Chapter 2

Methods
2. METHODS

2.1. Estimation of protein

Protein content was determined by the method of Lowry et al. (1951).

Stock solutions

Solution A was made by dissolving 4.5 gm sodium carbonate in 100 ml distilled water. When estimating membrane proteins, 1 gm of sodium dodecyl sulfate was added to this solution.

Solution B was made by dissolving 2 gm anhydrous copper sulfate in 100 ml distilled water.

Solution C was made by dissolving 4.5 gm sodium potassium tartarate in 100 ml distilled water.

All the solutions were used within two months.

Reagent A :

10 ml of this reagent was made by mixing 9.8 ml of solution A, 0.1 ml of solution B and 0.1 ml of solution C.

Reagent B :

This was made by diluting one volume of Folin-Ciocalteau phenol reagent with one volume of distilled water. Reagent A and B were prepared fresh, just prior to use.

Procedure :

Sample proteins and standard protein, bovine serum albumin (BSA) containing between 4 and 20 μg of protein, were taken in separate tubes and the volume
in each was adjusted to 100 µl. A tube containing 100 µl distilled water was also processed with the samples.

One ml of reagent A was added to each tube. The solutions were thoroughly mixed by vortexing and incubated at 37°C for 20 min. After this, 100 µl of reagent B was added to each tube, the contents mixed well and again incubated at 37°C for 20 min. The absorbance of each protein solution was recorded at 740 nm in a LKB Ultrospec II spectrophotometer. A standard curve was prepared using the absorbance values of the standard protein solutions, and the concentration of the unknown protein was determined from the standard curve.

2.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were separated on the basis of their molecular mass by SDS-PAGE according to the method of Laemmli (1970).

Stock solutions

Acrylamide solution (A)
Acrylamide solution (30%) was made by dissolving 29.2 gm acrylamide and 0.8 gm N,N'-methylene bis-acrylamide in 30 ml distilled water. The volume of the solution was made upto 100 ml with glass distilled water. The solution was filtered and stored in an amber-colored bottle.

Separating gel buffer (B)
1.5 M Tris-HCl, pH 8.8, was made by dissolving 18.13 gm Tris base in 90 ml distilled water. 0.4 gm SDS was dissolved in this. The pH was adjusted to 8.8
with 3 M HCl and the final volume of the solution was made up to 100 ml with glass distilled water.

Stacking gel buffer (C)

0.5 M Tris-HCl, pH 6.8, was made by dissolving 6.0 gm Tris base in 80 ml distilled water. 0.4 gm SDS was dissolved in this. The pH was adjusted to 6.8 with 3 M HCl and the final volume of the solution was made up to 100 ml with glass distilled water.

Glycerol

A 50% solution was made by mixing 50 ml glycerol with 50 ml glass distilled water.

TEMED - N, N, N', N' - Tetramethylethylenediamine.

Ammonium persulfate (APS)

A 5% solution was made by dissolving 20 mg APS in 400 µl glass distilled water.

For best results, APS was prepared fresh, while the other stock solutions were stored in the refrigerator and used within one month.

Working solutions

Separating gel solution

The stock solutions were mixed in the following proportions to obtain a 10% separating gel solution:

- Solution A: 3000 µl
- Solution B: 2250 µl
Glass distilled water 1250 μl
50% glycerol 2500 μl
TEMED 5 μl
5% APS 70 μl

Stacking gel solution

The stock solutions were mixed in the following proportions:

Solution A 900 μl
Solution C 1500 μl
Glass distilled water 3600 μl
TEMED 6 μl
5% APS 36 μl

APS and TEMED were added to each solution just prior to polymerization.

Electrophoresis buffer

Electrophoresis buffer containing 25 mM Tris-HCl, 190 mM glycine and 0.1% SDS was made by dissolving:

- Tris-base 3.0 gm
- Glycine 14.3 gm
- SDS 1.0 gm

Glass distilled water was added to give a final volume of 1000 ml.
**SDS sample denaturing buffer**

100 mM Tris-HCl, 25 mM dithiothreitol (DTT), 50% glycerol, 10% SDS and 0.025% bromophenol blue, was made by dissolving:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution C</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>DTT</td>
<td>48 mg</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>240 mg</td>
</tr>
<tr>
<td>Glass distilled water</td>
<td>0.75 ml</td>
</tr>
<tr>
<td>Bromophenol Blue (12.5%, w/v)</td>
<td>5 μl</td>
</tr>
</tbody>
</table>

Sample denaturing buffer was stored upto one month at -20°C.

**Procedure:**

**Polymerization:**

Gel electrophoresis was carried out in a Miniprotein gel apparatus (Bio-Rad). The glass plates were assembled according to the manufacturers' instructions. The separating gel solution containing APS and TEMED was poured between the two glass plates up to a height of three-fourths of the total height. On the top of the separating gel, the stacking gel solution was poured very slowly so that the level of the separating gel was not disturbed. A 0.75 mm thick 10-well teflon comb was inserted into the stacking gel solution. The gel solutions were allowed to polymerize at room temperature for 20-30 minutes, after which the comb was gently removed. The wells were flooded with water, cleaned thoroughly to remove unpolymerized acrylamide and refilled with water till they were ready to be loaded.
Electrophoresis buffer was added to the lower and upper chambers of the apparatus. The gels were placed in the chamber without making any bubbles underneath the glass plates.

**Sample preparation:**
Samples were mixed with one fifth of their volume of SDS sample denaturing buffer and boiled for 3 min in a water bath for complete denaturation.

**Sample loading and electrophoresis:**
Different samples were loaded into individual wells in the stacking gel. Standard marker proteins of known molecular mass were loaded to obtain molecular masses of sample proteins. Each well was then gently filled up with electrophoresis buffer.

The electrophoresis chamber was connected to a power pack and electrophoresis was carried out at a constant voltage of 200 volts for 45 min i.e. till the bromophenol blue dye front was 0.5 cm above the base of the gel.

**Protein staining**

**Coomassie blue staining solution:**
Staining solution (0.3%) was made by dissolving 0.3 gm of brilliant blue R 250 in 100 ml destaining solution. The solution was filtered through a Whatman no. 1 filter paper and stored in an amber-colored bottle.

**Destaining solution:**
50% methanol, 10% acetic acid, 40% water was made by mixing 500 ml methanol, 100 ml acetic acid and 400 ml of glass distilled water.
Procedure:
After electrophoresis, the gel was placed in 0.3% Coomassie blue solution and gently shaken for 20 min at room temperature. The gel was then destained in destaining solution. The destaining was continued until the background became sufficiently clear and the protein bands became distinctly visible. The gel was then finally transferred into glass distilled water.

2.3. Fluorography of radioactive SDS gels
The radiolabeled protein samples contained in SDS gels, were visualized by fluorography according to the method of Bonner and Laskey (1974). The details are given below.

Working solutions:

Scintillation solution for \(^{14}\text{C}\)- or \(^{3}\text{H}\)- labeled proteins
This was made by dissolving 20 gm PPO (2,5-diphenyloxazole) in 100 ml dimethyl sulfoxide (DMSO).

Developer solution
Developer solution was prepared as specified by the manufacturer (Agfa-Gevaert). The contents of packet A were first dissolved and then those of the packet B in 6.75 l of glass distilled water with continuous stirring. Sufficient water was then added to make up 13.5 l working solution and stirred vigorously again.

Fixer solution
This solution was also prepared as specified by the manufacturer (Agfa-Gevaert). The powdered contents of the pack were dissolved in 6.5 l of glass
distilled water with vigorous stirring. After the powder had dissolved completely, sufficient water was added to make up 9 l working solution.

Both these solutions were stored in the dark.

**Procedure:**

**Scintillator treatment**

The \(^{14}\text{C}\)-labeled gels were processed by soaking the gels successively in dimethyl sulfoxide (twice) for 30 min, in scintillation solution B for 3 hours and in glass distilled water for 1 hour.

**Gel drying**

The gels were wrapped with cellophane paper and then dried on a Whatman 3 mm filter paper at 80°C for 1 hour under vacuum using a gel dryer (Model 583, Bio-Rad).

**Sensitization of the X-ray film**

X-ray film (Kodak XAR-5) was pre-flashed in order to sensitize it. An orange Kodak Wratten 22 filter was placed over a standard flash unit (Cannon Speedlite 166A) and the distance of the flash unit from the film was adjusted so that after ‘pre-flashing’ the absorbance of the developed film increased to 0.15 \((A_{540})\) above that of the unexposed film.

**Exposure of the gel to pre-flashed X-ray film**

The dried gel was sandwiched between a pre-flashed X-ray film and a screen within an autoradiogram cassette in the dark room. The cassette was then kept at -70°C for one week or for longer periods.
Development of X-ray film

After the required exposure, the cassette was removed from the -70°C freezer and opened in a dark room under a safety light. The X-ray film was placed in the developer solution for 3-4 min with gentle shaking, removed from the solution, washed with clean water and again placed in the fixer solution for 3 min. Finally, the film was washed under running water, air-dried and the radioactive bands were visualized against light.

2.4. Growth of the organisms and determination of minimum inhibitory concentrations (MICs)

*Shigella dysenteriae* C152 was obtained from the School of Tropical Medicine, Calcutta. Bacteria were routinely grown in Tryptic Soybroth (Difco) on a rotary shaker at a temperature of 37°C.

Minimum inhibitory concentrations (MICs) were determined by the standard serial dilution technique in Mueller Hinton broth (Difco) or Mueller Hinton Agar (Difco). For the first method, an overnight culture was used as preinoculum for inoculating 10 ml of Mueller Hinton broth at a concentration of 1% of preinoculum. Cells were grown up to an OD_{600} of 0.6 and 10^5 cells were added to each well of a microtitre plate containing serially diluted antibiotics present in a total volume of 150 μl. Results were read after incubation at 37 °C overnight. The MIC was defined as that concentration at which 90% inhibition of growth occurred. For determination of MIC on Mueller Hinton agar, antibiotics were added after serial dilution to 2 ml Mueller Hinton agar in 35 mm
tissue culture petri plates. The plates were inoculated with $10^8$ colony forming units (CFU) and incubated overnight at 37°C, before reading MICs.

2.5. Mutagenesis using N-methyl-N'-nitro-N-nitrosoguanidine (NTG)

This was carried out as described by Adelberg et al. (1965). 15 ml of Tryptic Soy broth was inoculated with 1% of an overnight culture of S. dysenteriae C152 and grown to a density of $5 \times 10^8$ cells/ml. 10 ml of the culture was spun down at 4,000 x g for 10 min. The cells were washed in 5 ml of 0.1(M) sodium citrate, pH 5.5 and resuspended in 4.5 ml of the same buffer. Cells were chilled on ice for 10 min and then placed in a water bath at 37°C. 0.5 ml NTG was added to a final concentration of 100 μg/ml and incubated for 30 min at 37°C. Cells were spun down at 4,000 x g for 5 min at 4°C and the pellet was washed twice with 5 ml of 0.1 (M) phosphate buffer, pH 7.0. Cells were resuspended in 5 ml of Tryptic Soy broth and grown overnight at 37°C. Cells were subsequently screened for cefoxitin resistance, by plating on Tryptic soy agar plates containing cefoxitin (starting with a cefoxitin concentration of 5 μg/ml). Mutants obtained after the first screening, were subsequently adapted to increasing concentrations of cefoxitin by sequential transfers to plates containing two-fold higher concentrations of cefoxitin. Isolated mutants were finally stored in 20% glycerol at -70°C.

2.6. Selection of imipenem- and norfloxacin-resistant mutants

Imipenem-resistant mutants were isolated by adaptation in the presence of imipenem. 100 μl of an overnight culture of strain C152 was inoculated on a 20 ml Mueller Hinton agar plate containing imipenem at a concentration equal to
imipenem at a concentration equal to its MIC against C152. After incubation for 48 h at 37°C, colonies were transferred to plates containing the same concentration of antibiotic. After repeating this procedure thrice, colonies were transferred to the next two-fold higher concentration of imipenem and the entire procedure was repeated, for several generations. Colonies which grew stably at a particular concentration of imipenem for several generations were chosen for further studies.

Spontaneous norfloxacin-resistant mutants were isolated from the sensitive strain C152 by plating these cells on MacConkey agar plates containing 1, 2 or 4 μg of norfloxacin per ml. Mutants showing resistance to norfloxacin could be obtained at frequencies of $10^{-7}$ to $10^{-8}$.

2.7. Preparation of membranes

Cells were grown up to mid-log phase and harvested by centrifugation at 7,000 x g for 5 min at 4°C. The cells were then washed twice with 0.01 (M) HEPES buffer (pH 7.5) containing 0.1 mM phenylmethyl sulfonyl fluoride (PMSF) and disrupted on ice in a sonicator (Labsonic-2000, B. Braun, Melsungen, Germany) employing four 1 min pulses. After sonication, the broken cell suspension was centrifuged at 7,000 x g for 10 min at 4°C to remove the unbroken cells. Membranes were pelleted by centrifugation at 16,000 x g for 30 min, washed with 0.01 M HEPES (pH 7.5) and stored at -70°C.

2.8. Kinetic analyses of the interaction of beta-lactams with PBPs
The interaction of the PBPs with beta-lactam antibioitcs was interpreted on the basis of the following three-step scheme (Frere and Joris, 1985)

\[
\begin{align*}
E + D & \xrightarrow{k_{+2}} E \cdot D \rightarrow E \cdot D^* \rightarrow E + P
\end{align*}
\]

where 
- \(E\cdot D\) = Henri-Michaelis complex, 
- \(E\cdot D^*\) = acyl enzyme, 
- \(K\) = dissociation constant of \(E\cdot D\), 
- \(k_{+2}\) = first order rate constant for the acylation step, 
- \(k_{+3}\) = first order rate constant for the deacylation step, and 
- \(P\) = reaction product.

2.8.1. **Determination of the second-order rate constant of acyl-enzyme formation (\(k_{+2}/K\))**

The reaction obeys the following equations:

\[
\frac{k_a}{k_2} = \frac{1 + K/[D]}{1 + K/K} \quad \text{................. (1)}
\]

which for \([D] \ll K\) simplifies to,

\[
\frac{k_{+2}}{K} = \frac{k_a}{[D]} \quad \text{................. (2)}
\]

where \(k_a\) = pseudo first-order rate constant of the acyl enzyme complex \(E\cdot D^*\) formation at a given \([D]\) value,

\(k_{+2}/K\) = second-order rate constant of enzyme acylation by the carbonyl donor substrate.

Again at a given substrate concentration \([D]\), the time necessary for the acyl-enzyme to reach a certain percentage of the enzyme is given below by,

\[
\ln \left(1 - \frac{[E-D^*]}{[E_o]_t}\right) = k_a \cdot t \quad \text{................. (3)}
\]
Here, \([E-D^*]\) is the concentration of the acyl enzyme complex and \([E]_0\) is the total enzyme concentration. The equation (3) is justified if the time of incubation is much shorter than the half life of the acyl-enzyme and \(k_a >> k_{+3}\).

From the \(k_a\) values measured at various beta-lactam concentrations, the kinetic parameters of enzyme acylation can be determined graphically. If the plot of \(k_a\) versus \([D]\) is a straight line, the condition \([D] << K\) is fulfilled, then the slope of the line gives the value of \(k_{+2}/K\), the second order-rate constant which can be obtained by combining equation (3) with equation (1)

\[
\ln \left(1 - \frac{[E-D^*]}{[E]_0}\right) = \frac{k_{+2}}{K} [D] \quad \text{(4)}
\]

Therefore, determination of \(k_{+2}/K\) involves measurement of the pseudo first order rate constant \(k_a\) as a function of time.

The values of the pseudo first order-rate constant \(k_a\) of protein acylation are determined by monitoring the disappearance of the enzyme activity at different concentrations of the beta-lactam at three or four incubation periods.

In all the cases the \(k_a\) values were 10 times larger than the \(k_{+3}\) values as shown in equation (3) and the plot of \(k_a\) versus concentration of beta-lactam compound gave rise to a straight line. Consequently the \(k_{+2}/K\) values are calculated from the slopes of the lines thus obtained from equation (4).

**Procedure:**

For each assay, 100 \(\mu g\) membrane protein was incubated for time periods of 30 60, 120, 300 and 600 sec with \(^{[3]}H\) benzylpenicillin at different
concentrations (25, 50, 100 µM) at 30°C, and the reactions were terminated with a ten-fold molar excess of nonradioactive benzylpenicillin. Membrane protein was extracted using 1% sodium lauroyl sarcosinate (sarkosyl) followed by denaturation, SDS-PAGE, autoradiography and densitometric scanning of the fluorograms as described by Chambers et al (1994). Densitometric scanning was carried out using a model 2202 ULTROSCAN Laser Densitometer (LKB, Bromma, Sweden).

Plots of In $A_t/A_0$ vs. time [for determination of $k_a$] and $k_a$ vs. concentration of antibiotic [for determination of $k_{a2}/K$], were obtained by linear curve fitting using Sigma Plot version 4.0. $A_t$ was the experimentally derived fluorographic density of the PBP after incubation for time, $t$, with radiolabeled benzylpenicillin at a given concentration, and $A_0$ was the density at which all binding sites had been saturated with radiolabeled benzylpenicillin.

2.8.2 Determination of the first-order rate constant of acyl-enzyme breakdown ($k_{33}$)

The deacylation reaction in which inactive drug is released from the PBP is described by the first-order rate constant, $k_{33}$ which was determined from the following equation.

$$\ln \left( \frac{PBP_t}{PBP_0} \right) = -k_{33}t$$

where $PBP_t$ is the intensity of the radioactive band at time $t$ after removal of PBP from radiolabel and $PBP_0$ is the intensity of the band when the PBP is saturated.
**Procedure for determination of k+3:**

1.5 mg membrane protein was radiolabeled with saturating concentration of $[^3]$H benzylpenicillin for 10 min at 30°C. Excess penicillin was destroyed by incubating the reaction mixture for 5 min at 30°C with *Bacillus cereus* beta-lactamase I. Samples containing 100 µg of membrane protein were removed at 30 sec, 60 sec, 2 min, 5 min, 10 min, 20 min, 40 min, 60 min, 2 h, 3 h, 4 h, 5 h and 7 h, extracted with 1% (w/v) Sarkosyl, denatured, run on SDS gels followed by fluoro­graphy and densitometric scanning of the fluorograms.

2.8.3. **Determination of the kinetic constant k$_{+2}$/K for interaction of the PBPs with non-radioactive beta-lactams**

Knowing the $k_{+3}$ value of acyl enzyme breakdown using radioactive benzylpenicillin, the $k_{+2}$/K value for non-radioactive beta-lactams was determined in a one-step competition with radioactive benzylpenicillin [for which the $k_{+2}$/K value i.e. $(k_{+2}/K)_2$ was determined] as described by Frere et al. (1976) from the equation:

$$\frac{(k_{+2}/K)_1}{(k_{+2}/K)_2} = \frac{[E-D^*]_1[D_2]}{[E-D^*]_2[D_1]}$$

$[E-D^*]$, the amount of acyl enzyme formed with the radioactive beta-lactam 2 in the presence of the non-radioactive beta-lactam 1 and $[E-D^*]_2$, the amount of acyl enzyme formed with the beta-lactam 2 in the absence of the beta-lactam 1 were determined by densitometric scanning of the fluorograms. $[E-D^*]_1 = [E-D^*]_2 + 2$. Incubations of the enzyme with antibiotics...
were carried out for three different time periods, with a maximum incubation time of 3 min so that the time of contact between the enzyme and the antibiotic was in each case short compared with the lifetime of the ED* adducts. Under these conditions the ratios of \([E-D^*][E-D^*]_2\) were independent of the time of contact.

2.9. Immunoblotting

Immunoblotting was performed by the method of Towbin et al. (1979). The details are given below.

**Immunoblotting solutions**

- **Tris-buffered saline (TBS):** 20 mM Tris, 500 mM NaCl, pH 7.5
  
  4.84 gm Tris was added to 58.48 gm NaCl in 1 l of distilled water and the pH was adjusted to 7.5 with HCl. The volume was then made up to 2 l.

- **Tween-20 wash solution (TTBS):** 20 mM Tris, 500 mM NaCl, 0.05% Tween 20: 0.5 ml of Tween-20 was added to 1 l TBS.

- **Antibody buffer:** 1% gelatin in TTBS.

  2 gm of gelatin was added to 200 ml of TTBS.

- **Blocking solution:** 3% gelatin in TBS. 3 gm of gelatin was added to 100 ml of TBS and warmed to 37°C to dissolve gelatin.

- **Transfer buffer:** 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3.

  3.02 gm Tris, 14.4 gm glycine and 200 ml of methanol were added to 500 ml of glass distilled water. The pH was adjusted to 8.3 with HCl and the volume was made up to 1 l.
Procedure for transfer of protein onto nitrocellulose:

Protein samples were run on a 10% SDS polyacrylamide gel. After the run was over, the gel was transferred to transfer buffer and kept at 4°C for 30 min. Whatman No. 1 filter paper was soaked in transfer buffer and 9 pieces were placed over the cathode of a semi-dry blotting apparatus (Novablot, LKB). A nitrocellulose membrane was soaked in transfer buffer and placed on top of the filter papers. The gel was then placed over the membrane and covered with 9 more pieces of filter paper soaked in transfer buffer. Transfer was carried out in "constant current" mode for 1 h at 0.8 mA/sq. cm. of the gel. After one hour, the assembly was dismantled and the membrane was transferred to blocking solution for 30 min, to prevent non-specific binding of IgG. The membrane was washed in TTBS for 5 min with two changes, transferred to the first antibody (raised against the protein of interest) solution and kept at room temperature for 2 h with constant shaking. Dilutions of antibody used varied from 1:250 to 1:2000 depending on the titre of the antibody. After 2 h, the membrane was washed with TTBS for 5 min with two changes. The membrane was then transferred into the second antibody solution containing horse-radish peroxidase (HRP)- conjugated goat anti-rabbit IgG at a dilution of 1: 1000; and kept at room temperature for 1 h. After 1 h, the membrane was washed twice with TTBS and once with TBS. 60 mg of HRP color developing reagent (1-chloro-4-naphthol) was dissolved in 20 ml chilled methanol. 60 μl of 30% H₂O₂ was added to it just before use, and the membrane was immersed into the solution for color development. After the bands became prominent, the
membrane was washed in distilled water several times and then dried on a filter paper.

2.10. Beta-lactamase activity

This was assayed in crude cell-free extracts of the strains which were prepared by centrifuging the sonicated cell suspension at 16,000 x g for 40 min. The hydrolysis of the chromogenic cephalosporin nitrocefin was monitored spectrophotometrically at 482 nm as described by O’Callaghan et al. (1972).

2.11. Preparation of outer membranes

Membranes suspended in 10 mM HEPES, pH 7.5 containing 100 µg of protein were treated with sarkosyl at a final concentration of 2% at 30°C for 1 h with vigorous shaking on an Eppendorf Thermomixer, followed by centrifugation at 48,000 x g for 30 min at 4°C. The supernatant was discarded. The pellet was washed twice with 100 µl of 0.5% sarkosyl and resuspended in 20 µl of HEPES buffer. Samples were subjected to electrophoresis on SDS polyacrylamide (12.5%) /urea( 8 M) gels (Mecsas et al., 1993).

2.12. Liposome swelling assay

This was carried out as described by Nikaido and Rosenberg. 2.5 µmol of egg phosphatidylcholine and 0.1 µmol of dicetyl phosphate were dried as a thin film under nitrogen. Tubes were kept for two hours in a vacuum dessicator to remove the remaining solvent. The film was suspended in 0.1 ml of buffer in which 30 µg outer membrane protein was added. The buffer used was 10 mM Tris-HCl, pH 8.0 when studying the uptake of sugars and zwitterionic
compounds, or 1 mM Na₂ NAD/1 mM imidazole, pH 7.0 when studying the uptake of monoanionic and dianionic beta-lactams. The suspension was mixed briefly in a cyclomixer, sonicated for two min in a water bath sonicator, dried under vacuum and left overnight in a vacuum dessicator. Finally, the film was reconstituted with 100 μl of a solution containing 20 mM stachyose in the respective buffer. 20 mM was recorded as the isotonic concentration of stachyose since neither shrinkage nor swelling of the liposome suspension (without protein) was observed. Similarly the isotonicity of the control liposome with the sample (whose permeability was to be determined) was estimated. As an example, if the same batch of liposomes showed isotonic behavior with 20 mM stachyose and 11.5 mM of a particular solute; and the porin-containing liposomes showed neither swelling nor shrinkage when diluted into 19 mM stachyose, then the solute was used in an 11.5 x (19/20)=10.9 mM nominal concentration with the porin-containing liposomes (Yoshimura and Nikaido, 1985).

Permeabilities of small molecules were measured by recording the swelling of liposomes which was accompanied by a time-dependent decrease in absorbance at 400 nm.

2.13. Accumulation of chloramphenicol and tetracycline

Accumulation of chloramphenicol was studied by using the procedure of McMurry et al. (1994). Cells were grown upto an OD₅₃₀ of 0.8 in Luria broth at 37°C, washed twice with 50 mM potassium phosphate buffer (pH 6.0) at room temperature and resuspended at 30°C at an OD₅₃₀ of 10 in the same buffer
containing 0.2% glucose, followed by incubation for 20 min at 30°C, in order to energize the efflux pump system. [3H] chloramphenicol (10.5 mCi/nmol, final concentration, 1μM) or [3H] tetracycline (0.5 mCi/μmol, final concentration, 10 μM). Accumulation was measured by diluting 50 μl of the cell suspension at different time points up to 20 min into 10 ml 100 mM LiCl, 50 mM potassium phosphate (pH 6.0) and immediately collecting the cells on Whatman GF/C filters (0.45 μm) using a Millipore filtration apparatus, followed by thorough washing with the same buffer and determination of the radioactivity on the dried filters in a LKB Minibeta liquid scintillation counter. Binding of radiolabel to the filters in the absence of cells was subtracted.

At 20 min, the proton motive force inhibitor, carbonyl cyanide meta-chlorophenyl hydrazone (CCCP) was added at a concentration 50 μM and the suspension was further incubated at 30°C. Aliquots were withdrawn at different time points (21, 25, 30, 35 and 40 min) and chloramphenicol accumulation was measured as described above.

2.14. Accumulation of norfloxacin and ciprofloxacin
This was done as described by Charvalos et al. (1995) with some modifications. Cells were grown up to an OD_600 of 0.4, harvested and washed with 50 mM phosphate buffer, pH 7.2 (PB). Cells were resuspended in the same buffer maintaining an OD_600 of 20 per ml, and energized with 0.2% glucose for 20 min at 30°C. Norfloxacin or ciprofloxacin was added at a concentration of 10 μg/ml. Aliquots of 0.5 ml were withdrawn at different time intervals and dispensed into tubes containing 1.5 ml PB. For one set of
experiments, CCCP (100 μM) was added at 20 min and further aliquots were withdrawn. Cells were immediately harvested, washed with 2 ml ice-cold PB and finally resuspended in 2 ml of 0.1(M) glycine hydrochloride, pH 3. After incubation for 60 min at 37°C, the suspension was centrifuged and the fluorescence of the supernatant was monitored in a spectrofluorimeter (for norfloxacin: excitation wavelength 282 nm, emission wavelength 448 nm; for ciprofloxacin: excitation wavelength 275 nm, emission wavelength 440 nm).

2.15. Accumulation of ampicillin

Cells from 50 ml cultures (grown to OD_{600} of 1.0) were harvested, washed twice with PB and resuspended in 16 ml PB. 160 μl of 20% glucose solution was added to make a final concentration of 0.2% glucose and the cell suspension was incubated at 30°C for 20 min. Ampicillin was added to a final concentration of 1 mM. Aliquots of 1 ml of the suspension were removed at different time points, filtered through a 0.22 μm membrane filter disc and ampicillin remaining in the supernatant was estimated from its hydrolysis by R-TEM beta-lactamase (followed by the decrease in optical density at 235 nm). In one set of experiments, CCCP was added at 20 min, further samples were withdrawn at different time intervals, and ampicillin was estimated in the supernatant.

2.16. Preparation of lipopolysaccharide (LPS)

Lipopolysaccharides (LPS) were prepared from acetone-dried cells by the phenol-water extraction method of Westphal & Jann (1965). Bacteria were
grown overnight in Tryptic Soybroth at 37°C with constant shaking, harvested by centrifugation at 12,000 x g and washed with phosphate-buffered saline (PBS), pH 7.4. The cells were incubated with acetone for 1 h at 37°C with vigorous shaking to extract water and phospholipids and washed thrice with acetone. The pellet was dried in air. A mixture of 17.5 ml of water and an equal volume of phenol, warmed to 65°C was added to 1 g of acetone-dried cells. The mixture was incubated at 65-70°C for 15 min with stirring and then cooled in an ice bath to allow phase separation between the aqueous and phenol-rich layers. Most of the LPS molecules, except those with very short oligosaccharide chains were present in the aqueous phase, and the denatured protein and cell debris collected at the interface. The mixture was centrifuged at 3,000 x g for 15 min, the aqueous layer was aspirated and exhaustively dialyzed against water. The dialyzate was centrifuged to remove insoluble matter, if any, and concentrated ten-fold under reduced pressure. Nucleic acids were precipitated by addition of 0.1 ml of a 2% (w/v) solution of cetyltrimethylammonium bromide (Cetavlon). The precipitate was discarded by centrifugation at 6,000 x g for 10 min and the supernatant was lyophilized. The freeze-dried material was dissolved in a minimum volume of 0.5 M NaCl and the crude LPS was precipitated by the addition of ten volumes of ethanol. The crude LPS was dissolved in 100 mM Tris-HCl buffer containing 5 mM CaCl₂, pH 7.6 and digested sequentially with DNase (10 μg/ml), RNase (10 μg/ml) and pronase (100 μg/ml) at 42°C for 16 h to remove contaminating protein and
nucleic acids. The digested material was heated in a water bath for 30 min, exhaustively dialyzed against water and lyophilized. The purity of the LPS was checked by monitoring absorbances at 260 and 280 nm for nucleic acid and protein contamination, respectively.

2.17. Polyacrylamide gel electrophoresis of LPS

After electrophoresis of the LPS preparation on a SDS/polyacrylamide (12.5%)/8M urea gel, the gel was stained with silver nitrate following the method of Hitchcock & Brown (1983).

**Working solutions:**

Fixative: 25% (v/v) isopropanol, 7% (v/v) acetic acid

Ammoniacal silver stain: This contained 0.1 N NaOH (28 ml), concentrated (25%) ammonium hydroxide (1.2 ml), 20% (w/v) silver nitrate (5 ml), and distilled water (115 ml). The solution was prepared just prior to use with vigorous agitation while mixing.

Developer: 50 mg citric acid and 600 μl of formaldehyde was added to 1 l of glass distilled water.

**Procedure:**

After electrophoresis, the gel was fixed in the fixing solution overnight. The fixing solution was replaced by fresh fixative containing 0.7% (w/v) periodic acid (prepared fresh). The gel was immersed in this solution for 5 min, then washed with water by gentle shaking eight times, each wash being of 30 min duration. The gel was stained using the ammoniacal silver stain for 10 min with vigorous shaking at room temperature. After washing the gel thrice, each time
for 10 min, the gel was immersed in formaldehyde developer solution (prepared fresh) till the bands were visible. The developing reaction was stopped by double distilled water containing 7% (v/v) acetic acid, washed and dried.

2.18. Assay for 2-keto-3-deoxyoctonate (KDO) in LPS

This was done as described by Karkhanis et al. 1 ml of 0.2 N H₂SO₄ was added to a test tube containing 2 mg of LPS-containing material. This amount was sufficient to determine 0.05 to 1% KDO accurately from the standard curve. For material containing higher than 1% KDO, 1 mg of starting material was sufficient. The reaction mixture was heated at 100°C for 30 min, cooled and centrifuged at maximum speed in a clinical centrifuge for 5 min. 0.5 ml of the supernatant was transferred into another test tube. 0.25 ml of 0.04 M HIO₄ in 0.125 N H₂SO₄ was added to this, vortexed, and allowed to stand at room temperature for 20 min. 0.25 ml of 2.6% NaAsO₂ in 0.5 N HCl was added, vortexed, and kept until the brown color disappeared. 0.5 ml of 0.6% thiobarbituric acid (TBA) was added, vortexed, and the mixture was heated at 100°C for 15 min. While hot, 1 ml of DMSO was added, the mixture was allowed to cool to room temperature and the optical density was read at 548 nm against a blank, treated as above, without KDO. The amount of KDO in the material was calculated from a standard curve. To obtain a standard curve, known concentrations of KDO from a stock solution (0.1 mg/ml) were taken in 0.5 ml of 0.2 N H₂SO₄ instead of 1 ml and treated as above. Following hydrolysis at 100 °C for 30 min, the reaction mixture was treated with HIO₄.
without centrifugation. The optical density at 548 nm was corrected for the total volume of 2.5 ml.

2.19. Agarose gel electrophoresis of DNA

*Electrophoresis buffer:*

*Working solution of Tris-acetate (pH 8) [TAE]:* 0.04 M Tris-acetate, 0.001 M EDTA, pH 8

*Concentrated (50x) stock solution (per liter):* 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA (pH 8).

*Gel-loading buffer:*

*6x buffer:* 0.25% bromophenol blue, 0.25% xylene cyanol FF and 40% (w/v) sucrose in water.

*Ethidium bromide solution:* Ethidium bromide was usually prepared as a stock solution of 10 mg/ml in water, which was stored at room temperature in dark bottles. Gels were immersed after electrophoresis in ethidium bromide solutions (0.5 µg/ml) for 30 min before visualization.

*Casting of the gel:*

The open ends of the plastic tray supplied with the electrophoresis apparatus were sealed with autoclave tape so as to form a mold. The mold was placed horizontally on the table. The required amount of powdered agarose was added to a measured quantity of electrophoresis buffer (1x TAE) (usually to obtain a 1% solution of agarose) in an Erlenmeyer flask. The slurry was heated till the agarose dissolved and then cooled to 60°C. Using a pasteur pipette, the edges of the mold were sealed with a small quantity of the agarose
solution. The seal was allowed to set. The comb was positioned 0.5 to 1 mm above the plate so that a complete well was formed when the agarose was added. The remainder of the agarose was poured into the mold ensuring that there were no air bubbles under or between the teeth of the comb. The gel was between 3 and 5 mm thick. After the gel was set (30 to 45 min at room temperature), the comb and the autoclave tape were removed carefully and the gel was mounted in the electrophoresis tank.

**Electrophoresis of DNA in agarose**:

Just enough electrophoresis buffer was added to cover the gel to a depth of about 1 mm. The samples of DNA were mixed with gel-loading buffer. The mixture was slowly loaded into the slots of the submerged gel using a micropipette. Marker DNAs of known size (e.g., λ DNA digested with Hind III) were loaded into slots on both the right and left sides of the gel. The lid of the gel tank was closed and the electrical leads were attached so that the DNA could migrate toward the anode. A voltage of 1 to 5 V/cm (measured as the distance between the electrodes) was applied. The gel was run until the bromophenol blue and xylene cyanol FF migrated to the appropriate distances through the gel. The gel was removed from the tank, immersed into a solution of ethidium bromide and finally examined by ultraviolet light and photographed.

**2.20. Polyacrylamide gel electrophoresis of DNA**

**Stock solutions**:

30% Acrylamide : acrylamide, 20 g; N,N’-methylenebisacrylamide, 1 g; water to 100 ml. This solution was stored in dark bottles and used within one month.
1 x TBE (electrophoresis buffer): 89 mM Tris-borate, 2 mM EDTA (pH 8)

TBE was made and stored as a 5x stock solution: 54 g Tris base, 27.5 g boric acid, 20 ml 0.5 M EDTA (pH 8) per liter.

10x ammonium persulfate: 1 g ammonium persulfate in 10 ml water.

**Polymerization and running of gels:**

The stock acrylamide solution was mixed with water and 5x TBE to yield the appropriate percentage, volume and buffer concentration (1x), filtered and gently deaerated by swirling. 50 μl TEMED and 1 ml 10% ammonium persulfate solution were added per 100 ml of acrylamide solution. Gels were polymerized and run on a Bio-Rad Miniprotean electrophoresis apparatus. Gels were run at 8 V/cm or less for 45 min, stained with ethidium bromide and examined by ultraviolet light.

**2.21. PCR amplification of 5'end region of gyrA of S. dysenteriae**

*Shigella* spp. are genetically related to *E. coli* and considered taxonomically as members of the genus *Escherichia*. Two primers from the conserved regions of *gyrA* of *E. coli* K-12 were selected for PCR amplification of the 5’end coding region comprising the putative quinolone resistance-determining region of *gyrA* of *S. dysenteriae* type 1. The two primers were 20-mer oligonucleotides, 5’-TACACCGGTCAACATTGAGG-3’ and 5’-TTAATGATTGCCGCGCTCGG-3’, which were identical in sequence to nucleotide positions 24 to 43 or complementary in sequence to positions 652 to 671 of *E. coli* K-12 *gyrA* Rahman et al. (1994). Chromosomal DNA was prepared from each strain by boiling one colony in 1 ml of water for 8 min followed by centrifugation in an...
Eppendorf centrifuge for 10 s. One microliter of the supernatant was used as template DNA for PCR amplification.

Amplifications were performed in 100 µl of reaction mix in capped Perkin Elmer Cetus polypropylene microcentrifuge tubes (0.5 ml) using a Perkin-Elmer Cetus DNA Thermal Cycler (GeneAmp 2000). Aerosol-free tips were used in order to avoid any contamination during PCR. The following reaction mix was prepared according to the manufacturers' protocol.

**Reaction Mix:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Order of Addition</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile distilled water</td>
<td>1</td>
<td>•</td>
<td></td>
</tr>
<tr>
<td>10X PCR Buffer</td>
<td>2</td>
<td>10 µl</td>
<td>1X (1.5 mM MgCl₂)</td>
</tr>
<tr>
<td>dATP</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dCTP</td>
<td>dNTP</td>
<td>3</td>
<td>2 µl 200 µM of each</td>
</tr>
<tr>
<td>dGTP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dTTP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer 1</td>
<td>4</td>
<td>5 µl</td>
<td>1.0 µM</td>
</tr>
<tr>
<td>Primer 2</td>
<td>5</td>
<td>5 µl</td>
<td>1.0 µM</td>
</tr>
<tr>
<td>Experimental template</td>
<td>6</td>
<td>•</td>
<td>1 µg/100 µl</td>
</tr>
<tr>
<td>AmpliTaq @ DNA Polymerase</td>
<td>7</td>
<td>0.5 µl</td>
<td>2.5 Units/100 µl</td>
</tr>
</tbody>
</table>

---

| Total Mix                     | 100 µl            |                                      |

•, variable volume.
10X PCR Buffer: 100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl₂

The above reagents were mixed gently and spun down in a microcentrifuge. Thirty cycles were used for each reaction, with the following temperature profiles: 90°C, 1 min; 64°C, 1 min; 72°C, 2 min. A final cycle of 10 min at 72°C was performed. PCR was performed thrice per strain.

The samples were stored at -20°C until subsequent analysis. The PCR products were analysed by 10% polyacrylamide gel electrophoresis followed by staining with ethidium bormide.

2.22. Purification of DNA fragments from PCR reactions

The PCR Clean Up Kit (Boehringer Mannheim) was used for this purpose.

The kit contains:
1. Silica Matrix containing sodium perchlorate
2. Nucleic acid binding buffer containing sodium perchlorate
3. Washing buffer.

Procedure:
1. The PCR reaction was extracted with an equal volume of chloroform, centrifuged for 5 min at 5,000 rpm in a table-top centrifuge and the upper phase was transferred to a new reaction tube.
2. TE buffer was added to the PCR reaction tube to obtain a final volume of 100 µl, followed by the addition of 400 µl of nucleic acid binding buffer.
3. 10 µl of the homogeneous silica suspension was added to the PCR reaction mixture and incubated for 10 min at room temperature with occasional vortexing.
4. This was followed by centrifugation in a table-top centrifuge for 30 sec at maximum speed. The supernatant was discarded.

5. The matrix containing the DNA was resuspended in 400 μl nucleic acid binding buffer on a vortex mixer, centrifuged and the supernatant was discarded.

6. The pellet was washed with 400 μl washing buffer, centrifuged and the supernatant was discarded. This step was repeated once.

7. All the liquid was finally removed with a pipette, the tube was inverted on an absorbent tissue and was dried at room temperature for 15 min.

8. 50 μl of water (pH 8-8.5) was used for the elution of DNA. After the application of elution buffer, the tube was vortexed and incubated for 5 min at 56-60°C. It was centrifuged at maximum speed in a table-top centrifuge for 30 sec, the DNA-containing supernatant was removed to a new reaction tube, and stored at -20°C.

2.23. Digestion of DNA fragments

PCR amplified and purified DNA (20 μg) was digested with 50 units of Sma I in a volume of 200 μl at 30°C for 2 h. DNA was then purified using the PCR clean up kit (Boehringer Mannheim) and it was further digested with 60 units of Sac I in a volume of 400 μl at 37°C for 2 h. The digested DNA was purified using the PCR Clean Up Kit (Boehringer Mannheim).

2.24. Restriction enzyme digestion of plasmids

5 μg of pK19 (Pridmore, 1987) was digested completely with Sma I and Sac I as described above. Digested plasmids were purified from the reaction mix by
extraction once with phenol: chloroform: isoamyl alcohol (50:49:1, v/v/v) and twice with chloroform:isoamyl alcohol (24:1, v/v), followed by precipitation with chilled ethanol. The precipitated DNA was washed twice with 70% ethanol and dried. The dried DNA was reconstituted in an appropriate volume of TE buffer.

2.25. Insertion of DNA into plasmid vectors by ligation

Ligation of DNA fragments was performed using the Rapid DNA Ligation Kit (Boehringer Mannheim). The kit contains:

- T4 DNA ligation buffer (2x)
- DNA dilution buffer
- T4 DNA ligase (5 units/μl)

The vector DNA and insert was dissolved in 1x DNA dilution buffer. The molar ratio of vector DNA to insert DNA in a total volume of 10 μl was usually 1:3.

Procedure:

10 μl DNA (vector + insert) in 1x DNA dilution buffer was mixed thoroughly with 10 μl T4 DNA ligation buffer (2x). 1 μl T4 DNA ligase was added, mixed thoroughly and incubated at 22°C for 5 min. The ligation mixture was used directly for transformation of competent cells. A maximum of one-tenth of the volume of the ligation mixture containing a maximum of 200 ng DNA was used for transformation.

2.26. Preparation of competent E. coli cells

Escherichia coli DH5α was grown overnight in Luria broth at 37°C. 1% of this culture was used to inoculate 100 ml Luria broth. Cells were grown at 37°C upto an absorbance of 0.5 - 0.6 at 600 nm. Cells were then transferred
asceptically in a centrifuge tube and harvested at 4°C for 5 min at 4,000 rpm (Sorvall SS-34 rotor). Cells were resuspended slowly in 80 ml chilled 10 mM Tris-HCl pH 8.0 containing 0.1 M CaCl₂ (sterilized by filtration through a sterile 0.22 μm membrane filter disc). This suspension was kept on ice for 30 min followed by centrifugation. The pellet was finally resuspended in 5 ml chilled 0.1M CaCl₂ (filter-sterilized) and kept on ice for 3 to 16 h. The competent *E. coli* cells were used within 16 h.

2.27. Transformation of competent *E. coli*

All steps were performed strictly under asceptic conditions. For each transformation, 100 μl of a chilled suspension of competent cells were taken in a pre-chilled sterile Falcon tube (17 mm x 100 mm). DNA (50 to 200 ng) was added in a volume of 5 μl to each tube by slowly dispensing the DNA into the cell suspension. The tubes were stored on ice for 30 min. The tubes were placed on a non-circulating waterbath preheated to 42°C for 1 min. After heat treatment, the tubes were placed on ice for 2 min. 900 μl Luria broth was then added and the tubes were placed in a 37°C shaker for 1 h in order to allow the cells to recover and express the antibiotic (kanamycin) resistance marker encoded by the plasmid. 50-100 μl of this culture was then plated on Luria agar containing 50 μg/ml kanamycin. The plates were then inverted and kept in an incubator at 37°C for 12 to 16 h.

2.28. Small-scale preparation of plasmid DNA
Plasmid DNA was prepared on a small scale from overnight cultures in order to examine the transformants and select for recombinants. The Promega Wizard™ Plus Minipreps DNA Purification System was used.

**Components:**

*Cell resuspension solution*: 50 mM Tris (pH 7.5); 10 mM EDTA, 100 µg/ml RNase A.

*Cell lysis solution*: 0.2 M NaOH and 1% SDS.

*Neutralization solution*: 1.32 M potassium acetate

*Wizard™ Minipreps DNA Purification Resin*

*Column wash solution*: 80 mM potassium acetate, 8.3 mM Tris-HCl, pH 7.5, 40 µM EDTA and 55% ethanol.

*TE buffer*: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA (not supplied by Promega).

**Procedure:**

1. 1-3 ml of an overnight culture of cells were centrifuged at 10,000 x g in a microcentrifuge. The supernatant was poured off and the tube was blotted upside-down on a paper towel to remove excess media.

2. The cell pellet was carefully resuspended in 200 µl of cell resuspension solution.

3. 200 µl of cell lysis solution was added and mixed by inverting the tube four times. The cell suspension cleared immediately.

4. 200 µl of neutralization solution was added and mixed by inverting the tube.

5. The lysate was centrifuged at 10,000 x g in a microcentrifuge for 5 min or longer in order to obtain a tight pellet.
6. For each miniprep, one Wizard minicolumn was prepared. The plunger was removed from a 3 ml disposable syringe and set aside. The syringe barrel was attached to the Luer-Lok extension of the Minicolumn and 1 ml of the resuspended resin was pipetted into the barrel.

7. The cleared lysate was removed from each miniprep and transferred to the barrel of the minicolumn/syringe assembly containing the resin.

8. The syringe plunger was inserted carefully and the slurry was pushed gently into the Minicolumn.

9. The syringe was detached from the Minicolumn and the plunger was removed from the syringe barrel. The syringe barrel was reattached to the Minicolumn.

10. 2 ml of Column Wash Solution was pipetted into the barrel of the Minicolumn/syringe assembly. The plunger was inserted into the syringe and the Column Wash Solution was gently pushed through the Minicolumn.

11. The syringe was removed and the Minicolumn was transferred to a 1.5 ml microcentrifuge tube. The Minicolumn was centrifuged at 10,000 x g for 2 min to dry the resin.

12. The Minicolumn was transferred to a new 1.5 ml microcentrifuge tube. 50 µl of TE was added. After 1 min, the tube was centrifuged at 10,000 x g in a microcentrifuge for 20 seconds to elute the DNA. The Minicolumn was discarded and the plasmid was stored in a microcentrifuge tube at -20°C.

2.29. Preparation of plasmid DNA on a large scale
Recombinant and non-recombinant plasmids were isolated from transformed *E. coli* cells using a modification of the method of Birnboim and Doly (1979) and the Nucleobond AX kit from Macherey-Nagel, Germany. Plasmid isolation was performed following the procedure provided by the manufacturers. Transformed *E. coli* cells were grown overnight in Luria broth containing the appropriate antibiotic. Cells grown overnight were harvested by centrifugation at 5,000 rpm (Sorvall SS-34) for 5 min at 4°C. The pellet was carefully resuspended in 4 ml buffer S1 (50 mM Tris-Cl, 10 mM EDTA, 100 µg/ml RNase A, pH 8.0). To this suspension, 4 ml of buffer S2 (200 mM NaOH, 1% SDS) was added and mixed gently, without vigorous shaking or vortexing. The mixture was kept at room temperature for 5 min followed by the addition of 4 ml of buffer S3 (2.8 M potassium acetate, pH 5.1). After mixing gently by inverting the tube 6-8 times until a homogeneous suspension was formed, the suspension was incubated on ice for 5 min and centrifuged at >12,000 x g at 4°C for 38 min. The clear supernatant was loaded on a Nucleobond AX cartridge (containing ion-exchange silica between two inert filter elements in a polypropylene cartridge), preequilibrated with 2 ml of buffer N2 (100 mM Tris, 15% ethanol, 900 mM KCl pH 6.3, adjusted with H₃PO₄). After loading was over, the cartridge was washed twice with 4 ml each of buffer N3 (100 mM Tris, 15% ethanol, 1150 mM KCl, pH 6.3 adjusted with H₃PO₄). The plasmid DNA was eluted from the cartridges using 2 ml buffer N5 (100 mM Tris, 15% ethanol, 1 M KCl, pH 8.5 adjusted with H₃PO₄). The purified plasmid DNA was precipitated with 0.7 volumes of
isopropanol at room temperature and centrifuged at high speed (>12,000 x g)
at 4°C for 30 min. The precipitated DNA was washed with 70% chilled ethanol,
twice and dried briefly in a Savant Speed Vac centrifugal evaporator. The dried DNA was reconstituted in 60 μl of TE buffer for further manipulations.

2.30. DNA Sequencing

DNA sequencing was carried out using Sanger's chain-termination DNA sequencing method (Sanger et al., 1977) and the Sequenase Version 2.0 T7 DNA sequencing Kit from United States Biochemicals, USA.

2.30.1. The sequencing reaction

Components:

Sequenase buffer (5x concentrate) : 200 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, 250 mM NaCl.

Primer (~40) (0.5 pmol/μl)

5'GTTTTCCCAGTCACGAC-3'

Dithiothreitol solution : 0.1 M

Labeling mix(dGTP) 5x concentrate :

7.5 μM dGTP, 7.5 dCTP, 7.5 μM dTTP

ddG Termination mix : 80 μM dGTP, 80 μM dATP, 80 μM dCTP, 80 μM dTTP, 8μM ddGTP, 50 mM NaCl.

ddA Termination mix : 80 μM dGTP, 80 μM dATP, 80 μM dCTP, 80 μM dTTP 8 μM ddATP, 50 mM NaCl

ddT Termination mix : 80 μM dGTP, 80 μM dATP, 80 μM dCTP, 80 μM dTTP, 8 μM ddTTP, 50 mM NaCl
**ddC Termination mix**: 80 μM dGTP, 80 μM dATP, 80 μM dCTP, 80 μM dTTP, 8 μM ddCTP, 50 mM NaCl

**Stop solution**: 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF

**Sequenase version 2.0 T7 DNA polymerase**: 1.3 units/μl in 20 mM KPO₄, pH 7.4, 1 mM DTT, 0.1 mM EDTA, 50% glycerol

**Glycerol enzyme dilution buffer**: 20 mM Tris-HCl, pH 7.5, 2 mM DTT, 0.1 mM EDTA, 50% glycerol.

All nucleotide mixtures were stored frozen at -20°C and kept on ice when thawed for use. The Sequenase version 2.0 enzyme was also stored at -20°C. [³⁵S]dTTP (10 mCi/ml) was a product of Amersham, Buckinghamshire, U.K.

**Procedure**:

1. **Denaturation of double-stranded DNA template** - 3.5 μg of plasmid DNA was denatured by addition of 0.1 volume of 2 M NaOH-2 mM EDTA and incubation for 30 min at 37°C. The mixture was neutralized by adding 0.1 volume of 3 M sodium acetate (pH 4.5 - 5.5) and the DNA was precipitated with 4 volumes of ethanol (-70°C, 15 min). After washing the pelleted DNA with 70% ethanol, it was dissolved in 7 μl of distilled water.

2. **Annealing of template with primer (total volume: 10 μl)** -
   
<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>7 μl</td>
</tr>
<tr>
<td>Reaction Buffer</td>
<td>2 μl</td>
</tr>
<tr>
<td>Primer</td>
<td>1 μl</td>
</tr>
</tbody>
</table>
The mixture was heated for 2 min at 65°C and then cooled slowly to <35°C over 15-30 min, centrifuged briefly and chilled for use during the labeling reaction.

3. While cooling, 2.5 μl of each Termination Mixture (G, A, T and C) were dispensed in separately labeled tubes.

4. Labeling mix was diluted 5-fold to its working concentration.

5. The 4 termination tubes from step 3 were kept in a 37°C bath (G, A, T and C).

6. **Labeling reaction** -

   To ice-cold annealed DNA mixture (10μl), was added:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT, 0.1 M</td>
<td>1 μl</td>
</tr>
<tr>
<td>Diluted Labeling Mix</td>
<td>2 μl</td>
</tr>
<tr>
<td>[35S] dATP</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Diluted (1:8) Sequenase</td>
<td>2 μl</td>
</tr>
<tr>
<td>polymerase</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>15.5 μl</td>
</tr>
</tbody>
</table>

   The mixture was incubated at room temperature for 2-5 min.

7. **Termination reaction** -

   3.5 μl of labeling reaction was transferred to each termination tube (G,A,T and C), mixed and incubation of the termination reactions was continued at 37°C for 5 min.

8. The reactions were stopped by adding 4 μl of Stop Solution.

9. Samples were heated to 75°C for 2 min immediately before loading onto the sequencing gel. 2-3 μl was loaded in each lane.
2.30.2. Preparation and running of polyacrylamide gels for DNA sequencing

Buffers and gel recipes:

5x TBE (2 liters)
Tris base 108 g
Boric acid 55 g
0.5 M EDTA 40 ml

Urea mix
Ultra-pure urea 117 g
5x TBE 50 ml
Water to 250 ml

20% T, 3.35% C Acrylamide Monomer Solution
Acrylamide 48.25 g
Bisacrylamide 1.67 g
5x TBE 50 ml
Water to 250 ml

8% T Sequencing Gel
20% T, 3.35% C Monomer solution 20 ml
Urea mix 30 ml
10% Ammonium persulfate 0.4 ml
TEMED 50 μl
Procedure:
Gels were run using a Hoefer SQ3 sequencing unit. To set the gel, the plates between which the gel was to be cast, were cleaned. The larger plate (with no notch) was siliconized. The plate was laid on a pad of paper in a chemical hood. A small amount of siliconizing fluid (Sigmacote) was poured onto it. The fluid was wiped over the surface of the plate and the plate was dried with a hair dryer. It was then laid flat on the bench and two spacers were arranged in place along the sides of the plate. A small amount of petroleum jelly was used to keep the spacers in position while placing the smaller (notched) glass plate on top of the bigger one. After placing the smaller plate on the bigger one, resting on the spacers, the plates were clamped together with several large bulldog binder clips. The bottom was sealed with gel-sealing tape to make a water-tight seal. The acrylamide solution was filtered through a 0.45 μ sterile membrane filter unit. The urea mix was added and warmed to dissolve the urea. This was followed by the addition of ammonium persulfate and TEMED. The gel mix was poured gently from one corner of the gel-mold, holding the mold at an angle of approximately 45° to the horizontal. To avoid formation of air bubbles the solution was poured in a continuous stream. The flat side of a shark’s tooth comb was placed immediately, approximately 0.5 cm into the gel solution. The gel was allowed to polymerize and kept up to 16 h before use.
**Loading and running sequencing gels**

When polymerization of the gel was complete, excess dried polyacrylamide or urea were cleaned from the outside of the gel mold. The sealing tape was removed from the bottom of the mold. The gel mold was attached to the electrophoresis apparatus with bulldog binder clips and bubbles (if any) were removed from the bottom of the gel after it was dipped in 1x TBE buffer. The shark’s tooth comb was inserted with its teeth just penetrating the surface of the gel. The gel was run at 45 W for 1 h prior to loading of the samples. 2 µl of each sample was loaded into adjacent slots (keeping the order of the templates on record) in triplicate at intervals of 90 min. The gel was run at a constant power of 45 W and the migration of the bromophenol blue and xylene cyanol FF were followed in order to determine the duration of the run.

**Autoradiography of sequencing gels**

After completing electrophoresis of sequencing gels, the gel mold was removed from the apparatus. The mold was layed flat on a bench with the smaller plate on the top. Using the end of a metal spatula, the plates were slowly taken apart ensuring that the gel was on the bottom plate. A Whatman 3 MM filter paper was placed on top of the gel and it was removed from the plate onto the filter paper in one smooth action. After placing a Saran Wrap on top, the gel was dried, removed and exposed to an X-ray film (Kodak XAR-5) for 16-24 h before development. Distinct bands were observed. The sequence was read from the bottom of the film following the upward ladder of the bands.
2.31. Scanning electron microscopy (SEM) of cells

The cells were harvested by centrifugation at 5,000 x g for 10 min and washed with 0.15 M cacodylate buffer. The cells were then prefixed with 0.15 M cacodylate buffer containing 1% osmium tetroxide for 1 hour at room temperature. The prefixed cells were collected by centrifugation at 600 x g for 2 min, washed with 0.15 M cacodylate buffer and post-fixed with 0.15 M cacodylate buffer containing 2% glutaraldehyde for 2 hours at room temperature. The cells were again collected by centrifugation at 600 x g and fixed with 0.15 M cacodylate buffer containing 1% osmium tetroxide at 4°C overnight. The cells were subsequently dehydrated using graded ethanol solution (10-100%), spread on glass slides, air-dried and then gold-coated under vacuum for 30 min. The cells were examined under a scanning electron microscope (PSEM-500, Phillips, Holland).