condition I is the most favorable for *V. cholerae* HGT compared to the other two conditions. However, the $\Delta chiA2$ *V. cholerae* did not show HGT under any of the conditions except in presence of (GlcNAc)₂. The experiment was performed thrice and the data presented as mean $\pm$ SEM.

Figure 5. HPLC analysis of end products of ChiA2-treated chitin. The chitin-ChiA2 Reactions were carried as mentioned in the materials and methods section. The end products were analyzed using a 4.6 mm $\times$ 250, 5 μ m Zorbax carbohydrate analysis column (Agilent Technologies, Waldbrown, Germany) connected to a Shimadzu Prominence 20A; HPLC system. **A.** This figure shows the HPLC chromatogram of the end products of ChiA2 treated chitin. The chromatogram shows four distinct peaks. Peak 1 with retention time 5.5 minutes indicates presence of GlcNAc. Peak 2 indicates presence of (GlcNAc)₂ which is divided into two separate peaks: peak 2A and peak 2B. Peak 2A with retention time 6.2 minutes probably is for the β enantiomer and peak 2B with retention time 6.5 minutes is for α enantiomer of (GlcNAc)₂. Peak 3 with retention time 7.4 minutes indicates the presence of (GlcNAc)₃. **B.** This is the HPLC chromatogram of standards of GlcNAc, (GlcNAc)₂, (GlcNAc)₃ and (GlcNAc)₆. The chromatogram shows 4 distinct peaks. Peak 1 with retention time 5.5 minutes is for GlcNAc. Peak 2 with retention time 6.6 minutes is for α -(GlcNAc)₂. Peak 3 with retention time 7.5 minutes is for (GlcNAc)₃ and peak 4 with retention time 11.1 minutes is for (GlcNAc)₆.

Figure 1,
Mondal et al, 2015

Figure 1. A. Graphical representation of the effect of environmental conditions on *chiA2* expression. Effect of environmental conditions on *chiA2* expression was studied under the three mentioned conditions (Table 1). *V. cholerae* cells were cultured in sea water media supplemented with chitin separately under the conditions mentioned in Table 1. After 24 h growth the total RNA was isolated and *chiA2* expression was analyzed by quantitative PCR. Maximum expression was observed under condition I which was 3.6-fold higher compared to the expression under condition II. Lowest expression was observed under condition III. B. Graphical representation of effect of environmental factors on *tfoX* expression. Under varying environmental conditions the expression of *tfoX* was studied following the similar method used to study the *chiA2* expression.

Quantitative analyses of *tfoX* expression by qPCR showed condition I is the most favorable for *tfoX* expression and condition III least favorable for *tfoX* expression. Under condition I the *tfoX* expression was 4.5- fold higher compared to the expression under condition II. All the experiment was carried out thrice and the data represented as mean $\pm$ SEM.

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Figure 2,
Mondal *et al*, 2015

Figure 2. Graphical representation of the effect of environmental conditions on ChiA2 activity. Activity of *V. cholerae* C6709 ChiA2 was studied under the varying environmental conditions. Activity assay of ChiA2 was performed separately under the mentioned environmental conditions (Table I) using 2% chitin as assay substrate. Under condition I *V. cholerae* C6709 ChiA2 showed maximum activity and under condition III the activity was minimum while, under condition II ChiA2 showed moderate activity. Under condition I the activity of ChiA2 was 4.3-fold higher compared to the activity under condition III. Under condition II ChiA2 showed significant activity but it was 1.2- fold less compared to the activity under condition I. The experiment was performed thrice and the data presented here as mean $\pm$ SEM.

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Figure 3,
Mondal *et al.*, 2015

Figure 3. Graphical representation of the effect of environmental conditions on *V. cholerae* C6709 growth. Effect of environmental conditions on *V. cholerae* C6709 growth was studied in sea water media supplemented with 2% chitin separately under the mentioned three environmental conditions (Table 1). The growth curves were generated by plotting the number of viable cells (CFU/ml) in Y-axis again the time interval plotted in X- axis. The wild type *V. cholerae* C6709 was cultured in sea water media supplemented with 2% chitin separately under the three mentioned environmental conditions (Table 1). The number of viable cells after the time intervals (0 h, 2 h, 4 h, 8 h, 10 h, 12 h, 24 h, 30 h, 48 h and 72 h) was enumerated by plate count method using TCBS plates. The Δ chiA2 *V. cholerae* was cultured in sea water media supplemented with 2% chitin or 6 mM (GlcNAc)2 and was grown separately under the three mentioned environmental conditions (Table 1). The number of viable cells after the time intervals (0 h, 2 h, 4 h, 8 h, 10 h, 12 h, 30 h, 48 h and 72 h) was enumerated by plate count method using TCBS plates. Figure presents the growth curve of *V. cholerae* C6709 wild type and the Δ chiA2 strains under A. condition I, B. condition II, C. condition III.

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Figure 4,
Mondal *et al*, 2015

- I- environmental condition I
- II- environmental condition II
- III- environmental condition III

Figure 4. Graphical representation of the effect of environmental conditions on *V. cholerae* Horizontal Gene transfer (HGT). Effect of environmental factors on *V. cholerae* HGT was studied in sea-water media supplemented with chitin or (GlcNAc)₂. 1×10⁹ CFU of overnight cultures of *V. cholerae* and Δ chiA2 *V. cholerae* were inoculated with sea-water media and the transformation reaction was performed under three different environmental conditions (Table 1) with 3µg/ml transforming ampicillin resistant GFP plasmid. The graph shows that condition I is the most favorable for *V. cholerae* HGT compared to the other two conditions. However, the Δ chiA2 *V. cholerae* did not show HGT under any of the conditions except in presence of (GlcNAc)₂. The experiment was performed thrice and the data presented as mean ± SEM. 170x154mm (300 x 300 DPI)

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