Involvement of in vivo induced *cheY-4* gene of *Vibrio cholerae* in motility, early adherence to intestinal epithelial cells and regulation of virulence factors

Rajat Banerjee[a], Soumita Das[a], Kanchan Mukhopadhyay[b], Sanjay Nag[a], Amit Chakrabortty[a,1], Keya Chaudhuri[a,*]

[a] *Biophysics Division, Indian Institute of Chemical Biology 4, Raja S.C. Mullick Road, Jadavpur, Calcutta-700 032, India*
[b] *Electron Microscopy Division, Indian Institute of Chemical Biology, Jadavpur, Calcutta-700 032, India*

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Abstract **Using a global transcription profile approach *cheY-4* of *Vibrio cholerae* was identified as an in vivo induced gene. In the present study, duplication of the gene in the chromosome resulted in increased motility, increased chemotactic response towards isolated intestinal mucus layer and stronger adhesion to human intestinal epithelial cell line at an early phase of infection compared to wild type and a null mutant strain. In contrast to the *cheY-4* null mutant, duplication of *cheY-4* gene resulted in increased expression of *ctxAB* and *tcpA*, the two major virulence genes of *V. cholerae*.**

Key words: Chemotaxis; Motility; Adherence to epithelial cells; Virulence factors; *Vibrio cholerae*

1. Introduction

Cholera is endemic in the Indian subcontinent and continues to re-emerge elsewhere in Asia, Africa and the Americas with the incidence estimated to exceed 5 million cases each year. The causative organism *Vibrio cholerae* can exist as a free-living environmental organism in aquatic reservoirs, estuaries, coastal water and also as a significant intestinal pathogen within the human host. To identify the genes that are expressed following infection to the host, several approaches like in vivo expression technology [1], signature tagged mutagenesis [2] and RNA arbitrarily primed PCR fingerprinting [3] have already been used. Besides, comparison of global transcription profiles of *V. cholerae* grown in vitro and in vivo revealed that 20% of the genome was repressed and about 5% was induced under in vivo condition when a cosmid library covering almost 90% of the *V. cholerae* 569B genome was used [4]. In addition to the virulence genes *ctxAB, toxR, toxT* and *tcpA* that are induced under in vivo conditions, one of the in vivo induced cosmid after dissection, subcloning and sequencing showed homology with the *cheY-4* gene of the *V. cholerae* El Tor strain N16961 [4]. Several chemotaxis-related gene homologs are present in the *V. cholerae* genome, including four *cheY* genes. Among all *cheY* genes of *V. cholerae* only *cheY-4* belongs to the chemotaxis cassette present in chromosome II, whereas the other three *cheY* homologs are present in chromosome I. Previously using *tnpR* operon fusions encoding a site-specific DNA recombinase, it was shown that three transcriptional units of *V. cholerae* related to bacterial motility and chemotaxis were induced during infection in an infant mouse model of cholera [1]. One of them encoded CheV present in chromosome II of *V. cholerae* N16961. Also in recombinase-based in vivo expression technology (RIVET) *cheY-3* was obtained as a responsive regulator that contributes to colonization of the infant mouse small intestine and thereby involved in infection [5]. Very recently, by using Microarray technology a marked number of genes involved in chemotaxis showed repression in human stool-derived *V. cholerae* [6].

V. cholerae is a highly motile organism and exhibits chemotactic behavior in its interaction with the intestinal mucosa [7]. Evidence suggests that motility and chemotaxis are required for full virulence of *V. cholerae* in various animal models [8,9]. In the rabbit ileal loop, the growth rate of chemotactic vibrios were significantly faster than that of the non-chemotactic mutants and this was correlated with a higher degree of association with the mucosa by the chemotactic vibrio [10]. In vitro studies with rabbit mucosal slices supported the same conclusion [11]. In contrast, in the infant mouse model of cholera, non-chemotactic mutants survive in greater numbers than wild type chemotactic vibrios and produce a more rapid and severe disease [12]. It has been shown that affecting flagellar rotation by increasing the viscosity of the medium elevated the expression of the major transcriptional activator of *V. cholerae* [13]. Although the roles that chemotaxis and motility play in virulence of *V. cholerae* are not fully understood, the above results suggest that there is an intimate relationship between motility and virulence gene expression.

Chemotaxis has been extensively studied in enterobacteria and a model has been proposed for this important adaptation [14–16]. Sensing of external stimuli or repellent ligand is achieved via methyl-accepting chemoreceptor proteins (MCPs), which traverse the inner membrane. The effect of binding a ligand causes a conformational change in MCP,

*Corresponding author. Fax: (91)-33-473 5197, (91)-33-473 0284.
E-mail address: keya@cal2.vsnl.net.in (K. Chaudhuri).

[1] Present address: Cellular and Molecular Medicine West University of California, San Diego 9500 Gilman Drive, La Jolla, CA 92093-0648, USA

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which is sensed by the CheA–CheW complex and alters the activity of the CheA protein kinase, and the susceptibility of MCP to methylation or demethylation. CheA has an autokinase activity, phosphorylated CheA can donate the phosphate to CheY and PCheY interacts with FliM in the flagellar basal body to influence the direction of flagellar rotation.

The present study is the first report of molecular genetic analysis of *cheY-4*. The full-length *cheY-4* gene was cloned, sequenced and the phenotypes of a duplicated *cheY-4* in the chromosome were compared to *cheY-4* null mutant and to the wild type. From our study it is clear that *V. cholerae* chemotaxis, motility and adherence to intestinal epithelial cells are required early during the infection process and thereby initiates expression of two major virulence factors, cholera toxin (CT) and toxin-co-regulated pili (TCP).

2. Materials and methods

2.1. Bacterial strains and plasmids

The strains and plasmids used in this study are described in Table 1. *V. cholerae* and *Escherichia coli* strains were maintained at −70°C in Luria–Bertani (LB) medium containing 20% (v/v) glycerol. *E. coli* cells were grown in LB or M9 medium and *V. cholerae* cells were grown in nutrient broth (NB) or LB medium. Ampicillin was used at 100 μg/ml for *E. coli* and 50 μg/ml for *V. cholerae* wherever appropriate. Streptomycin concentration was 1 mg/ml for *V. cholerae*. All cloning experiments were done in pBluescript KS+ and pTAdv vector. The plasmids were maintained and amplified in *E. coli DH5α* and *XL1 Blue* strains.

2.2. DNA preparation and manipulation

Genomic DNA from *V. cholerae* 569B was prepared from proteinase K digested and cetyl-trimethyl-ammonium-bromide precipitated cell lysates by using standard procedures [17]. Plasmid DNA was prepared by the alkaline lysis method [18]. DNA probes were labeled with [α^{32}P]dCTP (Amersham International, Amersham, UK) by using a random primed labeling kit (New England BioLabs). Subgenomic libraries of *V. cholerae* DNA were constructed in plasmid pBluescript. Genomic DNA was digested with appropriate restriction enzymes, and the fragments within the size range of interest were excised from agarose gels, purified with agarose gel extraction column (Qiagen) and ligated with linearized and dephosphorylated plasmid pBluescript, and transformed into *E. coli* according to standard recombinant DNA techniques [18].

2.3. Construction of 569BY and 569BYN

Full-length *cheY-4* gene with 372 bp was generated from pRB600 by PCR, cloned (pRB372) and re-sequenced. An *Eco*RI–*Eco*RI fragment from plasmid pRB372 containing full-length *cheY-4* gene was cloned in pGP704 to give the plasmid pRB372Y, which was transformed into a λ pir lysogen of *E. coli* SM10 [19]. Ampicillin resistant transformants containing recombinant plasmids were selected. pRB372Y was then conjugally transferred from *E. coli* SM10 to *V. cholerae* 569B (Sm^r). Transconjugants resistant to both ampicillin and streptomycin were selected, and Southern blot analysis was done to confirm the specific integration of pRB372Y into the chromosomal *cheY-4* gene. From pRB600, 120 nucleotides (40 amino acids) from the beginning of the coding region of *cheY-4* gene were excluded out of digesting with *Hin*dIII and was religated to generate pRB900. From pRB900 an *Xba*I–*Sal*I fragment containing part of the *cheY-4* gene was then cloned to vector pGP704 to generate pRB901. Null *cheY-4* mutant strain was then generated with chromosomal integration of pRB901 by the same method described above.

2.4. RNA isolation and analysis

For isolation of RNA, cells were grown to the late logarithmic phase in LB (pH 7.2) or at the specified condition. Total RNA was extracted and purified by using guanidium isothiocyanate as described elsewhere [17]. RNA samples (15–25 μg/well) were electrophoresed in duplicate in 1% agarose-2.1 M formaldehyde-morpholinepropanesulfonic acid gels, and one part was stained with ethidium bromide and visualized with UV light to confirm equal loading of all samples. The other part of the gel was blotted onto nylon membranes by using 20×SSC and hybridized with labeled probes as described elsewhere [17]. The Northern blots were finally quantitated with respect to the 23S rRNA internal control in Quantity-One software (BioRad, USA).

2.5. DNA sequencing

Nucleotide sequence was determined with double stranded plasmid DNA as the template by cycle sequencing with the Applied Biosystem Prism dye system (Perkin-Elmer) in ABI prism 377 automatic DNA sequencer (Applied Biosystem, Perkin-Elmer), using company supplied kit reagents and protocols.

2.6. Swarm plate assay

Wild type *V. cholerae*, 569BY and 569BYN cells were inoculated with a toothpick into semi-solid agar plates containing LB (pH 7.2) solidified with 0.3% Bactoagar (Difco) [20]. The motility or chemotaxis agar plates were incubated at 37°C for 16–18 h and the swarm diameter was measured for each strain. For chemotactic assay, an about 8 mm diameter hole was bored at the center of the motility agar plate and filled with 150 μl of 10% glucose or 5 mM glutamine, which were used as positive controls for standardization. For mucous

Table 1
Bacterial strains and plasmids used in this study

Strain or plasmids	Relevant genotype or phenotype	Source or reference
Vibrio cholerae:		
569B	Prototroph, highly toxinogenic, biotype classical, serotype Inaba	[23]
569B St ^{R}	Streptomycin resistant, prototroph highly toxinogenic, biotype classical, serotype Inaba	lab collection
569BY	569B St^R containing duplicate *cheY-4* gene	present study
569BYN	569B St^R mutated *cheY-4* gene	present study
Escherichia coli:		
XL1 Blue	*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17, *supE*44, *relA*1, *lac*[F′*proAB*, *lacI*^{q}*Z*Δ15, Tn10 (tet^R)]	Strata Gene
DH5α	F$^-$f80d/*lac*Z DM15 D(*lac*ZYA *arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_k^-, m_k^-) *sup*E44l$^-$ thi-1 *gyr*A *rel*A1	Bethesda Research Laboratories, USA
SM10	*thi-1 thr*, *leu*, *tonA*, *LacY*, *SupE*, *recA*::RP4-2-Tc::Mu, λ-pir	[19]
CSR603	*thr-1*, *ara-14*,*leuB6Δ*(*gpt*-ProA)*62*, *lacY1*, *tsx33*, *suppE44*, *phr-1*, *galK2*, *λ*$^-$, *rac*$^-$, *recA1*, *gyrA98*, *rpsL31*, *kdgK51*, *xyl-5*, *mtl-1*, *uvrA6*	[24]
Plasmids:		
pBluescript KS$^+$	2.96 kb, Amp^R	Strata Gene
pTAdv	3.9 kb, Km^r, Amp^r, with 3′ T overhang	Clontech
pGP704	Amp^r *oriR6K mobRP4* MCS of M13 tg 131	[19]
pRB600	*Bam*HI-*Pst*I fragment from cosmid clone A606 of length 908 bp, containing full *cheY-4* gene cloned in pBluescript	present study
pRB372	PCR amplified 372 bp *cheY-4* gene from pRB600 cloned in pTAdv vector	present study
pRB372Y	*Eco*RI fragment of *cheY-4* gene from pRB372 cloned in pGP704 vector	present study
pRB900	pRB600-derived plasmid containing part of *cheY* gene	present study
pRB901	*Xba*I-*Sal*I fragment of pRB900 cloned in pGP704	present study

layer preparation, the internal wall of a section of guinea pig intestine was scraped and sterilized by UV followed by passage through 26-gauge sterile needle and placed in the central well. *V. cholerae* 569B, 569BY and 569BYN were then inoculated on each plate. The swarming area of each plate was quantitated by the Quantity-One software (BioRad) and the average of at least five experiments was taken.

2.7. Adherence to intestinal epithelial cells

Int407 cells were cultured at 37°C under 5% CO_2 in MEM (Gibco BRL) and supplemented with 10% heat inactivated FBS. For quantitative adherence assays, epithelial cells were seeded in a six-well culture plate. Two days later when the cells were confluent, the medium was put off, washed with PBS and bacteria suspended in fresh MEM medium without antibiotic were added to each well at an multiplicity of infection of about 100. Int407 cells were released from the plates after 5 h of incubation with *V. cholerae*, possibly due to the cytotoxic effect of CT. After 3 h of incubation the monolayers were washed with PBS several times, scraped and fixed with 2.5% glutaraldehyde in phosphate buffer, pH 7.2. Monolayers were then post-fixed with 1% OsO_4 for 1 h and in uranyl acetate for 1 h. After dehydration with a graded series of alcohols, samples were embedded in Epon and thin sectioned. The sections were stained with uranyl acetate and lead citrate and observed in a JEOL 100CX electron microscope. For adherence assay, the monolayers were infected in a similar fashion, incubated for 5, 20 and 60 min at 37°C under 5% CO_2. Cells were then washed vigorously thrice with PBS to remove non-adherent bacteria. The number of cell associated CFU was determined after lifting off the monolayer by scraping and vortexing the cells to dissociate bacteria. The percentage of adhesion was calculated as 100× cell-associated CFU/(cell-associated CFU+CFU present in the supernatant).

2.8. Nucleotide sequence accession number

The nucleotide and deduced amino acid sequence of the *V. cholerae* 569B *cheY-4* gene appear in the EMBL, GenBank and DDBJ databases under the accession no. AF171076.

3. Results and discussion

3.1. Cloning nucleotide sequence and predicted protein of cheY-4 gene of V. cholerae

In an earlier study using global transcription approach, a fragment homologous to *cheY-4* gene of *V. cholerae* N16961 was found to be induced under in vivo condition [4]. pRB600 containing the full-length *cheY-4* gene of *V. cholerae* 569B was screened from a subgenomic library using the *Bam*HI–*Hin*dIII fragment of the clone Sb3 [4]. The sequence of the insert in the plasmid pRB600 contained an open reading frame of 372 nucleotides which codes for a 123 amino acid protein that is

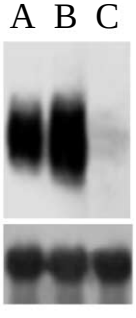

Fig. 1. Northern blot analysis of *V. cholerae* 569B, 569BY and 569BYN cells. *V. cholerae* strains 569B (lane A), 569BY (lane B) and 569BYN (lane C) were grown in LB (pH 7.4) at 37°C, total RNA was isolated, Northern blots prepared and probed with [^{32}P]α-dCTP labeled fragments of *cheY* gene (upper panel) and 23SrRNA (lower panel). The values indicated below are the relative ratio between the intensities of *cheY-4* expression to that of optimized 23SrRNA expression.

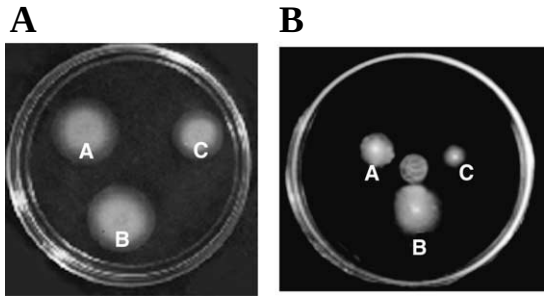

Fig. 2. a: Swarming behavior of *V. cholerae* 569B (A), 569BY (B) and 569BYN (C) cells grown on motility agar. b: Swarming behavior of *V. cholerae* 569B (A), 569BY (B) and 569BYN (C) cells grown on motility agar, the central well being filled with isolated mucous layer from intestinal wall of guinea pig.

about 55% identical to *E. coli* CheY-1 protein and 98% identical to the recently published putative CheY-4 protein from *V. cholerae* ElTor strain N16961 located in chromosome II [21]. The five residues (Asp12, Asp13, Asp56, Thr87 and Lys109) found in all CheY proteins to date were conserved in CheY-4. Asp56 is the site of phosphorylation by CheA, Asp12 and Asp13 help to coordinate Mg^{+2}, an essential element in phosphorylation and dephosphorylation, Lys109 is involved in the conformational change of phosphorylated CheY and Thr87 is proposed to generate and stabilize this conformational change.

Maxicell analysis was carried out to examine the protein encoded by the *V. cholerae* fragment in pRB600. The plasmid pRB600 was transformed into the *E. coli* CSR603 cells and the transformants were irradiated with UV light and labeled with ^{35}S methionine for 1 h and the soluble proteins were assayed by SDS–PAGE and autoradiography. The 908 bp DNA fragment was found to encode a protein of about 12.5 kDa (data not shown), which is in agreement with the predicted amino acid sequence of 123 amino acids.

3.2. Construction of V. cholerae strain duplicating cheY-4

To study the effect of induction of *cheY-4* gene of *V. cholerae*, a strain (569BY) containing duplication of *cheY-4* gene was constructed by chromosomal integration of the mobilizable suicidal plasmid pGP704 containing full-length *cheY-4* gene. A null *cheY-4* mutant (569BYN) was also constructed by similar strategy using an internal *Xba*I-*Sal*I fragment from the plasmid pRB900 derived from pRB600. That the mobilized plasmid has integrated into the genome at the desired site in the *cheY-4* gene was confirmed by Southern blot analysis. The expression level of cheY-4 in 569BY containing duplicated *cheY-4* was about two-fold of that of the wild type, while *cheY-4* specific transcript was barely detectable in 569BYN when examined by Northern blot analysis (Fig. 1).

3.3. Swarming ability of 569BY

The swarming capability of 569BY was examined in semi-solid LB motility plates made with 0.3% Bactoagar (Difco) motility and compared with that of 569B and 569BYN. Compared to wild type the swarming area increased to about 172% in 569BY, while the corresponding area for 569BYN was 60% (Fig. 2a). Since the swarming ability of bacteria depends on its growth, the growth rates of the three strains were determined in LB and were found to be similar. Thus the increased expression of *cheY-4* is accompanied by increased swarming

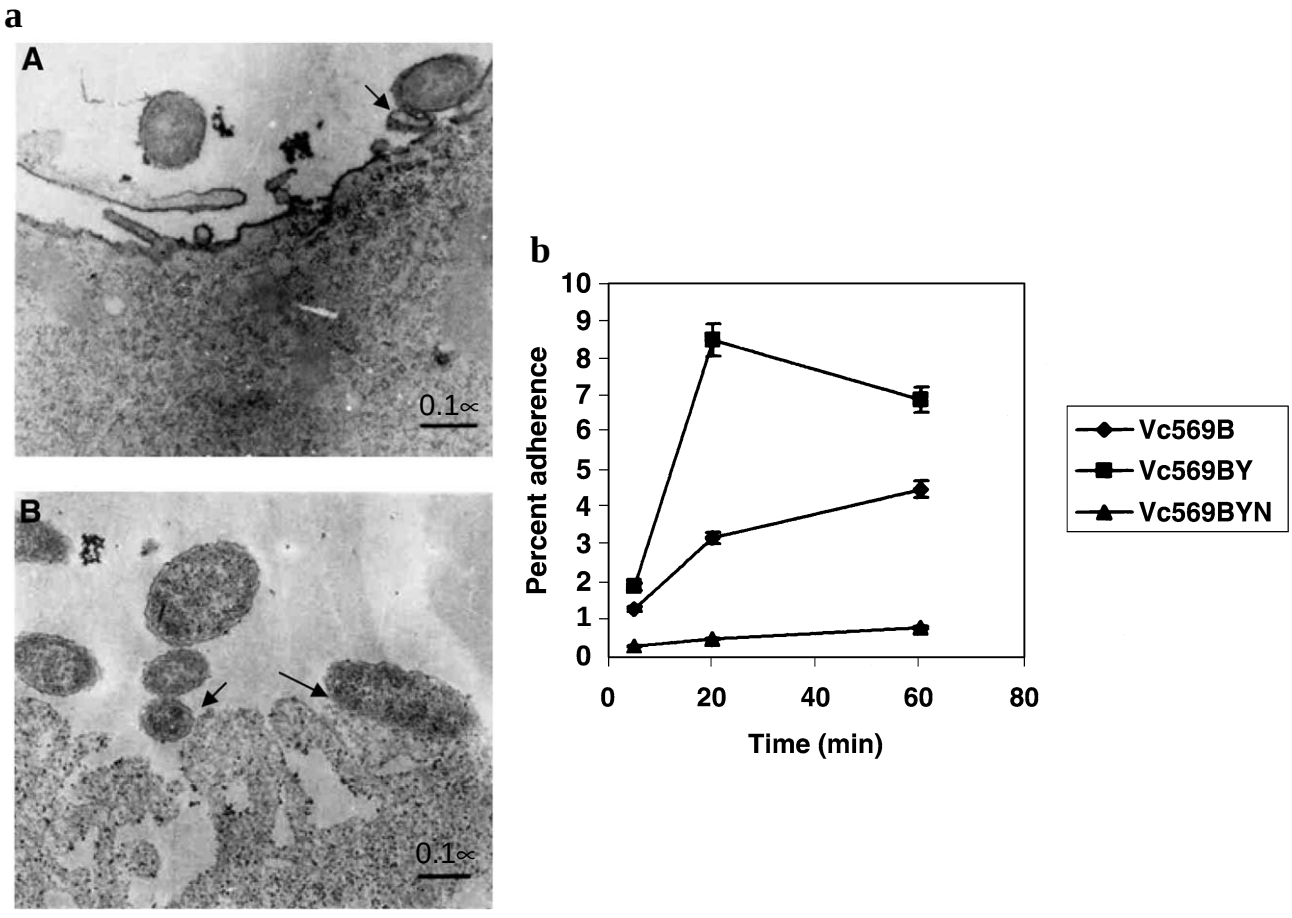

Fig. 3. a: Ultrathin sections of Int407 cell monolayers infected with *V. cholerae* 569B at magnification of 60 000× (A), *V. cholerae* 569BY at 60 000× (B). b: Adherence of Int407 intestinal epithelial cell monolayer by *V. cholerae* 569B, 569BY and 569BYN. Each *V. cholerae* strain (about 2×10^8 CFU) was added to a separate well of a six-well tissue culture plate, each of which contained confluent epithelial cells. The adherence was monitored at 5, 20 and 60 min, respectively, as described in Section 2. The values shown are means ± S.E.M. from five different experiments.

ability. Since *V. cholerae* penetrates the mucous coat of the small intestine and adheres to it, it is possible that the chemotactic genes might be involved in such a process. To explore this possibility the mucus layer was used in the central well of a motility agar plate and the three strains were examined for their ability to swarm towards the mucus solution. Interestingly, 569BY was found to be attracted towards the central well (as evident from the oval shape of the swarming bacteria) and at the same time the swarm area increased to about 243% compared to the wild type. In contrast, the swarm diameter of 569BYN decreased to about 36% (Fig. 2b).

3.4. Adherence to intestinal epithelial cell line

To address the role of increased *cheY-4* expression in the interaction of *V. cholerae* with intestinal epithelial cells, adherence of 569BY to human intestinal epithelial cell line Int407 (NCCS, Pune, India) was compared with that of the wild type by transmission electron microscopy as described in Section 2. Intimate attachment could be observed with both wild type and 569BY (Fig. 3a). Interestingly, the number of *V. cholerae* cells associated per Int407 cell after infection with 569BY was much more than that of the wild type (Fig. 3a). This might be due to an increased adherence of 569BY to epithelial cells or might be due to the chemotactic effect of mucous layer. No differences, however, were observed in the CFU of adhered 569B and 569BY with monolayer of Int407 at 37°C under 5% CO_2 after 2 h and 4 h of incubation (data not shown). The interaction of 569B, 569BY and 569BYN with the Int407 cell lines were then examined at early time points (up to 60 min) as at early time points the adherence due to chemotaxis would be more pronounced. Int407 associated CFU was much higher in 569BY compared to wild type, while that of 569BYN was much lower. A sharp increase in the cell associated CFU of 569BY was observed up to 20 min, which declined at 60 min. In contrast, the CFU of adhered 569B wild type showed a steady increment up to 60 min (Fig. 3b).

Adherence and colonization of the intestinal epithelial tissues lining the small bowel is a crucial step in *V. cholerae* pathogenesis. Electron microscopic examination of ultrathin sections of human intestinal cell line infected with *V. cholerae* 569B and 569BY have suggested that adherence increases in *cheY-4* duplicated strain. Moreover, the measurement of cell-associated CFU suggested that adherence is much higher in 569BY compared to wild type and the null mutant and is pronounced at an early time point (up to 20 min), suggesting that the adherence might be chemotaxis mediated. This is supported by the fact that isolated mucous layer could act as a chemoattractor for 569BY. It is possible that *cheY-4* mediated chemotactic attraction towards the mucus layer cov-

ering the upper surface of intestinal epithelium might be responsible for initial adherence in vivo. More experiments are needed to find out whether such mechanisms occur in vivo.

3.5. Expression of virulence factors in 569BY

In case of *V. cholerae*, the roles of motility and chemotaxis in pathogenesis have not been clearly established. However, the production of the two major virulence factors cholera toxin (CT) and the toxin co-regulated pili (TCP) is known to be affected by the motility phenotype of the bacterium [20]. CT is an ADP-ribosylating toxin largely responsible for eliciting the profuse diarrhea characteristics of this disease, and TCP is a type IV bundle forming pilus that is essential for intestinal colonization in humans and in animal models of cholera [22]. To find out whether duplication of the *cheY-4* gene is associated with increased or decreased expression of *ctxAB* and *tcpA*, RNA was isolated from 569B, 569BY and 569BYN and Northern blot hybridization was performed using cloned *ctxAB* as well as with cloned *tcpA* gene (laboratory collection) as probe. An increased expression of both *ctxAB* (~1.8-fold) and *tcpA* (~1.9-fold) was observed in 569BY compared to the wild type, but the reduced expression of both *ctxAB* (0.67) and *tcpA* (0.77) was observed in 569BYN (Fig. 4a,b). CT production was also investigated by GM1 ganglioside-dependent ELISA. The strains were grown in LB medium (pH 6.5) at 30°C for 16–18 h ($4–5\times10^9$ CFU per ml), conditions favorable for optimum CT production

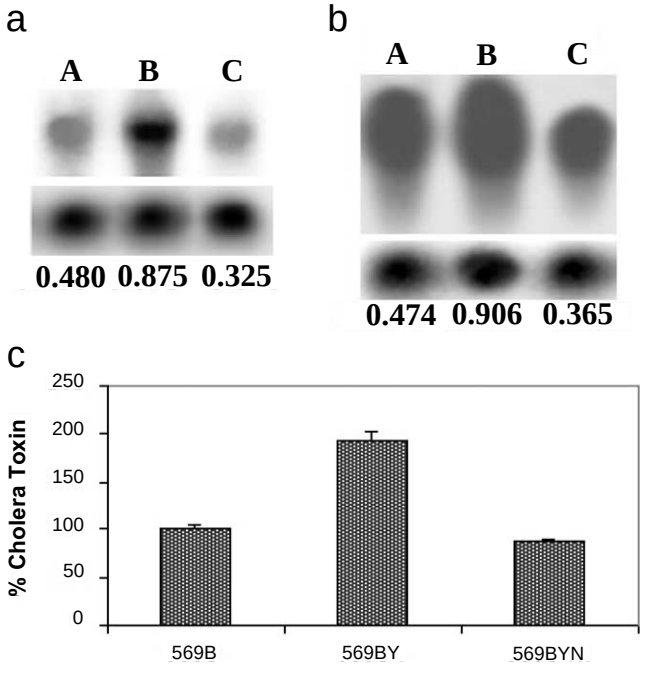

Fig. 4. Northern blot analysis of expression level of *ctxAB* (a) and *tcpA* (b) gene of *V. cholerae* strains 569B (lane A), 569BY, (lane B), 569BYN (lane C). Total RNA was isolated from the cells grown in 37°C in LB (pH 7.4) and Northern blot was prepared, probed with [^{32}P]dCTP labeled cloned *ctxAB* and *tcpA* gene, respectively. Lower panel in each case represents the 23SrRNA as an internal standard. The values indicated below are the relative ratio between the intensities of the corresponding gene expression to that of optimized 23SrRNA expression. c: Cholera toxin production in *V. cholerae* cells 569B, 569BY and 569BYN. Cells were grown in LB at 30°C, pH 6.5, and CT was measured in culture supernatants by GM1-ELISA.

[19], and the amount of toxin in culture supernatant was determined for each strain. About 1.12 μg/ml of CT could be detected in the culture supernatant of the wild type strain 569B. In contrast, the culture supernatant of *cheY-4* duplicated mutant 569BY produced 2.167 μg/ml of CT and that of *cheY-4* disrupted mutant 569BYN produced 950 ng/ml of CT (after normalization of CFU of mutant and wild type strains). Thus, compared to wild type, CT production is increased by more than 93% for 569BY and that is reduced by 14% for 569BYN (Fig. 4c).

Random non-motile mutants of *V. cholerae* have shown increased expression of CT and TCP. Moreover, defined mutations viz. deletions in *cheY-3* or *cheA-2* have indicated that there is no direct link between lack of chemotaxis and virulence gene expression. Interestingly, Lee et al. [5] reported that several *V. cholerae* chemotaxis genes, including *cheA-2*, regulate virulence gene expression in an in vivo model. It is therefore clear that no definite regulation of virulence factors by chemotaxis genes has so far been proposed. In this context the regulation of virulence factors by in vivo induced *cheY-4* might be important.

3.6. Organization of cheY-4

Analysis of upstream and downstream sequences in the published complete genome sequence of *V. cholerae* reveals that the *cheA-3* gene is present immediately downstream of *cheY-4* at a distance of 49 bp. In silico promoter analysis through http://www.fruitfly.org/seq_tools/promoter.html predicts the presence of a promoter at 85–130 bases from the translation start site of *cheY-4*. No promoter could be detected between *cheY-4* and *cheA-3*. A conserved hypothetical protein with STAS motif designating anti-sigma factor is present immediately upstream of *cheY-4*. This anti-sigma factor has its own promoter. The organization of this region of *cheY-4* [21] is: anti-sigma factor-CheY-4-CheA-3-CheW-3-CheW-2- putative methyl-accepting chemotaxis protein-CheR-3-CheD-CheB-3-putative methyl-accepting chemotactic protein-anti-sigma factor-putative response regulator-conserved hypothetical protein.

The present study is the first report in which the in vivo induced chemotaxis gene *cheY-4* present in chromosome II of *V. cholerae* has been characterized. The strain containing duplicated *cheY-4* gene showed increased motility, increased chemotactic response towards isolated intestinal mucus layer and stronger adhesion to human intestinal epithelial cell line at an early phase of infection compared to wild type and a null mutant strain. The duplication of *cheY-4* was associated with enhanced expression of the two major virulence genes *ctxAB* and *tcpA*, suggesting a yet to be defined role of chemotaxis genes in virulence factors regulation. When the motility, early adherence to intestinal epithelial cell line and expression of *ctxAB* and *tcpA* were compared between wild type and null mutant of *cheY-4* the observed phenotypes were not repressed in the null mutant as expected. It is likely that the other three *cheY* gene(s) might also contribute to maintaining the above phenotypes in the null mutant. Very recently, using cDNA microarray technology, it has been shown that many chemotactic genes are down-regulated in stool-derived *V. cholerae* [6]. However, among the four *cheY* genes, only *cheY-4* has been repressed, which probably suggests that the regulation of *cheY-4* is time-dependent and is probably required during the early stage of infection. Our future direction

will involve further experimentation of temporal expression pattern of *cheY-4* along with the roles played by other chemotaxis genes of *V. cholerae*.

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