Chapter 4

Materials and Methods
### Strains used during this study

<table>
<thead>
<tr>
<th><strong>E. coli</strong> strain</th>
<th><strong>Genotype</strong></th>
<th><strong>Reference</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>DH10B</td>
<td>F-, mcrA (mrr-hsdRMS-mcrBC) f80 d lacZ M15 lacX74 endA1 recA deoR (ara,leu)7697 GraD139 galU galK nupG rpSL.</td>
<td>Lorow and Jessee (1990)</td>
</tr>
<tr>
<td>JH139</td>
<td>F' tif-1 sfia11 thr leu pro his arg ilv gal str Pro+ lac U169 dinD1::MudI1734 (Km lac)</td>
<td>Heitman and Model (1991)</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F'ompTgal[dcm][lon]hsdS_B (rB-mB-) an <em>E. coli</em> B strain with DE3, a λ profage carrying the T7 polymerase gene.</td>
<td>Studier <em>et al.</em>, (1990)</td>
</tr>
<tr>
<td>BL21(DE3) pLysS</td>
<td>F'ompTgal[dcm][lon]hsdS_B (rB-mB-) pLysS,Cm'</td>
<td>Studier <em>et al.</em>, (1990)</td>
</tr>
</tbody>
</table>
### Plasmids used during this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMAP6</td>
<td><em>BamHI</em> methylase gene cloned at <em>EcoRI</em> of pACYC184,Tet&lt;sup&gt;r&lt;/sup&gt;</td>
<td>From our Laboratory</td>
</tr>
<tr>
<td>pPMBamR.0</td>
<td><em>BamHI</em> endonuclease gene under <em>T7</em> promoter of pT7-6, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>From our Laboratory</td>
</tr>
<tr>
<td>pRSET A</td>
<td><em>E.coli</em> expression vector</td>
<td>InVitrogen</td>
</tr>
<tr>
<td></td>
<td><em>T7</em> promoter, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Corporation</td>
</tr>
<tr>
<td>pAABRw</td>
<td><em>BamHI</em> endonuclease gene cloned at <em>NdeI-EcoRI</em> site of pRSET A, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pAABR37p</td>
<td>Mutant (Cys54Ile) <em>BamHI</em> endonuclease gene cloned at <em>NdeI-EcoRI</em> site of pRSET A, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pAABR23a</td>
<td>Mutant (Cys54Trp) <em>BamHI</em> endonuclease gene cloned at <em>NdeI-EcoRI</em> site of pRSET A, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pAABR24d</td>
<td>Mutant (Cys54Ala) <em>BamHI</em> endonuclease gene cloned at <em>NdeI-EcoRI</em> site of pRSET A, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pAABR47a</td>
<td>Mutant (Cys54Asp;Cys64Arg) <em>BamHI</em> endonuclease gene cloned at <em>NdeI-EcoRI</em> site of pRSET A, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
</tbody>
</table>
Antibiotic solutions used

Antibiotic solutions were prepared as stock and filter sterilised through 0.2 \( \mu m \) cellulose acetate filter (Millipore):

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Stock solution concentration</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (Sigma)</td>
<td>100 mg/ml in water</td>
<td>100 ( \mu g/ml )</td>
</tr>
<tr>
<td>Kanamycin (Sigma)</td>
<td>50 mg/ml in water</td>
<td>50 ( \mu g/ml )</td>
</tr>
<tr>
<td>Tetracycline(Sigma)</td>
<td>10 mg/ml in water</td>
<td>10 ( \mu g/ml )</td>
</tr>
<tr>
<td>Nalidixic acid (Sigma)</td>
<td>15 mg/ml in water</td>
<td>15 ( \mu g/ml )</td>
</tr>
<tr>
<td>Streptomycin (Sigma)</td>
<td>50 mg/ml in water</td>
<td>50 ( \mu g/ml )</td>
</tr>
<tr>
<td>Chloramphenicol (Sigma)</td>
<td>50 mg/ml in ethanol</td>
<td>50 ( \mu g/mg )</td>
</tr>
</tbody>
</table>

Growth and storage of bacteria

Bacterial strains were grown in Luria Broth (LB) media (Himedia). Two gms of Luria Broth was dissolved in 100 ml double distilled water and sterilised by autoclaving at 15 lb/sq. inch. for 15 minutes. For short term storage (one month at 4\( ^o \)C), the strains were maintained on LB agar plates containing appropriate antibiotic. For long term storage, fully grown cultures were stored frozen in 15% glycerol at -70\( ^o \)C.

Plasmid DNA isolation: Mini and Maxipreps

Minipreps

Plasmid DNA from transformants were isolated by miniprep method of alkaline lysis protocol (Birnboim and Doly, 1979).
The cultures were grown in tubes containing 3 ml LB with appropriate antibiotics. The cells were pelleted down by centrifugation at 10,000 rpm for 2 minutes at 4°C in Sigma cooling centrifuge (2K15). The supernatant was discarded and the pellet was suspended in 100 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0).

To the well vortexed cells, 200 µl of freshly prepared lysis buffer (0.2 N NaOH, 1% SDS) was added, mixed gently and incubated on ice for 2 minutes at room temperature. Then 150 µl of ice cold 3M KOAc (pH 5.2) was added, mixed properly and kept on ice for 10 minutes. The white precipitate containing genomic DNA and proteins were pelleted down at 13000 g for 20 minutes at 4°C.

The supernatant was carefully taken and mixed with equal volume of phenol:chloroform:isoamyl alcohol mixture (25:24:1). The mixture was spun at 12000 g for 10 minutes and the upper aqueous layer was taken and extracted with equal volume of chloroform:isoamyl alcohol mixture (24:1). To the extracted DNA sample, 0.6 volumes isopropanol was added and incubated for 10 minutes at room temperature. The precipitated DNA along with RNA was pelleted by centrifugation at 14,000 rpm for 15 minutes at room temperature. The pellet was washed with 70% ethanol, dried in 'Vacuum Concentrator' (Heto Corporation, Japan), dissolved in 50 µl TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and stored at 4°C for further analysis.

**Maxipreps**

Medium scale isolation and purification of plasmid DNA (100-500 µg) for characterisation by restriction analysis etc. was done using alkaline lysis method (Birnboim and Doly, 1979).

Cells were grown in 100 ml LB with appropriate antibiotics. The cells were pelleted down at 8000 rpm for 5 minutes at 4°C in SS34 rotor of Sorvall Centrifuge.
The cell pellet was resuspended in 4 ml TE buffer and dispersed completely, leaving no clumps. To this, 8 ml of freshly prepared lysis buffer (0.2 N NaOH, 1% SDS) was added and mixed gently and incubated over ice for 2 minutes. After the cells have completely lysed, 6 ml of chilled 3 M KOAc (pH 5.2) was added, mixed immediately and incubated over ice for 10 minutes.

The precipitate containing genomic DNA and proteins was then pelleted down at 14,000 rpm in SS34 rotor of Sorvall centrifuge RC5C at 4°C for 20 minutes. The supernatant was carefully collected and the plasmid DNA was precipitated by adding 0.6 volume isopropanol. The precipitate was pelleted down at 14,000 rpm at room temperature for 20 minutes. The pellet was washed with 70% ethanol, air dried and dissolved in 500 µl TE buffer.

10 µl RNase (Sigma) was added from a stock of 20 mg/ml and incubated at 37°C for 1 hour. The DNA solution was then extracted twice with equal volume of phenol:chloroform:isoamylalcohol mixture (25:24:1) and finally with chloroform:isoamylalcohol mixture (24:1). To the aqueous solution containing the plasmid DNA, 0.1 volume of 3 M NaOAc (pH 5.2) was added. The DNA was finally precipitated with 2.5 volume absolute ethanol, kept at -70°C for an hour and centrifuged at 14,000 rpm at 4°C for 15 minutes. The pellet was washed with 70% ethanol, air dried and dissolved in 200 µl TE buffer. The DNA was checked both on agarose gel and spectrophotometrically to determine the quality and quantity.

Preparation of plasmid DNA for sequencing

Plasmid DNA for use in sequencing of the cloned genes was prepared using the Qiagen Plasmid Mini Kit.

Inoculated single colony into 3 ml LB media containing appropriate antibiotics and grew the cells by shaking at 37°C overnight. The cells were pelleted down at 1000
rpm for 2 minutes in Sigma centrifuge. The cell pellet was resuspended in 0.3 ml of buffer P1. Then added 0.3 ml of buffer P2, mixed gently and incubated at room temperature for 5 minutes. To the lysed cells added 0.3 ml of ice cold buffer P3, mixed immediately and incubated on ice for 5 minutes. The suspension was centrifuged at 14,000 rpm for 10 minutes at 4°C. Meanwhile, the Qiagen-tip 20 was equilibrated by applying 1 ml of buffer QBT. After the buffer QBT had passed through the column, applied the supernatant got after centrifugation was loaded on the Qiagen-tip 20 and allowed it to enter the resin through gravity. The column was then washed four times with 1 ml of buffer QC. Finally, the DNA was eluted using 0.8 ml of buffer QF and collected into an eppendorf tube. Precipitated the DNA by adding 0.56 ml (0.7 volumes) of isopropanol. Pelleted down the precipitate at 14,000 rpm for 30 minutes at room temperature, washed the pellet with 70% ethanol and air dried.

**Agarose gel electrophoresis**

DNA samples were analysed by electrophoresis on 0.8-1.2 % agarose gel, as required. Agarose gel was prepared by heating appropriate amount of agarose (SRL) in 1X TAE buffer (1 litre 10X buffer contained 48.4 gm Tris-base, 11.4 ml glacial acetic acid and 20 ml 0.5 M EDTA, pH 8.0) and ethidium bromide was added at a concentration 0.5 µg/ml after it cooled and just before setting. The gel slabs were prepared by pouring the dissolved agarose into the required casting tray fitted with appropriate comb. The samples were loaded after mixing with 0.1 volume 6X gel loading buffer (30% glycerol, 0.25% xylene cyanol and 0.25% bromophenol blue). The gel was run in horizontal electrophoresis tank in 1X TAE buffer at constant voltage of 100 volts using power pack (Hoefer Scientific Instruments). The DNA bands were visualised under UV transilluminator (Photodyne).

**Restriction digestion**

The restriction enzymes used during this study were from New England
Biolabs, USA, Promega, Boehringer Mannheim and MBI Fermentas. For digestion of plasmid, 1-2 units of appropriate enzyme was used for 1 μg DNA (plasmid or PCR fragment) for 2 hours at 37°C. The buffer used was supplied by the respective companies in 10X concentration. BSA was supplemented when required. The digestion was stopped either by heat inactivation or by adding 1-5 μl of 0.5 M EDTA (pH 8.0) to the reaction mixes.

Purification of DNA fragments from agarose gel

The DNA sample was electrophoreised in the agarose gel. The desired DNA fragment was excised and placed in a microfuge tube. The gel piece was frozen by keeping it at -70°C for 10-15 minutes. The frozen gel was crushed extensively, 100-200 μl of TE and an equal amount of phenol (pH 8.0) was added and vortexed. The aqueous layer containing the extracted DNA fragment was separated by centrifugation at 8000 rpm for 5 minutes at room temperature. The upper aqueous layer was carefully collected and extracted with equal volume of chloroform: isoamylalcohol (24:1) mixture. DNA was then precipitated by addition of 0.1 volume of 3M NaOAc (pH 5.2) and 0.6 volumes of isopropanol. DNA precipitate was pelleted down at 14,000 rpm for 20 minutes. The pellet was washed using 70% ethanol and air dried.

Dephosphorylation of vector DNA

Shrimp alkaline phosphatase (SAP) from Boehringer Mannheim was used to dephosphorylate the linearised vector molecules before ligation. This enzyme removes 5' phosphate group from linear DNA which is required by ligase, thus preventing self ligation of the vector. The DNA sample was incubated with SAP (5 U) in 1X SAP buffer at 37°C for 10 minutes. The enzyme was inactivated by heating at 65°C for 15 minutes.
Ligation of DNA

Ligation of the vector with insert was carried out in 1:3 vector: insert molar ratio in presence of 1U of T4 DNA Ligase from Bangalore Genei in 1X ligase buffer and incubated at 16°C for 16 hours.

Preparation of competent cells

The procedure followed for making competent cells is given below. The host cell culture was streaked on a LB plate containing the appropriate antibiotic from the frozen glycerol stock at -70°C. A single colony was inoculated into 5 ml LB containing antibiotics. One ml of the overnight grown culture was further inoculated into 100 ml LB and allowed to grow for 2-3 hours until O.D. at 600 nm reached to 0.4. The flask was then kept over ice for 15-30 minutes to cool down the culture to 4°C.

The cells were transferred aseptically into sterile ice cold SS34 tube and centrifuged at 4000 rpm for 5 minutes at 4°C in SS34 rotor of Sorvall centrifuge. The supernatant was discarded and the pellet was resuspended in 30 ml ice-cold 0.1 M CaCl₂ and kept on ice for 30 minutes. The cells were recovered by centrifuging at 4000 rpm for 5 minutes at 4°C. The supernatant was decanted completely and the pellet was resuspended in a total volume of 4 ml ice cold 0.1 M CaCl₂ and 1 ml 75% ice cold sterile glycerol (Sigma).

200 µl aliquots was taken for checking viability, contamination and efficiency of transformation. The rest of the suspension was kept at 4°C for 6 hours to increase the efficiency of transformation and stored in aliquots of 200 µl at -70°C.
Transformation

Transformation protocol was followed according to Sambrook et al., (1989). The competent cells aliquot of 200 μl was thawed over ice and 10 ng of plasmid DNA or 50% of the ligated DNA was added, mixed by tapping and kept on ice for 15 minutes. The tube was given heat shock at 42°C for 75 seconds and immediately plunged into ice, kept for 2 minutes and then added 800 μl LB and allowed to grow at 37°C for 1 hour. The cells transformed with the ligation mix were pelleted down at 5000 rpm for 5 minutes. The supernatant was discarded leaving about 100 μl inside the tube to suspend the pelleted cells which were then plated on the selective antibiotic plate. In case of cells transformed with plasmid DNA preparation (10 ng), 100 μl of the transformation mix was plated on the selective antibiotic plate. For checking SOS response 40 μl of 20 mg/ml X-gal in DMF was plated on the plate before spreading the transformants. The plate was kept at 37°C for 12-16 hours.

Purification of Oligonucleotides through PAGE

Oligonucleotides required for cloning and sequencing were purchased from Yale University (40 nM scale, without any purification). Unpurified primers can be used for PCR amplification but for sequencing we need primers with single band purity. Electrophoresis is a technique for separation of mixtures of charged molecules example oligodeoxynucleotides. The mixture is applied to a gel and the components separate according to their charge / mass ratio when an electric field is applied between two electrodes at opposite edges of the gel.

1. *Stock solutions*

   **TBE 10X  pH 8.3**
   
   89 mM Tris base  (SRL)
   89 mM Boric acid (SRL)
   02 mM EDTA    (SRL)
Acrylamide stock, 40%

38% (w/v) acrylamide (SRL)
02% (w/v) N,N' methylene bisacrylamide (in deionised water) (Fluka)
Ammonium per sulphate (APS) (SRL)
20% (w/v) in water (freshly prepared)

Formamide  (deionised)

Dye mixture

1 mM EDTA (SRL)
10 mM NaOH (SRL)
80% (v/v) Formamide, deionised (Sigma)
0.1% (w/v) Xylene cyanol blue (Sigma)
0.1% (w/v) Bromophenol blue (Sigma)

Note: All solutions are made up with distilled water and filtered through a 0.45 µm filter prior to use.

2. Preparing the gel

For every 100 ml gelling solution the following were mixed
50 ml 40% Acrylamide mix (SRL)
10 ml TBE (10X) (SRL)
42 gm Urea (electrophoresis grade) (SRL)
distilled water to make the final volume to 100 ml.

Urea was dissolved and the solution was degased carefully because oxygen inhibits the polymerisation. Added 40 µl TEMED (SRL) and 200 µl APS solution. This initiates the polymerisation and casted the gel immediately.

3. Running the gel

Electrophoresis was conducted at an elevated temperature (50-60°C) in order to minimise the risk of problems due to interactions between sample molecules. The gel was prerun in 1X TBE buffer for about one hour to get rid of acrylic acid and to heat up
the gel uniformly. Mixed the sample with at least 50% formamide and denatured it by heating at 90°C for 3 minutes and then immediately plunged on to ice. Loaded the sample on the gel and electrophoreised at constant voltage till the dyes moved to an appropriate distance.

4. Detection and Recovery

To visualise the bands on the gel, a fluorescent thin layer chromatography plate (TLC) was used. The plate was placed under the gel. When the gel is illuminated with UV-light (254 nm) the TLC plate fluoresced, whereas the oligonucleotide, which absorb UV-light, appeared as dark band against the fluorescent background. For recovery, the areas containing the desired product were excised. The gel piece was then crushed and soaked in sterile water overnight at 37°C according to the diffusion method (Smith, 1980).

The soaking was performed at 37°C overnight. Centrifuged at 15000 rpm for 5 minutes and took the supernatant and dried. The dried sample was then desalted through Nap10 (Pharmacia) column (G-25) and dried and kept at -20°C till use.

Polymerase chain reaction (PCR)

PCR is a simple and powerful method, invented by Mullis et al. (1986), which allows amplification of DNA segments in vitro through a succession of incubation steps at different temperatures. Typically, the double stranded DNA is heat denatured, the two primers complementary to the 3' boundaries of the target segment are annealed at low temperature and then extended at an intermediate temperature. One set of these three consecutive steps is referred to as a cycle. The PCR is based on the repetition of this cycle and can amplify DNA segments by at least million fold and potentially as high as 109 fold (Saiki et al., 1988).
PCR was carried out in 0.5 ml tight capped polypropylene microcentrifuge tube and the machine used was PTC100 of MJ Research, USA. The components used are as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 10X PCR buffer</td>
<td>IX</td>
</tr>
<tr>
<td>2. dNTPs (Promega)</td>
<td></td>
</tr>
<tr>
<td>a. dATP</td>
<td>200 μM each</td>
</tr>
<tr>
<td>b. dCTP</td>
<td></td>
</tr>
<tr>
<td>c. dGTP</td>
<td></td>
</tr>
<tr>
<td>d. dTTP</td>
<td></td>
</tr>
<tr>
<td>3. Primer 1</td>
<td>100 p moles</td>
</tr>
<tr>
<td>4. Primer 2</td>
<td>100 p moles</td>
</tr>
<tr>
<td>5. Experimental template</td>
<td>10 ng</td>
</tr>
</tbody>
</table>

The final volume was generally 50 μl - 100 μl. The enzyme Taq DNA polymerase (Bangalore Genei) was added after complete denaturation of the mixture at 94°C for 3 minutes. This procedure is commonly known as "Hot start" and it prevents the unwanted annealing of the primer to the nonspecific region.

The cycling condition was varied depending on the sequence of the primer and the condition was optimised for each experiment.

**DNA Sequencing**

The sequence of the cloned genes were determined by the dideoxychain termination method (Sanger et al., 1977) using the T7 Sequenase Version 2.0 DNA Sequencing Kit (Amersham Life Science). T7 Sequenase version 2.0 DNA polymerase is a modified form of T7 DNA polymerase (Tabor and Richardson, 1987), which has high processivity, completely lacks 3'-5' exonuclease activity and can incorporate nucleotide analogs. The following steps were followed during sequencing.
**Preparation of alkali-denatured DNA**

3-5 µg of RNA-free supercoiled plasmid DNA, prepared using Qiagen Plasmid Mini Kit was taken and incubated with 0.2 M NaOH, 0.2 mM EDTA (pH 8.0) at 37°C for 30 minutes. The mixture was neutralised by adding 0.1 volumes of 3 M NaOAc (pH 5.2) and the DNA then precipitated with 2.5 volumes of ethanol incubating at -70°C for 15 minutes. The precipitate was pelleted down at 14,000 rpm for 30 minutes. The pellet was washed with 70% ethanol and air dried.

**Annealing of primer to template DNA**

For each set of four sequencing lanes, a single annealing reaction was used. The denatured DNA was dissolved in 7 µl sterile distilled water. Two µl sequenase reaction buffer (5X) and 1 µl of primer (1 pmole) were added and heated at 65°C for 2mins. The mixture was allowed to cool slowly over a period of 30 minutes. When the temperature reached 37°C the tube was chilled on ice.

**Labelling reaction**

Diluted the labelling mix (1:5 in ddH₂O) and sequenase enzyme (1:8 in sequenase buffer) before use. To the ice cold 10 µl annealed template-primer added the following

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>diluted labelling mix</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>([\alpha^{35}S] dATP)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>diluted sequenase</td>
<td>2.0 µl</td>
</tr>
</tbody>
</table>

Incubated for 2 minutes at 22°C.
**Termination reaction**

Took 2.5 µl each of ddGTP, ddATP, ddTTP and ddCTP in four different eppendorfs and preheated these tubes at 37°C. After 2 minutes of labelling reaction, 3.5 µl of labelling reaction mixture was added to each of the four termination tubes. Mixed and quickly returned to 37°C for 5 minutes. The reaction was stopped by adding 4 µl of stop solution. Mixed and kept on ice.

**Preparation of Sequencing Gel**

Cleaned the sequencing glass plates, the notched plate was siliconised using 5% dimethyl dichloro silane (SIGMA) in chloroform. The binding silane [30µl α-(methacryloxyl)-propyltrimethoxysilane (Pharmacia) in 30 ml methanol and 100 µl glacial acetic acid] was spread on the other plate to help the gel stick on the plate. 0.4 mm spacers were placed between the two plates, sealed using adhesive tape and clamped.

**Denaturing polyacrylamide gel electrophoresis**

The DNA fragments were separated using 6% Urea PAGE

Stock solutions needed:-

**TBE 10X pH 8.3**

Dissolve 54 gms Tris base (SRL)  
27.5 gms Boric acid (SRL)  
20 ml 0.5 M EDTA pH 8.0 (SRL) in 500 ml deionised water

**30% Acrylamide solution**

Dissolve 29 gms acrylamide (SRL) and 1 gm N,N’ methylene bisacryamide in 100 ml deionised water, filtered the solution and store in dark bottle at 4°C.
Preparation of 6% Urea Page gel mixture (50 ml)

The following were mixed

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>22.0 grams (7 M)</td>
</tr>
<tr>
<td>30% acrylamide solution</td>
<td>10.5 ml</td>
</tr>
<tr>
<td>TBE (10X)</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

and made the volume of the mixture to 50 ml with ddH2O.

To start polymerisation reaction, added 20 µl TEMED and 200 µl 20% APS in that order were mixed with the gel mixture and immediately poured in between the plates. The comb was inserted and the gel was allowed to polymerise for at least 2 hours.

When the gel had set, the tape and the clamps were removed and placed the plates in the electrophoresis apparatus (Bangalore Genei). The top and the bottom tank of the electrophoresis apparatus was filled with 1X TBE. The comb was then removed and the wells were rinsed with 1X TBE to remove any bubbles, acrylamide and urea using a syringe. A pre run (1250 V or 50 W) for 10-15 minutes was conducted before loading DNA samples.

Sample Loading

The samples were heated to 75°C for 2 minutes. Rinsed the wells to remove any bubbles and urea. Loaded 3 µl of four reaction mixture each in the order GATC. Switched on the power supply (1250 V or 50 W).

Fixing the Gel

After the gel was run to desired length it was soaked in the fixing solution (5% acetic acid, 15 % methanol in H2O) for 15 minutes to fix the DNA and to remove urea.
**Gel Drying**

After fixing, the gel was dried in the oven maintaining temperature at 80°C for 2 hours or at 60°C for 12 hours and exposed the dried gel on plate, for autoradiography, to the X-ray film (35 cm X 42 cm XAR, Kodak) for appropriate time. It was then developed and the sequence read.

**Detection of SOS response**

The SOS strain, JH139, was transformed with the plasmids containing the endonuclease gene. The transformants were plated on LB agar plates containing the antibiotic ampicillin (100 mg/ml), which was plated with 40 µl of X-gal (Bangalore Genei) (20 mg/ml in dimethylformamide, SRL). The plates were incubated at 37°C overnight.

**β - galactosidase assay**

As the transformants of JH139 harbouring the plasmid containing *BamH* gene (wild/ mutants) lysed easily on plates, β-galactosidase assay was done with slight variation as described by Miller (1972). Soon after transformation, the transformation mixture was inoculated into 5 ml LB containing ampicillin (100 µg/ml) and allowed to grow. The growth was monitored by withdrawing 0.5 ml and immediately diluting it with 0.5 ml chilled broth. This was done with as minimum disruption of the shaking of the cells as possible. The flask was also maintained at 37°C at all times throughout these manipulations. The turbidity of the sample was measured at 600 nm in the Hitachi Spectrophotometer. Optical density versus time was plotted to get the growth curve.

For the β-galactosidase assay, the cells were grown till late log phase. Just
before the short stationary phase began the culture was withdrawn, its absorbance at 600 nm recorded. 0.5 ml of culture was mixed with 0.5 ml of Z-buffer (60 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄ and 50 mM 2-mercaptoethanol). To the above 1ml mix added 20 μl of chloroform and 10 μl of 0.1% SDS, vortexed for 1 minute and incubated at 28°C for 5 minutes. Prepared ONPG (4 mg/ml in 0.1 M NaPO₄ buffer pH 7.0) and preincubated at 28°C for 5 minutes. To the above cell lysate added 200 μl of ONPG solution (4mg/ml) and incubated at 28°C till a yellow colour developed. The incubation time was noted before stopping the reaction by adding 0.5 ml of 1 M NaCO₃. The OD of the solution was recorded at 420nm and 550nm. The units of β-galactosidase was calculated according to the following formula.

\[
\text{Units} = \frac{1000 \times \text{O.D. at 420nm} - 1.72 \times \text{O.D. at 550nm}}{t \times V \times \text{O.D. at 600nm}}
\]

where \( t = \text{time of reaction in minutes} \) and \( v = \text{volume of culture taken for assay in ml} \).

**Purification of BamHI endonuclease**

General steps in the isolation of BamHI endonuclease from the recombinant E.coli strain used were:

a) Induction of the protein  
b) Sonication to lyse the cells  
c) Centrifugation to remove cell debris  
d) Phosphocellulose chromatography  
e) Hydroxyapatite chromatography  
f) Concentration through Centricon assembly
Induction of the protein

The plasmid containing the BamHI endonuclease gene (wild / mutant) was cotransformed along with the plasmid containing the methylase gene into BL21 (DE3) cells. The transformant (single colony) was inoculated into 3 ml LB containing antibiotics ampicillin (100 µg/ml) and tetracycline (10 µg/ml) and grown overnight at 37°C in the shaker. The overnight grown culture was used to inoculate 10 X 100 ml media containing appropriate amounts of the two antibiotics and grown at 37°C in the shaker. Absorbance of the culture at 600nm was monitored, when the absorbance reached 0.6, IPTG was added to a final concentration of 0.4 mM and again kept in the 37°C shaker for 3 hours. The cells were harvested by centrifugation at 8000 rpm for 5 minutes in the SS34 rotor of the Sorvall centrifuge (RC5C). The cells can be stored if needed at -70°C.

Sonication to lyse the cells

The cell pellet was suspended in 5 ml lysis buffer containing 10 mM KPO4 (pH 7.0), 1 mM DTT, 1 mM EDTA (pH 8.0), 200 mM NaCl, 1 mM PMSF and lysozyme 100 µg/ml.

The cell suspension was sonicated in continuous mode at microtip output 4 in Ultrasonics (model no W385) Sonicator. The pulses are given for 30 seconds with a gap of 30 seconds each, the cells were kept over ice all throughout. The complete lysis of cells was monitored by measuring O.D. at 280 nm from the sonicated aliquots at 1:100 dilution.
Centrifugation to remove cell debris

The sonicated cell suspension was centrifuged at 14,000 rpm for 1 hour at 4°C in SS34 rotor of Sorvall centrifuge (RC5C) to pellet down the cell debris.

Phosphocellulose chromatography

This is a cation exchange chromatography and used to separate biopolymers using NaCl concentration gradient. Phosphocellulose matrix needs to be activated before packing into the column. 6 gms of phosphocellulose resin was suspended in 200 ml of a solution containing 95% ethanol and 0.2 N HCl in 1:1 ratio. The suspension was gently stirred for 30 minutes. The slurry was allowed to settle down and the supernatant containing the fines was decanted. The slurry was washed with 400 ml of sterile distilled water. The pH was adjusted near to 7.0 with 1 N NaOH. The resin was then suspended in 200 ml of 0.1 N NaOH and stirred for 30 minutes at room temperature. The slurry was allowed to settle down and the supernatant was decanted. The resin was then suspended in 200 ml of 1 mM EDTA and stirred for 30 minutes at room temperature. The resin was again washed with sterile distilled water and the pH adjusted to near neutral with 1 M HCl. The supernatant was discarded and suspended in 200 ml extract buffer containing 10 mM KPO4 (pH 7.0), 1 mM DTT, 1 mM EDTA and 200 mM NaCl. A column with 20 ml bed volume was prepared, washed and equilibrated with equilibration buffer which is same as the extract buffer plus 10% glycerol. The cell lysate after centrifugation (supernatant) was loaded onto the column and the eluate recycled twice for efficient binding of the protein on the matrix. The column was then washed with equilibration buffer till O.D. at 280 nm of the eluate reached zero. The protein, BamHI endonuclease was eluted with a linear gradient of 0.2 N - 0.6 N NaCl in equilibration buffer. The eluants were collected in 1 ml fractions. All fractions having BamHI activity were pooled.
**Hydroxyapatite chromatography**

This is an example of adsorption chromatography where proteins are adsorbed by gels of crystalline hydroxyapatite, an insoluble form of calcium phosphate having empirical formula $\text{Ca}_5(\text{PO}_4)_3\text{OH}$.

The pooled fraction obtained from phosphocellulose chromatography was loaded on a 10 ml hydroxyapatite column at a flow rate of 5 ml/hour. The column was washed with equilibration buffer and the enzyme was eluted with 50 ml gradient of potassium phosphate from 0.01 M to 0.5 M, containing 1 mM EDTA, 1 mM DTT, 0.2 M NaCl and 10% glycerol. One ml fractions were collected and 1 µl of these was assayed for activity. The fractions showing activity were pooled, activity was assayed and protein concentration was determined by micro Bradford method.

**Concentration through Centricon assembly**

The pooled fraction from hydroxyapatite chromatography, was pipetted into the centricon YM-10 assembly (Millipore) and centrifuged down in the concentrating mode at 5000 g in Sorvall RC5C centrifuge using SS34 rotor till the sample volume reduced to 500 µl. The sample was diluted in 10 mM Tris-HCl, pH 8.0 and again concentrated so as to replace the phosphate buffer with Tris buffer. The concentrated sample was then recovered and stored in storage buffer [10 mM Tris-HCl (pH 8.0), 300 mM KCl, 1 mM EDTA, 1 mM DTT and 10% glycerol] at 4°C.

**Protein estimation**

Accurate quantitation of protein concentration was carried out by Micro-Bradford method (Bradford, 1976).

The Bradford reagent was prepared by dissolving 10 mg Coomassie Brilliant Blue G.250 (SRL) in 5 ml 95% ethanol. Dye was allowed to dissolve properly till the solution became prussian blue. To this 10 ml of 85% (v/v) phosphoric acid (SRL) was
added and stirred till it turned brown. The resulting solution was diluted to a final volume of 100 ml with double distilled water and stored in dark bottle at 4°C.

Bovine serum albumin (Sigma) was used to prepare standard curve. All protein solutions were prepared in 0.15 M NaCl. Known amounts of BSA (1-10 μg) in a volume upto 0.1 ml was pipetted into eppendorfs. Final volume of each was adjusted to 0.1 ml with appropriate buffer. One ml of Bradford reagent was added, mixed and kept for 2 minutes. Absorbance at 595 nm was measured against a reagent blank prepared from 0.1 ml of the appropriate buffer and 1 ml of Bradford reagent. Absorbance versus protein concentration was plotted for standard curve. The sample solution whose protein concentration had to be quantified was similarly treated and its protein concentration determined from the curve.

**BamHI endonuclease assay**

One μl of fraction was added to 10 μl digestion mix containing 100 ng pRSET A, which has a single site for BamHI, in BamHI buffer [10 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 100 mM NaCl and 1 mM DTT] and incubated at 37°C for 1 hour. Fractions which could linearize the plasmid contained the BamHI endonuclease. These fractions were pooled.

**Determination of BamHI enzyme units**

Lambda DNA (Promega) was used as substrate for determining units of BamHI endonuclease activity present in the pooled fraction as well as the specificity of mutant endonucleases. One μg of lambda DNA was digested with decreasing amounts of pooled fraction in 20 μl reaction mix containing BamHI buffer at 37°C for 1 hour. The minimum amount of fraction capable of complete digestion of 1 μg lambda DNA in 1 hour was defined as one enzyme unit.
SDS-PAGE analysis of protein

When proteins are denatured by heating in presence of excess SDS and 2-mercaptoethanol, polypeptides bind SDS in a constant weight ratio such that they have essentially identical charge densities and migrate in a polyacrylamide gel of the correct porosity according to polypeptide chain length. Under these conditions, a logarithmic plot of polypeptide molecular mass versus relative mobility reveals a straight line relationship (Weber and Osborn, 1969).

The following stock solutions were prepared for casting and carrying out SDS-PAGE.

a) Acrylamide-bisacrylamide (30%) was prepared by dissolving 29 gms acrylamide (SRL) and 1 gm bisacrylamide in a total volume of 100 ml, filtered through Whatmann No I and stored at 4°C in dark bottle.

b) SDS (10% w/v): prepared by dissolving 10 gms SDS (SRL) in 100ml.

c) 1.5 M Tris-Cl, pH 8.8 and 1.0 M Tris-Cl, pH6.8.

d) Electrophoresis buffer: TGS (pH 8.3)

   3 gms Tris base (SRL), 14.5 gms glycine (SRL) and 10 ml of 10% SDS in a final volume of 1 litre.

e) 10% Ammonium persulphate, prepared fresh by dissolving 100 mg in 1ml dH2O.

f) TEMED (Sigma)

g) Protein Marker from Bangalore Genei.

The gel apparatus from Hoefer Scientific Instruments was used. The glass plate and the notched metal plate were cleaned, the two spacers were sandwitched between the plates and assembled to the apparatus using clamps. The bottom of the gel was sealed with agarose. The separating gel mixture was prepared by mixing the following in the given order
30% Acrylamide 4 ml
1.5 M Tris-HCl, pH 8.8 2.5 ml
distilled water 3.3 ml
10% SDS 100 µl
10% APS 100 µl
TEMED 5 µl

The above composition is for 12% separating gel mixture. The mixture was poured carefully in between the plates to fill approximately 3/4 of the total height. It was gently layered with water to smoothen the gel surface. Once the gel was polymerised the water layer was decanted off and the stacking gel mixture was poured which was prepared by mixing the following

30% Acrylamide 0.830 ml
1.0 M Tris-HCl, pH 6.8 0.625 ml
distilled water 3.445 ml
10% SDS 50 µl
10% APS 50 µl
TEMED 5 µl

This 5% stacking gel mixture was poured over the separating gel. The comb was inserted and allowed the gel to polymerise.

The samples were loaded after boiling for 5 minutes with 1X gel loading buffer (5X loading buffer : 15% 2-mercaptoethanol (Sigma), 1.5% bromophenol blue, 15% SDS, 50% glycerol made final volume with 1 M Tris-HCl, pH 6.8).

The gel was run till bromophenol front reached the bottom of the separating gel. Disassembled the gel and stained it using the non-polar, sulphated triphenylamine dye Coomassie Brilliant Blue, R250 (CBB-R250). The gel was soaked in 0.1% w/v CBB-R250 in 45% v/v methanol, 10% v/v acetic acid in water and shaken for 10-15 minutes. The gel was destained using 30% methanol and 10% acetic acid and stored in 7% acetic acid.
Enzyme kinetics

Continuous spectrophotometric assay, based on the hyperchromic effect of DNA, was followed as per the published procedure (Waters and Connolly, 1992) to determine the $K_M$ and $K_{cat}$ values of the purified $Bam$HI endonucleases (wild as well as the mutant enzymes). For this purpose a 13 mer self complementary oligonucleotide of following sequence 5' AGTGCGGATCCGC 3' was used as substrate.

The oligonucleotide was purchased from Yale University (0.2 μM scale). It was dissolved in autoclaved distilled water and the concentration of the oligonucleotide solution was calculated by taking absorbance at 260 nm and calculating making use of the formula

$$c = \frac{\text{O.D.}_{260}}{\varepsilon \times 1}$$

where $c$ is the concentration in M, $l$ is the path length of the cuvette in cm and $\varepsilon$ the extinction coefficient. The concentration of the endonuclease ($Bam$HI) was determined using the micro Bradford method.

Determining the temperature at which the self complementary oligomer is double stranded

This was done by studying the UV melting profile of the oligomer in $Bam$HI buffer [10 mM Tris-HCl (pH 8.0), 10 mM MgCl$_2$, 100 mM NaCl and 1 mM DTT]. The UV absorption melting profile was recorded in UV-160A spectrophotometer (Shimadzu) fitted to a linear temperature program controller, CE-247 (Cecil Instruments). The thermal translations were recorded from 20°C to 90°C using stoppered cuvettes. Prior to the melting studies, the oligomer solution was heated to 80°C for two minutes, and then slowly annealed to 25°C. The melting temperature ($T_m$) was obtained from the plot of relative absorbance versus temperature.
Determination of increase of absorbance at 254 nm per nanomole of oligonucleotide cleaved

Oligonucleotide solution was diluted to a concentration of 4 μM in a final volume of 389.6 μl containing BamHI buffer without DTT in a stoppered cuvette of 0.7 ml capacity having path length of 1 cm. The solution was heated to 80°C for 2 minutes using a heating block and allowed to cool slowly. When the temperature came down to 25°C, the absorbance at 260 nm was recorded to calculate the exact concentration of the substrate. The temperature of the cuvette holder was held at 25°C. The assay was started by adding 0.4 μl of 1 M DTT (final concentration 1 mM) and an excess of BamHI endonuclease 10 μl (final concentration 150 nM) and immediately started monitoring the increase in absorbance at 254 nm with time. The final absorbance after the increase had ceased was noted. The difference in absorbance just after adding the enzyme and after complete digestion at 25°C was used to calculate the change in absorbance per nanomole of substrate.

Determination of \( K_M \) and \( K_{cat} \) values

The same procedure mentioned above was carried out except that the amount of enzyme was kept low, approximately 15 nM and varying the oligomer concentration ranging from 2-30 μM concentration. Assays was carried out at 25°C and the reaction was monitored for about 1 hour. The initial rate of cleavage was determined by the rate of increase in absorbance and the total absorbance increase per nanomole of product formed. \( K_M \) and \( V_{max} \) values were determined from Linerweaver Burk plot, a plot of \( 1/\text{velocity} \) versus \( 1/[\text{substrate concentration}] \).
Circular Dichroism (CD) Absorption Spectroscopy

CD is a particular kind of absorption spectroscopy that uses circularly polarised rather than unpolarised light. It can only be applied to molecules that are asymmetric. Since most biological molecules are asymmetric, such chiroptical spectroscopy has been applied extensively to the study of molecules found in biological systems particularly biopolymers like proteins and nucleic acids, since it gives information on secondary structures or their alteration.

An asymmetric molecule absorbs right circularly polarised light differently from left circularly polarised light. In other words, the radii of the circle traced out by the left and right electric vector (E_L and E_R) becomes different. This phenomenon is called Circular Dichroism. When these two opposite circularly polarised light waves join in the transmitted beam the result is an elliptically polarised light as the two components are of different amplitudes. Thus the measure of CD is a measure of ellipticity (θ) produced. At a given wavelength the ellipticity is proportional to the difference in extinction coefficient (E_L-E_R). θ_{obs} is the observed ellipticity of the sample and can be read from CD spectra. It is given by

\[ \theta_{obs} = \pm H \times S \]

where H is the instrument reading in cm, may be positive or negative and S is the sensitivity of the CD scale (m^o/ cm = x 10^{-3} deg/cm). Molecular ellipticity \([\theta]_M\) is given by

\[ [\theta]_M = \theta_{obs} \times 100 / C \times l \]

where C is the molar concentration (mol / lit) and l is the path length of the cell in cm.

CD spectra of the purified proteins (wild type as well as the mutant BamHI endonucleases) was recorded on Jasco J720 A spectropolarimeter, interfaced with a IBM PC compatible computer, calibrated with D-camphorsulphonic acid. Spectra was measured from 210 nm to 240 nm, at 25°C.
Melting profiles of the purified protein

The melting profiles of \textit{BamHI} and its variants was determined in UV-160A spectrophotometer (Shimadzu) connected to a linear temperature program controller CE 247 (Cecil Instruments). The purified proteins were diluted in 1X \textit{BamHI} buffer to about OD$_{280}$ = 0.3-0.4 The protein solutions were heated at the rate of 1°C per minute in a stoppered cuvette of 0.7 ml capacity and OD$_{280}$ were recorded every minute. The relative absorbance value was calculated and plotted against temperature.