Materials & Methods.....
3.1 MATERIALS

1. Bacterial strains and plasmids

All the bacterial strains used are listed in Table 3.1 and plasmids in Table 3.2 along with relevant markers, source and reference.

Table 3.1 Bacterial strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. cholerae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD81</td>
<td>Wild type, Sm', O1, classical biotype strain O395</td>
<td>Kumar et al., 1994</td>
</tr>
<tr>
<td>CD83</td>
<td>Derivative of CD81, salt sensitive, tcpR::TnphoA, Km', Sm'</td>
<td>This study</td>
</tr>
<tr>
<td>CD84</td>
<td>Derivative of CD83, Complemented with wild type tcpR, Km'</td>
<td>This study</td>
</tr>
<tr>
<td>KB619</td>
<td>Sm', O1 classical, Ogawa</td>
<td>Srivastava et al., 1980</td>
</tr>
<tr>
<td>CD85</td>
<td>Animal passaged culture of VBNC KB619</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM10</td>
<td>thi thr leu tonA lacY supE recA::RP4-2-Tc::mu carrying plasmid pRT291</td>
<td>Simon et al., 1983</td>
</tr>
<tr>
<td>MM294</td>
<td>F' endA1 thi-1 hsdR17 supE44λ' carrying plasmid pPH1J1</td>
<td>Bachmann, 1987</td>
</tr>
<tr>
<td>DH5α</td>
<td>F' φ80dlacZ ΔM15 recA1 endA1 gyrA96 thi-1 hsdR17 (rK'mK') supE44 relA1 deoRΔ(lacZYA'argF) U169 Host strain used for cloning</td>
<td>Raleigh et al., 1989</td>
</tr>
</tbody>
</table>
### Table 3.2  List of the plasmids utilized for this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC18</td>
<td>Cloning vector, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Bangalore Genei</td>
</tr>
<tr>
<td>pRT291</td>
<td>Derivative of plasmid pRK290, carrying TnphoA, Km&lt;sup&gt;+&lt;/sup&gt;, Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Taylor et al. (1989)</td>
</tr>
<tr>
<td>pPH1JI</td>
<td>Incompatible plasmid to pRT291, Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Beringer et al. (1978)</td>
</tr>
<tr>
<td>pSM1</td>
<td>N- and C- terminal ends of mutated gene of CD83 cloned in pUC18, Ap&lt;sup&gt;+&lt;/sup&gt;, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSM11</td>
<td>pUC18 containing a 456 bp BamHI PCR fragment <em>(tcpR)</em> inframe with lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>pJMK5</td>
<td>pBR322 containing TnphoA, Ap&lt;sup&gt;+&lt;/sup&gt;, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Dr. J. B. Kaper</td>
</tr>
</tbody>
</table>

### 2. Chemicals and Biochemicals

Analytical and molecular biology grade chemicals and general molecular biology products including antibiotics, specific enzyme tagged antibodies, protein molecular weight markers as well as enzymes including DNase, RNase, proteinase K were purchased from Sigma (USA). Restriction enzymes and modifying enzymes including T4 DNA ligase were purchased from Stratagene (USA), New England Biolabs (USA), MBI Fermentas, Promega, and Bangalore Genei (India). Non radioactive DIG-DNA labelling kit was purchased from Boehringer Mannheim, Germany. Custom
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oligonucleotides were synthesized by Sigma Genosys and PCR kits were from Promega.

Dehydrated media and media ingredients were purchased from Difco Laboratories, (USA). General biochemicals and solvents for routine use were purchased from E-Merck (Germany), BDH (England), BRL (USA) and local companies like Qualigens, Spectrochem, Hi Media and Ranbaxy.

3. Water

Desalted, water of milli RO grade was used for the preparation of media and general buffers. Milli Q grade water was used for the preparation of reagents/solvents for protein and DNA analysis. Millipore (USA) water system was used to get the high quality water.

4. Molecular weight markers

**DNA size markers**

\( \lambda \text{DNA/HindIII digest (NEB):} \)

23130, 9416, 6557, 4361, 2322, 2027, 564, 125 bp size fragments.

\( \lambda \text{DNA/HindIII + EcoRI digest (MBI Fermentas):} \)

21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125 bp size fragments.

**100 bp DNA ladder (NEB):**

1517, 1200, 1000, 900, 800, 700, 600, 500/517, 400, 300, 200, 100 bp size fragments.
**Protein molecular weight markers (kDa)**

SDS-7B (Sigma, USA):
180.0, 116.0, 84.0, 58.0, 45.0, 36.5, 26.6 kDa.

Colour marker for protein transfer (Sigma, USA):
205.0, 116.0, 66.0, 45.0, 29.0 kDa.

Rainbow™ coloured marker (Pharmacia):
45.0, 30.0, 20.1, 14.3, 6.5, 3.5, 2.5 kDa.

3.2 METHODOLOGY

(A) IDENTIFICATION AND CHARACTERIZATION OF THE GENE (S) RESPONSIBLE FOR SALT TOLERANCE IN SMALL INTESTINE

1. Construction and analysis of TnphoA insertional mutant library

(a) **Construction of TnphoA library of V. cholerae O1 classical strain CD81**

The plasmid pRT291 harboring TnphoA, a modified transposon Tn5, was used to construct the mutant library. The plasmid was transferred in CD81 by means of conjugation. SM10 strain of *E. coli* was used as the host strain for plasmid pRT291. The bacterial mating was performed on LB agar plates at 37°C by cross streaking the donor and the recipient strains at right angle to each other. Bacterial growth from the cross points (where the recipient and donor were in physical contact) were serially diluted in PBS and plated on LB agar plates containing streptomycin (Sm) and kanamycin (Km) antibiotics. Since the recipient strain CD81 was Sm resistant, the colonies obtained represent
transconjugants where pRT291 had been transferred to CD81. The transconjugants were transferred on LB agar plates containing Sm and Km. Transposition was allowed to occur at 37°C for 24 h. To select the transconjugants, where TnphoA was transferred on to the chromosome, pPH1JI, a plasmid, incompatible with pRT291 was mobilized in primary conjugants, and selected on LB agar plates containing gentamycin (Gm) and Km antibiotics. Final Km’ transconjugants represent those, which have acquired TnphoA on to their chromosome.

The final conjugants carrying TnphoA insertion were screened for alkaline phosphatase activity. The bacteria were serially diluted and plated on to LB agar plates containing selective antibiotics and 5-bromo-4-chloro-3-indolyl phosphate (BCIP), the substrate for alkaline phosphatase enzyme. Plates were supplemented with 0.2% glucose to suppress endogenous phosphatase level of CD81. Blue coloured colonies were selected and purified on LB agar plates containing BCIP. These represent the conjugants, in which the transposon was inserted inframe in a membrane spanning or secretary protein.

(b) Analysis of TnphoA mutant library

Selection of salt sensitive mutant

All TnphoA blue mutants, along with wild type strain CD81, were grown on LB agar plates with streptomycin containing different NaCl concentrations: 100 to 400 mM. Plates were incubated at 37°C for overnight. One of the mutants, designated CD83, could not grow on higher concentrations
of NaCl and was selected. Survival of CD83 on different concentrations of NaCl was determined and compared with that of parent strain CD81.

Both CD81 and CD83 were grown overnight on nutrient agar slants at 37°C and harvested in and washed with autoclaved distilled water to obtain $10^9$ cfu/ml. Different dilutions were made and 0.1 ml of appropriate dilution (to get isolated colonies) of each culture was plated on LB agar plates having 100-400 mM NaCl. Plates were incubated at 37°C for overnight. Next day number of colonies on each plate were counted and cfu/ml value of CD81 and CD83 in presence of 100, 200, 300 and 400 mM NaCl was calculated. A graph was plotted between cfu/ml and NaCl concentration.

**Adherence of CD81 and CD83**

Adherence of CD81 and CD83 to intestinal mucosa was measured according to Bhattacharjee and Srivastava (1978) by using freshly isolated rabbit intestinal discs of uniform sizes (10 mm). Bacteria were grown overnight on nutrient agar slants at 37°C and harvested in PBS to obtain cell density of $10^9$ cfu/ml. Viable counts of the bacterial cell suspensions were determined by preparing serial dilutions in PBS and plating 0.1 ml on nutrient agar plates containing selective antibiotics. Bacteria were diluted 100 times ($10^{-2}$ dilution) in 5 ml PBS in a 25 ml capacity sterile conical flask and a freshly isolated rabbit intestinal disc of 10 mm diameter was exposed to it for 30 min at 37°C with gentle shaking. The disc was washed twice with sterile distilled water to remove non-adherent bacteria. The disc was homogenized in 10 ml PBS. Viable counts of the adherent bacteria were determined by plating appropriate dilutions on nutrients agar plates containing selective antibiotics. Adherence index of the bacteria adhered to intestinal mucosa was determined as:
Total number of adherent bacteria

\[ \frac{\text{Total number of adherent bacteria}}{\text{Total number of bacteria to which intestinal disc was exposed}} \times 100 \]

Colonization of intestine by CD81 and CD83

Intestinal colonization was studied essentially as described by Singh et al. (1994) in 5-day-old infant mice. Briefly, the overnight grown culture of CD81 and CD83 was suspended in PBS to \(10^9\) cfu/ml. 0.1 ml inoculum containing \(10^6\), \(10^7\) and \(10^8\) cells were orally fed to different groups of infant mice. The control group was fed with 0.1 ml PBS. Mice were sacrificed at the interval of every 24 h and intact gut was removed, homogenized in PBS and different dilutions were plated on LB agar plates containing appropriate antibiotics. Colonization was observed upto 72 h of infection and colonization index was measured in terms of the ratio of the adherent bacteria to the initial input.

Virulence of CD81 and CD83

The virulence was measured by the ability to cause mortality of infant mice as described by Singh et al. (1994). Different dilutions of overnight grown culture were made in PBS and 0.1 ml inoculum containing \(10^6\), \(10^7\) and \(10^8\) bacteria were fed to different groups of infant mice with the control group being fed with PBS alone. Death of mice was observed upto 72 h.
Preparation and assay of cholera toxin

Toxin was obtained by growing CD81, CD83, and CD84 in syncase sucrose minimal medium supplemented with casamino acids at 30°C for 18 h in a shaker. Bacteria free culture filtrate was obtained by membrane filtration (Millipore, 0.22 µm pore size). The presence of toxin was assayed in two ways. (1) The presence of biologically active toxin was measured in vivo in adult albino guinea pigs. Culture filtrate was diluted in BHI and 0.1 ml was injected intradermally. Induration was recorded after 18 h. BHI alone caused no induration (Craig, 1971; Singh et al., 1994). (2) Toxin was estimated by ELISA essentially as described before (Harlow and Lane, 1988; Singh et al., 1994). Culture filtrate was serially diluted and coated in immunoplate wells (NUNC, Denmark). Titration was done using rabbit anti-cholera toxin IgG (1:500 dilution) and horseradish peroxidase conjugated goat anti-rabbit IgG (1:1000 dilution) (Sigma, USA) and O-phenylenediamine dihydrochloride as substrate. Absorbance was read at 492 nm.

2. DNA studies

DNA isolation and manipulation experiments were carried according to Sambrook et al. (1989), unless stated otherwise. The reagents, buffers and solutions used are listed in Appendix (chapter VII).

(a) DNA isolation

Isolation of Plasmid DNA

Plasmid DNA was isolated by alkaline lysis as described by Sambrook et al. (1989) with minor modifications. The plasmid containing culture of E.
coli was inoculated into terrific broth containing the appropriate antibiotic and incubated overnight at 37°C under shaking conditions. The cells were harvested by centrifugation at 6,000 g for 10 min at 4°C and the supernatant discarded. The pellet was resuspended in GTE buffer. Two volumes of NaOH-SDS mix was added, the mixture was incubated over ice for 5-7 min followed by the addition of 3.5 volumes of acetate mix and a further incubation of 1 h over ice. The debris was pelleted at 10,000 g for 15 min at 4°C twice, and the supernatant removed into another tube. The plasmid DNA was precipitated by the addition of equal volume of chilled isopropanol and incubation at –20°C for 1 h. The DNA was pelleted by centrifugation at 12,000 g for 15 min, washed twice with 70% and once with 96% ethanol and the supernatants discarded. The pellet was air dried and suspended in autoclaved Milli Q. After resuspension, a small amount of the DNA was checked by agarose gel electrophoresis and the rest stored at –20°C.

**Isolation of Genomic DNA**

Overnight grown bacterial culture was centrifuged at 6,000 rpm at 4°C for 10 min. Cell pellet was washed once with and suspended in TEN buffer. To this, SDS (1.5% final concentration) was added and cells were lysed by gently mixing at 65°C waterbath for 5 min. Proteins were precipitated by adding equal volume of buffered phenol and incubating in ice for 15 min. After centrifugation at 12,000 rpm at 4°C for 20 min, the upper aqueous phase was transferred in a fresh tube. The aqueous phase was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). To the final aqueous phase thus obtained, 0.8 volume of isopropanol was added and mixed properly. The DNA thus
precipitated was centrifuged. Pellet was washed twice with 70% and once with 96% ethanol, air dried, suspended in TE buffer and stored at -20°C.

(b) **Quantitation and purification of DNA**

The quantity of DNA was determined by measuring OD at 260 nm (1 OD = 50 μg/ml for double stranded DNA, 1 OD = 33 μg/ml for single stranded DNA). The DNA was purified either by simple phenol:chloroform:isoamyl alcohol extraction or ethanol precipitation (Sambrook *et al.*, 1989), depending on the need. After purification, DNA was air dried and suspended in appropriate volume of Milli Q for further analysis.

(c) **Digestion of DNA using restriction endonucleases**

DNA was digested with appropriate restriction enzymes in buffers recommended by their manufacturers. 1 μg of DNA was digested in a total reaction volume of 30 μl for 16 h at the optimal temperature for the enzyme(s). The reaction was terminated by heating at 75°C or by the addition of tracking dye and freezing at -20°C. The digestions were analyzed by agarose gel electrophoresis using proper controls and molecular size markers.

(d) **Agarose gel electrophoresis**

The gel electrophoresis of DNA was carried out using horizontal submarine gel electrophoresis apparatus (BRL or Genei) as described by Sambrook *et al.* (1989). Briefly, dehydrated agarose powder (required concentration) was weighed and suspended in 1X TBE/TAE buffer, melted by boiling and cooled to 45-50°C. The cooled agarose was poured into a casting tray fitted with comb and the gel allowed to solidify for 30-45 min. When gel
got properly solidified, comb was removed. The casting tray was then placed in an electrophoresis apparatus filled with 1X TBE/TAE buffer. The DNA samples to be analyzed were mixed with 6X tracking dye (final concentration 1X) and loaded into the wells. Electrophoresis was carried out at a constant voltage of 5-10 V/cm depending upon the buffer used for electrophoresis. After the required amount of separation, the DNA was stained using ethidium bromide and visualized under UV light. The electrophoresed DNA was analyzed on UVP gel documentation system and photographed. In cases where the DNA bands need to be eluted, preparative agarose gels were prepared using either routine or low melting point agarose.

(e) **Dephosphorylation of Plasmid DNA**

The vectors utilized for cloning and subcloning purposes were dephosphorylated using calf intestinal alkaline phosphatase (CIAP). The dephosphorylation was carried out in the same restriction digestion reaction mixture. Alternatively, purified fragments were dephosphorylated in CIAP buffer. One unit of CIAP was added to 0.15 pM of ends and incubated at 37°C for 15 min, followed by addition of another 1 unit and a further incubation step of 45 min at 37°C. The reaction was stopped by heating at 68°C for 15 min. The mixture was extracted with phenol and then with chloroform:isoamyl alcohol. The aqueous phase was mixed with sodium acetate (0.3 M final concentration, pH 5.2) and equal volume of isopropanol to precipitate the DNA. The DNA was pelleted, washed, air dried and suspended in Milli Q.
Elution of desired DNA

Electroelution of inserts from agarose gel

The agarose containing the desired fragment was excised and placed in a dialysis bag (6-8 kDa cut off) containing autoclaved 0.5X TBE buffer. The dialysis bag was placed in the electrophoresis apparatus containing 0.5X TBE and electrophoresis carried out at 100 V for 3 h. The current was reversed for 3 min and the buffer from the dialysis bag recovered. After extraction once with phenol and then with chloroform:isoamyl alcohol, the DNA was precipitated using sodium acetate (0.3 M final concentration) and equal volume of isopropanol. After incubation at -20°C, the DNA was pelleted by centrifugation, washed twice with 70% and once with 96% ethanol, air-dried and suspended in Milli Q.

Elution of DNA fragments using low melting point agarose (LMP)

The DNA was electrophoresed in LMP agarose gel of appropriate percentage in cold. The desired band was cut out and placed in an eppendorf tube. TE buffer was added, twice the volume of the gel, the agarose piece was melted at 65°C waterbath and extracted once with phenol and once with chloroform:isoamyl alcohol. The aqueous phase was separated, and the DNA precipitated using sodium acetate (0.3 M final concentration, pH 5.2) and twice the volume of 96% ethanol. The DNA precipitate was pelleted by centrifugation, washed twice with 70% and once with 96% ethanol, air-dried and suspended in Milli Q.
Elution of DNA fragments using DEAE cellulose paper

The DNA digest was electrophoresed on a routine agarose gel and stained using ethidium bromide. A slit was made in the agarose gel just ahead of the DNA fragment of the interest and a piece of DEAE cellulose placed in the slot. The electrophoresis was continued until the DNA impregnates on the paper. The paper was removed and washed once with low salt buffer. The paper was shredded into smaller pieces and the DNA eluted in a high salt buffer by incubation at 70°C for 30 min. The spent buffer was removed into another tube and another fresh aliquot was added and incubation continued another 15 min. This was repeated once more. The supernatants were pooled and extracted once with chloroform:isoamyl alcohol. The aqueous phase was separated and 0.3 volume of 7.5 M ammonium acetate and equal volume of isopropanol were added to precipitate the DNA. The DNA was pelleted by centrifugation, washed twice with 70% and once with 96% alcohol. The pellet was air dried and suspended in suitable volume of Milli Q.

(g) Ligation

(i) Cloning of insert (staggered ends)

Ligation was carried out according to manufacturer’s instructions (MBI Fermentas).
The insert to vector ratio was more than 2:1

| Insert DNA | 0.2-1 µg |
| Dephosphorylated linearized plasmid DNA | 0.1-0.5 µg |
| 10X T4 DNA ligase buffer | 2.0 µl |
**Materials & Methods**

10 mM ATP  
Milli Q to a total volume of 20 µl.

Add T4 DNA ligase (1-2 units), mix and incubate at 16°C overnight. Heat inactivate ligase at 65°C/15 min before transformation.

(ii) **Cloning of insert (blunt end ligation)**

Insert to vector ratio was more than 2:1

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert DNA</td>
<td>0.2-1 µg</td>
</tr>
<tr>
<td>Vector DNA</td>
<td>0.1-0.5 µg</td>
</tr>
<tr>
<td>10X T4 DNA ligase buffer</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>10 mM ATP</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>50% PEG 4000</td>
<td>2.0 µl</td>
</tr>
</tbody>
</table>

Milli Q to a total volume of 20 µl.

Add 5 units of T4 DNA ligase, mix and incubate at 16°C overnight. Heat inactivate ligase at 65°C/15 min before transformation.

(h) **Transformation of DNA in E. coli**

DH5α strain of *E. coli* was used for maintenance of all the plasmids and recombinant molecules. Transformation was done as described by Hanahan (1983) with minor modifications. Briefly, a single colony of *E. coli* DH5α was inoculated in LB broth and incubated overnight at 37°C under shaking conditions. The seed culture was inoculated at 1:50 dilution in fresh LB and incubated at 37°C under shaking conditions, till growth reached to $10^7$-$10^8$ cells/ml. Cells were centrifuged at 5,000 g for 10 min at 4°C and the pellet was washed with TFB I. The bacterial pellet was suspended in TFB II and
incubated over ice for 30 min to prepare competent bacteria. Cells were again centrifuged and pellet was suspended in about 1/12.5 th volume of fresh TFB II. The cells were aliquoted and kept over ice. DNA was added to the tubes and the mixture incubated over ice for another 30 min. Heat shock was given by immersing the mixture in a waterbath set at 43.5°C for 40 s. About 6.5 volumes of Z-broth was added and the mixture incubated at 37°C for 40 min. The transformation mixture was plated on LB agar plates containing appropriate antibiotics and incubated at 37°C.

(i) **Electroporation of V. cholerae (Schodel et al., 1991)**

Cultures were grown overnight at 37°C under continuous shaking in LB containing ampicillin (100 µg/ml). A dense overnight culture of *V. cholerae* was diluted 1:100 in 20 ml LB and allowed to grow to exponential phase for 1.25-1.5 h at 37°C under vigorous aeration. The cells were spun down for 10 min at 5000 rpm, and resuspended in 10 ml ice cold 2 mM CaCl₂, spun down for 10 min at 5000 rpm a second time and resuspended in 200 µl ice cold 1 mM CaCl₂. Plasmid DNA (10-100 ng in 5 µl TE) was added to 40-50 µl of this cell suspension, shaken down to a precooled electroporation cuvette (0.2 cm, Biorad) and electroporated once at 2.5 kV, 25 µF and 200 ohm in a gene pulser (Biorad). Immediately after the pulse the cells were gently resuspended in LB and allowed to grow without antibiotic pressure for 1 h at 37°C without shaking, before plating on LB agar plates containing 100 µg ampicillin/ml.
(j) **Confirmation of the clones**

Master plates of the transformants were made. Plasmid DNA was isolated from these clones by miniprep plasmid isolation method and the clones were confirmed by restriction digestion with appropriate restriction enzymes. The digested DNA was analyzed by agarose gel electrophoresis. Size of different DNA bands were determined by reference molecular weight markers. In addition to restriction patterns of the clones, hybridization with suitable probes was performed to conclusively establish the authenticity of the clones.

(k) **Southern hybridization**

The DNA digests were electrophoresed in an agarose gel prepared in suitable buffer, with proper controls and molecular size markers. The electrophoresis was continued until a suitable amount of separation was achieved. The gel was stained and photographed.

Agarose containing genomic DNA digests was pretreated with depurination solution for 15 min before denaturation and neutralization. Gels containing plasmid DNA digests were directly treated with denaturation solution for 45 min and neutralization solution for another 45 min. The DNA from the neutralized gels was transferred onto nitrocellulose paper by capillary blotting as described by Southern (1975).

**Capillary transfer of electrophoresed DNA**

The setup used for capillary transfer was as described by Sambrook *et al.* (1989). The two electrode buffer reservoirs of horizontal submarine electrophoresis unit were partially filled with 20X SSC solution. A wick of
Whatman No. 3 paper was soaked in 20X SSC solution and placed over the gel platform of the electrophoresis unit with its ends submerged into 20X SSC solutions in both reservoirs. One more sheet of Whatman 3 paper of slightly bigger size of the gel was presoaked in 20X SSC solution and placed over the wick. The gel was carefully placed onto the Whatman sheet in the inverted position taking care that no air bubble was entangled between the sheets and the gel. The water saturated nitrocellulose membrane of the gel size was soaked in 20X SSC solution and placed over the gel. The membrane was marked with a cut for orientation and the air bubbles in between the gel and membrane were smoothened out carefully. Two more sheets of Whatman 1 of the gel size were placed over the membrane. A stack (8-10 cm height) of dry blotting papers were placed on the Whatman sheets and approximately 1-2 kg weight was placed on the top of stacked blotting papers. The transfer of DNA was allowed for 18-20 h at 4-8°C. Once the transfer was terminated, the gel was stained in ethidium bromide and seen over UV light for the efficiency of the transfer process.

The transferred DNA was fixed on nitrocellulose paper by UV crosslinking for 2 min using Stratalinker. Exposure to UV light created nicks in the DNA at thymine positions and DNA was bound covalently to the nitrocellulose paper.

**Preparation of DIG-labelled probes**

The DIG labelling of DNA was carried out using random primed DNA labelling kit (Boehringer Mannheim) as recommended by the manufacturer. The DNA to be labelled was melted (denatured) over boiling water bath for 5
min and snap frozen over ice for another 5 min. The eppendorf was centrifuged to pellet the condensate

<table>
<thead>
<tr>
<th>DNA</th>
<th>10 ng-3 µg (in 15 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random hexanucleotide primers</td>
<td>2 µl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>2 µl</td>
</tr>
<tr>
<td>Klenow enzyme</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

Mix, spin and incubate at 37°C for 20 h. Before use, boil the probe and snap freeze and add an aliquot to prehybridization solution.

After UV crosslinking the blot was placed in thermostable hybridization tube wrapped in saran wrap into which standard prehybridization solution was added (20 ml solution/100 cm² membrane). Tubes were sealed and prehybridization was carried out at 68°C for 4 h followed by addition of the probe and carrying out hybridization at 67°C for another 16 h with gentle shaking. On incubation the following procedure was followed

<table>
<thead>
<tr>
<th>Solution</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X wash solution</td>
<td>RT/shaking/15 min/twice</td>
</tr>
<tr>
<td>0.1X wash solution</td>
<td>68°C/shaking/15 min/twice</td>
</tr>
<tr>
<td>GBI</td>
<td>Rinse</td>
</tr>
<tr>
<td>GBII</td>
<td>RT/shaking/1 h</td>
</tr>
<tr>
<td>GBII + DAC</td>
<td>RT/shaking/45 min</td>
</tr>
<tr>
<td>GBI</td>
<td>RT/shaking/15 min/thrice</td>
</tr>
<tr>
<td>GBIII</td>
<td>Rinse</td>
</tr>
<tr>
<td>GBIII + colour substrate</td>
<td>RT/ dark/ stationary conditions till signal appeared</td>
</tr>
<tr>
<td>GBIV</td>
<td>Rinse and wash after colour development</td>
</tr>
</tbody>
</table>

63
Positive hybridization was indicated by the development of brown coloration.

(I) **PCR amplification of ORF**

The reaction was set up as recommended by the manufacturer. In brief, the following was set up for 50 µl reaction.

- 10X Taq DNA polymerase buffer 5 µl
- dNTP mix 200 µM each
- Primers 200 ng each
- Taq DNA polymerase 1.5 units
- Milli Q to a total volume of 50 µl.

Add diluted DNA containing about 0.1-10 ng of target.

**Reaction conditions**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>5 min</td>
</tr>
<tr>
<td>96°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Annealing temperature</td>
<td>1 min</td>
</tr>
<tr>
<td>72°C</td>
<td>2 min</td>
</tr>
<tr>
<td>72°C</td>
<td>5 min</td>
</tr>
<tr>
<td>6°C</td>
<td>hold</td>
</tr>
</tbody>
</table>

1 cycle

30 cycles

Checked the amplified product on an agarose gel with appropriate molecular size markers.

**Primer**

**Sequence**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VcTcp1</td>
<td>5’-CgCggATCCCATgACTAgCATATggTTACATg-3’</td>
</tr>
<tr>
<td>VcTcp2</td>
<td>5’-CgCggATCCTCACATTAACAAAAATACgCC-3’</td>
</tr>
</tbody>
</table>
Primer | Tm  | TA  | 5'-end site |
--------|-----|-----|-------------|
VcTcp1  | 67°C | 55°C | *BamHI* |
VcTcp2  | 58°C | 53°C | *BamHI* |

(m) **Cloning and expression of the PCR product in E. coli**

The PCR amplified product was purified by ethanol precipitation and digested with appropriate restriction enzymes, recognition sites of which were tagged with primers. pUC18 plasmid was used for normal cloning purposes. The vector DNA was digested with restriction enzymes compatible to the sites present on both the ends of the PCR product, treated with CIAP to remove 5' phosphate group and purified by phenol:chloroform:isoamyl alcohol extraction method. Ligation reaction was set up and ligation mixture was used to transform *E. coli* DH5α. Transformants were selected over LB agar plates containing ampicillin and Xgal (the substrate of β-galactosidase enzyme) for the blue/white screening of the recombinants. White clones were selected and further confirmed by plasmid isolation and restriction digestion.

Orientation of the clones was checked by digestion with appropriate enzyme. Right oriented clone was used for expression. Culture was grown overnight in LB broth containing ampicillin at 37°C. Overnight culture was diluted 1:50 in fresh LB and allowed to grow at 37°C under shaking conditions, till growth reached to 0.4-0.5 OD. IPTG (1.0 mM) was added and again culture was grown for 5 h at 30°C. Protein samples were prepared and analyzed by SDS-PAGE (section 3).
(n)  **Sequencing of the cloned inserts**

The plasmid used as template for sequencing reaction was isolated using Qiagen plasmid midi kit (Qiagen, Germany) and used as recommended. Briefly, 20 ml of overnight culture of recombinant *E. coli* in LB broth with an antibiotic was centrifuged and the pellet resuspended in 4 ml of buffer P1. 4 ml of buffer P2 was added, mixed thoroughly and incubated at room temperature for 5 min. 4 ml of chilled buffer P3 was added, mixed thoroughly and the mixture incubated over ice for 15 min. The debris was centrifuged at 20,000 g for 30 min at 4°C, the supernatant was recentrifuged at the same speed for 15 more minutes at 4°C. The supernatant containing the plasmid was added to 4 ml of buffer QBT equilibrated Qiagen tip 100, and allowed to pass through the resin by gravity flow. The tip was washed twice with 10 ml of buffer QC and allowed to drain completely. The DNA was eluted from the resin in the column using 5 ml of buffer QF. The eluate containing DNA was mixed with 3.5 ml of room temperature isopropanol, and immediately centrifuged at 15,000 g for 30 min at 4°C. The supernatant was discarded and pellet washed twice with 2 ml of room temperature 70% ethanol and centrifuged. The washed pellet was air dried and suspended in suitable volume of Milli Q. The amount of plasmid was checked by agarose gel electrophoresis.

The plasmid DNA was used as a template for the sequencing reaction as follows:

- **Template DNA** 10 ng
- **Primer** 20 ng
- **Big Dye Terminator reaction mix** 4 µl

Milli Q to a total volume of 10 µl
Mix and cycle at the following conditions

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Time</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>3 min</td>
<td>1 cycle</td>
</tr>
<tr>
<td>94</td>
<td>20 s</td>
<td>25 cycles</td>
</tr>
<tr>
<td>50</td>
<td>20 s</td>
<td>25 cycles</td>
</tr>
<tr>
<td>60</td>
<td>4 min</td>
<td>25 cycles</td>
</tr>
<tr>
<td>4</td>
<td>hold</td>
<td></td>
</tr>
</tbody>
</table>

The amplified product was precipitated using 0.1 volume of 3 M sodium acetate and 2 volumes of 96% ethanol. The DNA was pelleted immediately at 20,000 g for 30 min and the supernatant discarded. The pellet was washed once with 100 µl 70% ethanol, air-dried and suspended in 20 µl of TSA (Template suppression reagent).

The sample was denatured at 94°C for 3 min, snap frozen over ice and transferred to 0.5 ml sample vials with rubber closure. The sample tubes were placed in the sample tray and loaded into ABI prism 310 Genetic Analyzer. The samples were run in 50 cm path length (61 cm total length) capillary containing POP 6 (Performance Optimized Polymer) and using 1X Genetic Analyzer buffer across a voltage of 1.4 kV for 2 h. The raw data was analyzed using a BDT matrix to obtain the sequence data.

**Primer** | **Sequence**
---|---
K36 | 5'-ATCGCTAAGAGAAATCACGCAG-3'
G2 | 5'-CCGAAGAGAAACACAGATTTAGC-3'

3. **Protein analysis**

The buffers and solutions used are listed in Appendix (chapter VII).
(a) **Preparation of whole cell proteins**

Bacteria were grown overnight on nutrient agar slants. The culture was harvested in Tris-HCl buffer (10 mM, pH 8.0) and centrifuged at 7,000 rpm for 10 min at 4°C. Bacterial pellet was washed twice with the same and finally suspended in appropriate amount of buffer. To this equal volume of 2X sample buffer was added and cells were lysed by incubating in boiling water bath for 10 min. Lysed mixture was centrifuged and the supernatant containing total soluble protein fraction was taken for analysis by SDS-PAGE.

(b) **Quantitative measurement of proteins**

Amount of protein was measured according to Lowry *et al.* (1951) by using Folin Phenol reagent. Samples were taken in test tubes and volume raised to 1 ml with distilled water. 1.0 ml of solution C was added to each tube and incubated at room temperature for 15 min. To this was added 100 µl of solution D and the tubes were incubated at room temperature for 45 min in dark. Absorbance of the blue coloured complex was measured at 650 nm in spectrophotometer (LKB). Amount of protein present in the test sample was determined from the linear curved obtained by the known concentrations of the standard protein (BSA) processed in parallel with the test samples.

(c) **Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)**

**Preparation of resolving gel**

The vertical slab gel unit (BRL or BioRad) was assembled as per the manufacturer’s instructions. The monomer solution of acrylamide-bis-
acrylamide of desired concentration was mixed with appropriate amounts of Tris-HCl buffer (pH 8.8), SDS and freshly prepared APS according to Laemmli (1970). TEMED was added to this mixture just prior to casting the gel. The monomer mixture was poured into the slab gel glass plate sandwich unit leaving appropriate space on the top for stacking gel. Water saturated n-butanol was layered on to it and allowed the gel to polymerize. Formation of a very sharp, visible liquid gel interphase was the indication of gel polymerization.

**Preparation of stacking gel**

The top of resolving gel (12.5%) was rinsed with distilled water to remove n-butanol. Stacking gel (4%) was laid over it by mixing all the components in appropriate conditions. A comb on the top was inserted and the gel was allowed to polymerize. Formation of visible well dividers beneath the comb was the indication of polymerization.

**Electrophoresis**

The sandwich containing SDS polyacrylamide gel was fixed with the electrophoresis apparatus and buffer reservoirs were filled with running (tank) buffer. Comb was removed and the wells were flushed gently with running buffer to remove the gel particles and unpolymerized acrylamide. Supernatant of the denatured protein samples was loaded in the wells. Appropriate molecular weight marker was also loaded in one of the wells for size analysis of the resolved proteins. The gel was electrophoresed at 80 V in stacking gel and 120 V in resolving gel.
Materials & Methods

Analysis of the gel

Electrophoresis was terminated after the tracking dye reached the bottom of the gel and the prestained molecular weight marker looked well resolved. The gel was separated from the gel plate sandwich and stained in warmed staining solution for 1 h followed by destaining with warm destaining solution with gentle shaking. Stained proteins were visualized with the help of visible light transilluminator and photographed.

(B) IDENTIFICATION OF THE ANTIGENS OF VBNC VIBRIOS AND UNIQUE PROTEINS APPEARING IN CULTURABLE STATE

1. Simulation of VBNC state in artificial media

(a) A culture of *V. cholerae* strain K.8619 was put in 50 ml LB broth with streptomycin and incubated at 37°C. Every week a loopful of above culture was taken and streaked on LB+Sm plate to check culturability of the culture.

(b) A culture of KB619 was put in 50 ml LB broth with some modifications (composition given in section A.1.7, Appendix) and incubated at 37°C with shaking for 16 h. After 16 h culture was taken out from 37°C and incubated at 4°C. Every week a loopful of culture from all 4 conditions were taken and streaked on LB+Sm plate to check their culturability.

(c) A culture of KB619 was put in 200 ml LB broth with streptomycin and incubated at 37°C with shaking for 16 h. Culture was taken out from 37°C, distributed in 4 sterile flasks (50 ml in each flask) and kept at 4 different temperatures: 4°, 20°, 30° and 37°C. A loopful of culture from each flask was streaked on LB+Sm plate at regular intervals to see their culturability.
2. **Analysis of proteins**

Analysis of proteins in different conditions was done by SDS-PAGE as described in section 3.2.A.3 (Materials and methods).

3. **Passage of VBNC bacteria into infant mice intestine**

0.1 ml of VBNC culture having approximately $10^9$ vibrios was orally fed to 5-day-old infant mice. Next day mice were sacrificed, whole intestine was taken out, homogenized and suspended in 10 ml PBS buffer. Debris was allowed to set. 0.1 ml of neat homogenate was plated on LB+Sm plate. Plate was incubated at 37°C for overnight. Bacteria growing on plate after 37°C incubation was checked by agglutination with anti-*V. cholerae* sera.

4. **Revival of VBNC bacteria by using different media**

Fresh culture of VBNC bacteria was put in different media viz. LB, TB, BHI and NB (1:20 ratio) and put at 37°C. Revival was checked by streaking a loopfull of culture on LB+Sm plate after 2-4 days.

5. **N-terminal amino acid sequence analysis**

The method described by Matsudaira’s (1987) was followed. The protein sample was analyzed on SDS-PAGE and then transblotted onto PVDF membrane (Immobilon-P, 0.45 µm, Millipore) using CAPS buffer (10 mM 3-cyclohexylamino-1-propanesulfonic acid, 10% methanol, pH 11.0). Protein sample was electroblotted for 1 h at 50 V. The blotted protein was visualized by ponceau S (Sigma) staining. Corresponding membrane area was excised, washed, air dried and used for N-terminal sequencing. Sequencing was done
using model 470A Gas phase sequenator (Applied Biosystems, USA), at Biomembrane Labs, Department of Chemistry and Biotechnology Centre, IIT, Mumbai (Courtesy, Prof. Anil K. Lala).

6. Preparation of anti-OmpT antibodies

Total cell proteins of KB619 in VBNC state were separated by SDS-PAGE. Protein band corresponding to OmpT protein was excised from the gel and homogenized in 3 ml PBS. 1 ml of protein (~100 µg) was injected subcutaneously 3 times at weekly intervals to adult rabbit. Rabbit was bled intracardially after 15 days of the last dose. The withdrawn blood was allowed to stand at room temperature for 1 h and then kept at 4°C for overnight. Serum was collected after low speed centrifugation of clotted blood.

7. Western blotting

The western blots were prepared by transferring the separated proteins from the polyacrylamide gel on to a nitrocellulose membrane (NCM) in a Transblot apparatus (Biorad Mini Protein III) as described by Towbin et al. (1979). After electrophoresis the gel was soaked in transfer buffer. The gel was sandwiched between whatman paper No. 3 on one side and NCM (Sigma, USA) on the other side. Additional sheets of Whatman paper No. 3 were kept on both sides and fixed in the Transblot apparatus. Care was taken to avoid any air bubbles to trap between gel and NCM. The apparatus was placed in such a way that the NCM faces the anode. The transfer was carried out at 60 V for 3-4 h. Complete transfer of proteins on the membrane was checked visually by transfer of the prestained molecular weight markers.
The membrane was incubated in blocking solution for 3-4 h followed by incubation in primary antibody solution (anti-OmpT, 1:500 dilution in TTBS solution) for 2 h with continuous shaking at room temperature (RT). The blots were washed 3 times with washing buffer, 15 min each. Blots were then incubated with secondary antibody (anti-rabbit IgG HRP conjugate, developed in goat, Sigma) for 2 h at RT. The blots were subsequently washed with washing solution, thrice, 15 min each. The blots were finally rinsed and soaked in developing solution in dark. After development the blots were rinsed with distilled water, dried and stored.

8. Scanning electron microscopy

Bacterial cells to be analyzed by scanning electron microscopy were washed 4 times in PBS buffer and finally suspended in fixative for overnight. 2-3 drops of each sample were put over a film of poly-L-lysine (Sigma) and allowed to adhere. After 30 min, excess sample was washed with 0.1 M Cacodylate buffer (Sigma). Sample was covered with osmium tetraoxide for 2 h for osmication. Excess osmium tetraoxide was drained out and sample was washed with distilled water. The cells were then dehydrated with ascending concentrations of ethanol (30, 50 and 70% for 10 min, 90% for 30 min and 100% for 1 h) and critical-point freeze dried in liquid CO₂. The cells were then mounted over stubs and finally gold plated. Observations were made with a model Philips XL9 scanning microscope.