MATERIALS AND METHODS
MATERIALS

MEDIA

Bactotryptone, Yeast extract and Bactoagar were obtained from Difco Laboratories, USA. Readymade Luria broth and Agar were obtained from Hi-media Laboratories, India.

ENZYMES

Restriction enzymes were purchased from New England Biolabs. DNA modifying enzymes T4 DNA ligase, Klenow fragment of DNA polymerase I, Calf Intestinal phosphatase, T4 Polynucleotide kinase, DNase I were purchased from New England Biolabs, USA, Boehringer Manheim, Germany, and Promega, USA. Proteinase K, Lysozyme and RNase were purchased from Sigma Chemical Company, USA.

RADIOACTIVE NUCLEOTIDES AND KITS

[α-32P] dCTP (specific activity>4000 ci/m mole) and [γ-32P] dATP (specific activity >5000 ci/m mole) were obtained from Bhabha Atomic Research Centre, Bombay.

Oligonucleotides used for labeling of DNA fragment by random priming method were synthesized and purified in the laboratory using nucleotides obtained from Sigma Chemical Company
Dideoxy DNA sequencing kit [Sequenase (version 2.0)] was purchased from United States Biochemicals, USA. Kit for plasmid isolation was from Qiagen Inc., USA.

CHEMICALS

Agarose (type II, medium EEO), Low melting agarose, deoxynucleotide triphosphates, Ethidium bromide, Calf thymus DNA, Calcium chloride, Ficoll, Polyvinylpyrolidone, Tris-base, Dithiothreitol, Cesium Chloride, X-gal, γ-(methylacryloxy)-propyltrimethoxy silane, Bovine serum albumin, all antibiotics, Acrylamide and bisacrylamide were purchased from Sigma Chemical Company, USA.

Other analytical grade, general-purpose laboratory chemicals and reagents were procured from E. Merck, India and Qualigens, India. Absolute alcohol was purchased from Bengal Chemicals and Pharmaceuticals Works Ltd., India.

PHOTOGRAPHIC MATERIAL AND X-RAY FILMS

X-ray films (XK-5), fixer and developer were obtained from Eastman Kodak Company, USA. Intensifying screens used in X-ray cassettes were from Kiran X-ray screens, India. Positive negative, Black and White instant films (type 665) were obtained from Polaroid Photographic Corporation, USA.
PREPACKED CHROMATOGRAPHY COLUMNS AND FILTER MATERIALS

Columns for synthesis and purification of oligonucleotides such as OPC and Sep-pak columns were purchased from Applied Biosystems Inc., USA and Millipore Corporation, Waters chromatography Division, USA, respectively. Prepacked one ml columns of S-Sepharose were obtained from Biorad, USA. Nitrocellulose membrane (0.45 μ) was purchased from Advanced Microdevices Pvt. Ltd., India. DEAE Cellulose paper (DE-81), 3 MM chromatography paper and Whatman paper no. 1 were purchased from Whatman Ltd., UK.

GROWTH AND STORAGE OF BACTERIA

All the media used for growth of bacteria were autoclaved at 15 psi, 121°C for 20 minutes. Solid media were prepared by addition of 2% agar prior to autoclaving. Heat labile components like antibiotics, ammonium acetate, X-gal were filter sterilized and added to the media after its temperature reached about 50°C. All bacterial strains were generally stored at −70°C either in 20% glycerol or 10% DMSO unless specified and streaked out freshly whenever required. 

E.coli strains were usually grown in Luria-Bertani medium (LB) at 37°C with shaking at 200 rpm. The constituents of LB were 1% tryptone, 0.5% yeast extract and 0.5% sodium chloride. Its pH was adjusted to 7.0.

Klebsiella pneumoniae is a diazotrophic bacterium capable of fixing nitrogen in absence of oxygen and fixed nitrogen. The cells are usually arranged singly or in pairs, producing dome shaped glistening colonies of varying degree of stickiness. Under laboratory conditions they grow well at 30°C on minimal media.
containing sucrose as a carbon source. It requires no special growth adjuvents and can utilize citrate and glucose as a carbon source and ammonia as a nitrogen source. *Klebsiella pneumoniae* strains are resistant to antibiotic penicillin and to a lesser extent ampicillin. Most strains produce 2,3-butenediol as end product of glucose fermentation giving the characteristic pungent odour to the colonies. *Klebsiella pneumoniae* strains were generally grown in LB medium or other media like the M9 minimal medium containing sucrose as a carbon source at 30°C.

*Azotobacter vinelandii* is a free living aerobic diazotroph found growing in soil and water. It is a gram negative bacteria capable of fixing molecular nitrogen in a nitrogen free medium in presence of an organic carbon source. The cells produce a water-soluble fluorescent pigment, which appears green. Optimal temperature for growth is 30°C and the optimum pH is 7-7.5. The cells carry multiple copies of genome ranging from 40-80 (Punita *et al.*, 1989; Sadoff *et al.*, 1979). *Azotobacter vinelandii* strains were grown in modified Burk’s nitrogen free medium (BNF) at 30°C with shaking at 200 rpm (Strandberg and Wilson, 1968). The medium was made by diluting stocks of 10X BNF salts (0.2% magnesium sulphate, 0.1% calcium sulphate), 1000X sodium molybdate (24 mg/100 ml), 1000X ferric citrate (95 mg/100 ml), and adding 2% sucrose. 10X phosphate buffer (50 mM potassium phosphate, pH 7.2) was autoclaved separately and added to the autoclaved medium. A final concentration of 0.11% ammonium acetate was added as a nitrogen source, if required.

**BNF salts (10X)**

0.1% CaSO₄

0.2% MgSO₄
BNF-PO₄ Buffer (10X) 50mM

0.68% KH₂PO₄
0.87% K₂HPO₄

M9 supplemented mixture (Rodriguez and Tait, 1983)

M9 salt mix - 100ml
20% glucose - 20ml
0.01M CaCl₂ - 10ml
0.1M MgSO₄ - 10ml
Deionized water - 860ml

M9 salt mixture (10X)

Na₂HPO₄ - 70g
KH₂PO₄ - 30g
NaCl - 5g
NH₄Cl - 10g
Volume made upto 1000ml

All antibiotic stocks were generally made 500X and diluted according to the requirement for both E. coli and A. vinelandii strains
ANTIBIOTICS | SOLVENT | Final concentration (for E.coli) | Final concentration (for A.vinelandii) 
--- | --- | --- | --- 
Ampicillin | Water | 100μg/ml | 50-100μg/ml 
Chloramphenicol | Ethanol | 30μg/ml | 170μg/ml 
Kanamycin | Water | 50μg/ml | 5-15μg/ml 
Nalidixic acid | 50mM KOH | 50μg/ml | - 
Tetracycline | 50% ethanol | 10μg/ml | 2-5μg/ml 

METHODS

The various standard protocols were followed from ‘Molecular Cloning: A laboratory manual’ (Maniatis et al., 1982), ‘Molecular Cloning: A laboratory manual’ (Sambrook et al., 1989), ‘Current protocols in molecular biology’ (Ausubel et al., 1987) or according to the instruction manuals provided with the kits.

ISOLATION OF PLASMID DNA

The protocol used was adapted from Birboim & Doly (1979). A bacterial culture grown overnight in LB with appropriate antibiotics was centrifuged at 5000 rpm for 10 minutes at 4°C in a microfuge. The cell pellet was suspended in 200 μl of T₅₀ E₅₀ (50 mM Tris-Cl, pH 8.0; 10 mM EDTA, pH 8.0) containing RNase at a final concentration of 100 μg/ml. 200 μl of alkaline SDS (0.2 N NaOH, 1% Sodium dodecyl sulphate) was added to the cell suspension, mixed gently and incubated for 5-10 minutes at room temperature. 200 μl of ice-cold Potassium acetate (3 M Potassium⁺, 5 M Acetate⁻, pH 4.8) was added and mixed thoroughly. It was kept on ice for 15 minutes after which it was centrifuged at 13000 rpm for 15 minutes at 4°C in a microfuge. The supernatant thus obtained was transferred into a fresh eppendorf tube and precipitated with 0.6 volumes of isopropanol at room temperature for 5
minutes followed by centrifugation at 10000 rpm for 15 minutes at room temperature in a microfuge. The DNA pellet was resuspended in an appropriate volume of sterile double distilled water or T10E1 (10 mM Tris-Cl, 1mM EDTA, pH 8.0). An additional purification step was employed for larger plasmids (>10kb), whose preparation was not expected to be clean. For this, the volume of dissolved DNA was made up to 50 μl or more and was mixed thoroughly with an equal volume of phenol-chloroform-Isoamyl alcohol (PCI) mix (Tris-saturated Phenol mixed with distilled chloroform and isoamyl alcohol in the ratio 25:24:1). It was centrifuged at 10000 rpm for 10 minutes at room temperature. The upper aqueous layer was extracted with chloroform-isoamyl alcohol mix (chloroform and isoamyl alcohol, 24:1) in the same manner. The aqueous layer was transferred to a fresh microfuge tube and the DNA precipitated by adding 1/10th volume of 3M sodium acetate (pH 5.2) and 3 volumes of cold ethanol. It was kept at 70°C for a minimum period of 30 minutes. The DNA was recovered by centrifugation at 13000 rpm for 15 minutes at 4°C in a microfuge. The pellet was washed with 70% ethanol, centrifuged again, dried in air and resuspended in either sterile water or T10E1.

**LARGE SCALE ISOLATION OF PLASMID DNA (MAXI PREP)**

The same alkaline lysis method was used. One litre LB medium with appropriate antibiotic was inoculated with freshly grown *E.coli* culture (10 ml) and further grown at 37°C on a rotary shaker. Bacterial cells were harvested by centrifugation at 5000 rpm for 10 minutes at 4°C in Sorvall GS3 rotor. The supernatant was discarded and the cells were washed in 100 ml 0.8% saline. The cells were resedimented as above and resuspended in 20 ml of solution I (50 mM glucose; 25 mM Tris-Cl, pH 8.0; 10 mM EDTA), containing 5 mg/ml lysozyme and kept on ice for 10-15 minutes. 40 ml of freshly prepared solution II (0.2 N NaOH,
1% SDS) was added and the contents were mixed gently and incubated at room
temperature for 10-15 minutes. To this, 30 ml of ice-cold sodium acetate (3 M Potassium+, 5 M Acetate-, pH 4.8) was added, mixed well and the contents were
incubated on ice for 30 minutes. The cell lysate was centrifuged in Sorvall GS3 rotor at 9000 rpm at 4°C for 15 minutes. The clear supernatant solution (containing
the plasmid DNA) was transferred to a fresh GS3 bottle and the pellet (containing
high molecular weight chromosomal DNA and cell debris) was discarded. To the
supernatant solution 0.6 volume of isopropanol was added, mixed well and allowed
to stand at room temperature for 5 minutes. The DNA was recovered by
centrifugation at 9000 rpm in Sorvall GS3 rotor at room temperature. The pellet was
air dried and dissolved in T10E1 (10 mM Tris-Cl, pH 8.0; 1 mM EDTA, pH 8.0). The
plasmid DNA was further purified by ethidium bromide–cesium chloride gradient.

ETHIDIUM BROMIDE-CESIUM CHLORIDE GRADIENT CENTRIFUGATION

In order to get highly purified DNA devoid of RNA, chromosomal DNA and
proteins, CsCl gradient centrifugation was carried out. To a 3 ml DNA solution, 3.4
g of cesium chloride and 0.5 ml of ethidium bromide solution (10 mg/ml) was added
and dissolved. The contents were transferred to a Beckman quick seal polypropylene
tube. The tubes were then balanced, sealed and centrifuged at 45000 rpm for 18
hours in VTi90 rotor in Beckman L7 ultracentrifuge at 15°C. After the
centrifugation, long wavelength (366 nm) UV light was used for visualization of
DNA bands. The solution containing the specific band was collected and transferred
to a fresh tube. The solution was extracted with water-saturated butanol to remove
ethidium bromide. The clear solution was then transferred to Corex tube and diluted
with two volumes of water. 2.5 volumes of ethanol was then added to precipitate the
DNA and left undisturbed at -20°C for 2 hours. Subsequently, it was centrifuged at 10000 rpm at 4°C in Beckman JS-13 rotor and the pellet obtained was redissolved in 0.5 ml water. DNA was re-precipitated and washed with 70% ethanol to remove excess salt and finally dissolved in T_{10}E_{1}.

**DIGESTION OF DNA WITH RESTRICTION ENDONUCLEASES**

Restriction digestion of up to 1 µg of DNA was carried out in a volume of 20 µl under suitable conditions of buffer (as specified by the manufacturer of the restriction enzymes) and temperature. Digestion was carried out for 1-2 hours, after which tracking dye (0.05% bromophenol blue, 0.05% xylene cyanol, 20 mM EDTA and 50% glycerol) was added to the sample and it was electrophoresed on an agarose gel for analysis.

Restriction digestion for larger amounts of DNA was scaled up appropriately. Digested DNA to be used for ligations was precipitated and dissolved in a small volume before ligation. DNA sample, which yielded more than one DNA fragment on restriction digestion was electrophoresed in a preparative low melting agarose gel and the band of interest was subsequently eluted out.

DNA with dissimilar staggered 5' overhangs could be filled in by the Klenow fragment and used for a blunt end ligation. The filling in reaction was carried out only for 5' overhangs. The reaction was carried out according to specified conditions in the presence of all four deoxy nucleotide triphosphates.

Vector DNA digested with single restriction enzyme was dephosphorylated (i.e. the 5' phosphate group removed) with calf intestinal phosphatase to reduce self-ligation of the vector during cloning. The reaction was carried out according to the specifications of the manufacturer.
AGAROSE GEL ELECTROPHORESIS

DNA samples were electrophoresed in 0.8% to 2% horizontal agarose gels in 1X TBE buffer. Agarose gel was prepared in 1X TBE buffer (89 mM Tris, 89 mM boric acid and 2.5 mM EDTA, pH 8.3) by boiling. It was cooled to 65°C and ethidium bromide was added to a final concentration of 0.5 µg/ml. The gel was then prepared by immediately pouring the agarose solution in a cast with a comb fixed onto it and allowed to solidify. The comb was removed after the gel solidified forming slots or wells in its place. The DNA samples were then electrophoresed. Electrophoresis was usually performed at 3-4 V/cm. The bands were visualized on an UV transilluminator (Fotodyne Inc.). The gel was photographed with a Polaroid MP4 camera using Polaroid type 665 (positive/negative) films or with UVP gel documentation system using thermal paper.

ELUTION OF A DNA FRAGMENT FROM LOW MELTING AGAROSE GEL

After the digestion of plasmid or genomic DNA, the digested DNA samples were electrophoresed on a 0.8% agarose gel. Gel piece(s) containing the DNA fragment to be eluted were cut under long wavelength UV and transferred to a microfuge tube. The tube was then incubated at 65°C till the gel piece had melted completely and 0.1 volume of 5M NaCl was added to the molten gel piece. The tube was incubated at 65°C again for 15 minutes after mixing thoroughly. The agarose was then removed by extracting with equal volume of phenol saturated with 100 mM Tris-Cl (pH 8.0). After centrifugation the upper aqueous layer was transferred to a fresh tube and extracted once with PCI mix (Tris-saturated Phenol mixed with
distilled chloroform and isoamyl alcohol in the ratio 25:24:1) followed by one extraction with chloroform-isoamyl alcohol mix (distilled chloroform and isoamyl alcohol in the ratio 24:1). Two volumes of ethanol were then added to the aqueous layer and the DNA was precipitated at \(-70^\circ C\) for 30 minutes. The DNA was then processed as before.

ELUTION OF DNA FRAGMENT FROM ACRYLAMIDE GEL

DNA fragments of smaller size (less than 200 bp) were eluted from acrylamide gels. Digested DNA samples were electrophoresed on a 5% acrylamide gel (acrylamide: bisacrylamide :: 29:1) in 1X TBE buffer at 10 V/cm at room temperature in a vertical gel apparatus (Atto corporation, Japan). Gel piece(s) containing the DNA fragment to be eluted were cut under long wavelength UV and transferred to a microfuge tube. The piece(s) were crushed to finer particles and elution buffer (ammonium acetate 500 mM; magnesium acetate 10 mM; SDS 0.1%; EDTA 1 mM) was added to the microfuge tube. It was then incubated overnight at 37°C followed by centrifugation at 3000 rpm for 5 minutes in a microfuge. To the supernatant thus collected, two volumes of ethanol was added, mixed well and the DNA was precipitated at \(-70^\circ C\) for 30 minutes. The DNA was sedimented by centrifugation at 12000 rpm at 4°C for 15 minutes. The pellet was then washed with 70% ethanol, dried and dissolved in water or Tris-

LIGATION OF DNA FRAGMENTS

The digested DNA fragments were mixed with the vector DNA having compatible ends. Generally vector DNA was used with 3-4 molar excess of insert DNA. Upto 1 µg of DNA was ligated in a reaction mixture containing 50 mM Tris-
Cl, pH 8.0, 10 mM MgCl$_2$, 1 mM ATP and 10 mM DTT. T4 DNA ligase (one unit per µg of DNA) was added and the reaction volume was made up to 20 µl. The reaction was carried out at 14°C for 10-16 hours. Ligated DNA was then used for transformation of *E.coli* or *A. vinelandii* as required.

For blunt end ligations, 5 fold excess of ligase and higher concentrations (1µg in 10µl of volume) of DNA were used.

**ISOLATION OF GENOMIC DNA**

Generally, genomic DNA was isolated from overnight grown or stationary phase 10 ml cultures. Cells were sedimented at 5000 rpm for 10 minutes at room temperature in a microfuge. The cell pellet was suspended in 1.5 ml of T$_{10}$E$_1$, followed by addition of 200 µl of 10% SDS and 150 µg of proteinase K. The sample was mixed thoroughly but gently inverting the tube several times. The solution was then incubated at 37°C for 16-18 hrs, after which the lysate was extracted once with PCI mix (Tris saturated Phenol mixed with distilled chloroform and isoamyl alcohol in the ratio 25:24:1) and once with chloroform-isoamyl alcohol mix (distilled chloroform and isoamyl alcohol in the ratio 24:1).

Proteinase K (100 µg) was again added to the supernatant solution and incubated at 37°C for 4-5 hrs. Extraction with phenol-chloroform and chloroform was repeated, after which 0.1 volume 3M sodium acetate (pH 5.2) and 3 volumes of ethanol were added to the supernatant. The DNA was then centrifuged down or spooled out with a curved capillary, washed with 70% ethanol, dried in air and left overnight for dissolving in T$_{10}$E$_1$ at 4°C.
Alternatively, following the chloroform extraction after the first proteinase K digestion, a CsCl density gradient centrifugation was performed. CsCl was added to the supernatant solution at a concentration of 1 mg/ml and dissolved gently. Ethidium bromide (100 µg/ml) was mixed with the DNA solution and it was filled into quick seal tubes. The centrifugation was done at 45000 rpm for 16-18 hours at 15°C in a Vti90 rotor in a L-7 Beckman centrifuge. The chromosomal DNA was visualized with long wavelength UV light from a hand monitor. A hole was made near the top of the tube and below the band with a needle. The DNA was allowed to flow out through the needle into a 5ml screw-cap tube. Ethidium bromide was removed from the DNA solution by several extractions with equal volumes of butanol saturated with water. The DNA was precipitated twice after dilution, as discussed in plasmid DNA isolation. Next, it was spooled out or centrifuged down, washed with 70% ethanol, dried and dissolved in sterile water.

PREPARATION OF COMPETENT CELLS

Competent E.coli cells were prepared following a protocol of Hanahan (1983). A single isolated colony of E.coli was inoculated in 10 ml of LB medium containing nalidixic acid and incubated on a rotary shaker at 37°C. One percent inoculum from this culture was added to a 100 ml LB medium and was incubated at 37°C on a shaker rotating at 200 rpm. When the O.D.600 attained a value of 0.3-0.4, the cells were chilled at 4°C for one hour. The cell culture was transferred aseptically into a sterile, pre-chilled GS3 centrifuge bottle and the cells were harvested by centrifuging at 5000 rpm for 10 minutes at 4°C in a Beckman JA10 rotor. The supernatant was discarded and the cell pellet was resuspended in 30ml of 0.1M MgCl₂ and centrifuged immediately at 5000 rpm for 10 minutes at 4°C in a
Beckman JA20 rotor. The cell pellet was resuspended in 25 ml of 0.1M CaCl₂ and incubated at 4°C for 30 minutes. The cells were pelleted as described earlier and resuspended in 4 ml of 0.1M CaCl₂ and incubated on ice for 10-12 hours. Glycerol was then added to the cells to a final concentration of 15%-20% and the cells were stored in 1.5 ml microfuge tubes at 70°C.

**TRANSFORMATION OF E.COLI**

About 50-200 ng of DNA was used for transformation of *E.coli* using plasmid DNA or ligated DNA. Competent cells were added to prechilled microfuge tube containing DNA sample in volume not more than 100 µl. The contents were gently mixed and incubated on ice for 30 minute following which a heat shock was given at 42°C for 3 minutes. The tube was then transferred to ice immediately and 1 ml LB medium was added to the transformed cells and incubated at 37°C for one hour. Cells were then plated on appropriate selection medium.

**TRANSFORMATION OF A. VINELANDII**

The *A. vinelandii* strain to be transformed was streaked on a BNF plate lacking Fe⁺⁺ and Mo⁺⁺ and incubated for two days at 30°C. The cells were picked up from the BNF plate (5 or 6 colonies) and inoculated into about 70 ml of BNF liquid medium lacking Fe⁺⁺ and Mo⁺⁺. The cells were grown at 30°C with shaking at 170 rpm for about 20 hours. 200 µl of cells were taken in a 5ml screw cap tube and to this 1 µg of DNA, 200 µl of 20 mM MOPS (pH 7.0) and 32 mM of magnesium ions (MgCl₂: MgSO₄: 1:1) were added. The tube containing the mixture was incubated at 30°C for 30 minutes. One ml of BNF medium was then added to the mixture and incubated at 30°C for 12-16 hours. The cells were pelleted and washed with one ml
of BNF medium. The cells were finally resuspended in 300 μl of BNF medium and plated on selection plates.

ELECTROPORATION OF *A. vinelandii* CELLS

*Azotobacter* strain to be electroporated was streaked out on BNF plate and incubated at 30°C for two days. Several loopfuls of cells were picked up and resuspended in 1.5 ml of cold sterile water in a microfuge tube. Cells were then sedimented down in a microfuge for one minute in the cold. The supernatant was discarded and the cells were again resuspended in one ml of cold water. The cells were washed with cold water 3-4 times. The cells were kept on ice all along and the washings performed quickly to minimize lysis of the cells. The cells were finally resuspended in an appropriate volume of (50-100 μl) of chilled water, to make final density of 10^{10} cells/ml. A cell sample of 40 μl was mixed with approximately 250 ng of DNA in a microfuge tube and transferred to a chilled sterile electroporation cuvette. The cuvette was placed in the cuvette holder of Bio-Rad Gene Pulsar transfection apparatus and subjected to a pulse given at 2.5 KV, 25 μF, 800 ohms. About 150μl of BNF-LB (containing 2% LB) was added to these cells immediately and then spotted on a BNF-LB plate. The plate was incubated at 30°C for 12-16 hrs. After which the cells were scraped off and resuspended in one ml BNF. 100 μl of the cell suspension was then plated on a selection plate. Plates were incubated at 30°C until the transformants appeared.

UV STREAK TEST

A single isolated colony of *A. vinelandii* was inoculated in 10ml of BNF medium and incubated at 30°C on a rotary shaker. The culture was streaked in
straight lines on a BNF plate. The BNF plate was exposed to ultra-violet (UV) light for a time period of zero second to forty seconds. The experiment was carried out in dark. The plate was covered with aluminium foil (taking care not to expose the plate to light at any time after UV exposure) and incubated at 30°C for 2-3 days. For UV exposure, the distance from the prewarmed UV lamp emitting at a dose of 3.5 J/m²/second was maintained at 25 cm.

GROWTH CURVE

For plotting the growth curve, late exponential culture was used to inoculate fresh medium. The inoculum was used at one percent. The shake flask cultures were allowed to grow at 30°C and at 175 rpm. OD₆₀₀ was measured at various time points and ln OD was plotted against time.

SOUTHERN BLOTTING

(A). Analysis of DNA by hybridization to specific probes requires that capillary action transfers the DNA onto nitrocellulose. Southern analysis involves hybridization of specific probes to DNA bound to nitrocellulose membrane (Southern, 1975).

DNA to be probed was electrophoresed on 0.8% agarose gel and transferred to nitrocellulose membrane. The detailed method was as follows:

The gel was photographed after electrophoresis of DNA, prior to any treatment. The gel was then soaked in 0.2 N HCl with constant, gentle agitation in an enameled tray for 20 minutes to allow partial depurination of the DNA. The
acid solution was decanted and the gel was rinsed for 5 minutes in distilled water. After rinsing, the gel was soaked in denaturing solution (0.5N NaOH, 1.5M NaCl) with continuous shaking for 45 minutes. Subsequently, the denaturing solution was discarded and gel was rinsed with distilled water again. Finally, the gel was neutralized by soaking in neutralization solution (1M Tris-Cl, pH7.4, 1.5 M NaCl) for 45 minutes. Meanwhile, a nitrocellulose membrane sheet and 3 pieces of 3MM Whatman paper cut to the same size as the gel were made ready. A wick of 3MM Whatman paper slightly larger than the gel and with overhangs to dip into the transfer buffer was also kept ready. The wick was wetted with 20X SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0), the transfer buffer and placed on a Perspex sheet with its ends dipping into 20X SSC in a tray. The gel was then placed on the wick. The nitrocellulose sheet was wetted with sterile water and then placed carefully over the gel. Strips of Saran wrap were cut out and put on the edges of the gel to avoid short-circuiting. The nitrocellulose membrane was covered with a sheet of wet Whatman (3MM) paper, followed by 2 sheets of dry Whatman (3MM) paper. About 2 inch high layer of blotting sheets, cut to the size of the gel was placed over the 3MM Whatman papers. Another Perspex plate was placed above the stack of blotting sheets and a weight was placed to impart uniform pressure and the arrangement was left undisturbed for 14-16 hours to allow complete DNA transfer. The blotting sheets and Whatman sheets were then carefully removed. The top left hand corner of the nitrocellulose blot was cut to mark its orientation and the positions of the sample slots were marked.

The blot was lifted and placed, DNA side up on a 3MM Whatman paper and crosslinked by UV in a Stratalinker in automode. The dried blot was either stored between Whatman sheets in cold or used immediately for hybridization.
(B). RADIOACTIVE LABELING OF DNA BY RANDOM PRIMING

Radiolabelling of DNA probe was carried out following a protocol of Fienberg and Vogelstein, (1983). Briefly, 50 ng of probe DNA was denatured by boiling for 5 minutes followed by instant chilling in ice bath for 2 minutes. The denatured DNA was then labeled in a reaction volume of 30 µl. The reaction was carried out in random priming buffer containing 2.5 µg of primers, 50 mM Tris-Cl, 5 mM MgCl₂, 10mM β- mercaptoethanol and 40 µg/ml of BSA. A final concentration of 20 µM of each dNTP was also added except dCTP for which about 40 µCi of its radioactive form (α-32P dCTP) was used. Klenow fragment of DNA polymerase I enzyme (4 units) was then added to allow extension. The reaction mix was incubated at 37°C for 2 hours and the reaction was stopped using stop buffer containing 250 µg/ml of Calf Thymus DNA, 2 mM EDTA and 1% SDS. The probe was precipitated with 3 volumes of ethanol after adding 2.5M ammonium acetate or alternatively the probes were purified using sephadex G-25 spin columns to remove unincorporated nucleotides. The desirable specific activity of the probe used was between 5x10⁸ to 2x10⁹ cpm/µg of DNA. The radioactivity was measured in a Beckman liquid scintillation spectrometer.

(C). HYBRIDIZATION OF DNA ON NITROCELLULOSE MEMBRANES

The nitrocellulose membrane was placed in hybridization bottles and wetted with sterile double distilled water. 10 ml of prehybridization solution [one ml 50X Denhardt's solution (5% Ficoll, 5% polyvinylpyrrolidone, 5% BSA), 3 ml of 20x SSC and 6ml sterile water] was added to the bottle and incubated in a Bachofer hybridization oven at 65°C for at least 3-4 hours. Prehybridization solution was decanted off and hybridization was set. Hybridization was generally carried out in a
5ml of hybridization solution for 16-18 hours at 65°C. 3ml of hybridization solution containing 0.25 ml of 10% SDS, 0.2 ml of 5 mg/ml calf thymus DNA, 2.5 ml of sterile water and probe (1x10⁶-2x10⁶ cpm/ml of hybridization solution) was boiled in a water bath for 10 minutes. To this 2 ml of solution containing 1.5 ml of 20X SSC and 0.5 ml of 50X Denhardt solution was added and incubated with the nitrocellulose membrane overnight at 65°C.

(D). WASHING OF THE BLOT

After hybridization, the solution was discarded. The blot was then washed with 50-100 ml of solution I (50X Denhardt, 0.5% SDS and 5X SSC) for 30 minutes followed by washing with the same volume of solution II (0.5% SDS, 1X SSC) for one hour. After 2 washes with solution II in a bottle, the blot was removed and washed with an excess of solution II in a tray. The nitrocellulose membrane was air dried partially and then fixed on a 3MM Whatman paper cut to the same size and covered with Saran wrap. The membrane was then fixed up in an X-ray cassette with intensifying screen and exposed to Kodak X-ray film at -70°C.

(E). REMOVAL OF THE PROBE FROM MEMBRANE

When it was necessary to repeat a probing, an already used nitrocellulose was stripped off of its probe (for this the nitrocellulose membranes were never dried completely prior to autoradiography). The semi dry membrane was washed with boiling solution of 0.05X SSC and 0.01M EDTA (pH 8.0). SDS (0.01%) was also added to this after boiling. Several such washes were carried out till no
detectable count was present. Finally the nitrocellulose membrane was rinsed with 0.01X SSC and used for hybridization.

OLIGONUCLEOTIDE SYNTHESIS AND PURIFICATION

The phosphoramidite method of oligonucleotide synthesis (Gait, 1984) was used in the laboratory. Synthesis was carried out in an Applied Biosystems DNA Synthesizer model 391. After synthesis was over, the crude mixture of oligonucleotides was extracted from solid support with 3 ml of fresh concentrated ammonium hydroxide. The extract was incubated at 55°C for 16-18 hours in a heating block. This resulted in deprotection of the bases and phosphate groups. Generally, the dimethoxytrityl (DMT) group was not retained on the 5' hydroxyl end. The oligonucleotide was then eluted from a polyacrylamide gel and purified through a Sep-pak column. The deprotected oligonucleotide was dried in a Savant Speed-vac concentrator and dissolved in 0.5 ml of sterile water. Approximately 5 O.D. 260 material was mixed with an equal volume of deionized formamide and electrophoresed on a 12-15% denaturing polyacrylamide gel in 1X TBE buffer in a vertical gel electrophoresis apparatus (Hoefer, USA) at 8V/cm at room temperature. An aliquot of tracking dye was also loaded into a side well to monitor the extent of electrophoretic run. The gel was removed from between the plates and either stained with ethidium bromide (1µg/ml for 20-30 minutes) or the oligonucleotide was visualized directly under ultraviolet light by using a UV shadowing membrane. The major visible band was excised and the acrylamide piece was crushed finely into a 5 ml solution of 1M sodium chloride and 10 mM EDTA (pH 8.0). The tube was left overnight on a rotary shaker at 37°C. The solution was transferred to Eppendorf tubes and centrifuged at maximum speed for 5 minutes in a microfuge to remove the debris. The supernatant solution was
recovered and passed through a Sep-pak column fitted with a 10 ml syringe and activated with 5 ml methanol. The methanol was then washed off with 10 ml of sterile water and the oligonucleotide loaded onto the column thrice over. The column was again flushed with 10 ml of sterile water to remove urea. The oligonucleotide was eluted with 3 ml of 70% methanol. The solution was evaporated to dryness, the oligonucleotide was resuspended in 100ul of water and its concentration was determined spectrophotometrically using the equation

\[
\mu \text{ moles/ml} = \frac{\text{O.D.}_{260}}{10 \times \text{length of the oligonucleotide}}
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**END LABELING OF A LINEAR DNA DUPLEX**

The DNA was first isolated from the plasmid it was already cloned in. The required amount of plasmid DNA was digested with appropriate restriction endonuclease. The fragment released was purified on a 5% acrylamide gel. About 50 ng of this DNA fragment was then 3'end labeled in a reaction mixture containing 10-20 μCi (specific activity 4000 ci/mmol) of [α-³²P] dCTP, 200 μM of the remaining unlabeled dNTPs, 1X Klenow buffer and 1U of Klenow fragment of DNA polymerase I. The reaction mixture was incubated at 30°C for 30 minutes and chased with unlabeled dNTPs for another 10 minutes at 30°C. The labeled DNA was extracted once with PCI mixture followed by purification using Sephadex G 25-50 spin column.
KINASING OF A LINEAR DNA DUPLEX

As described earlier, the DNA was first isolated from the plasmid it was already cloned in. The required amount of plasmid DNA was digested with an appropriate restriction endonuclease. The fragment released was purified and about 50 ng of it was then labeled in a reaction mixture containing 50 μCi (specific activity 5000 Ci/mmole) of [γ-32P] ATP, 1X Kinase buffer and 30 U of Polynucleotide kinase enzyme. The reaction mixture was incubated at 37°C for 30 minutes. The labeled DNA fragment was extracted once with PCI mixture followed by purification using Sephadex G25-50 spin column.

* In case of oligonucleotides, about 10 pmoles was labeled in one kinasing reaction.

PURIFICATION OF DNA BINDING PROTEINS

Preparation of cell extracts from Klebsiella pneumoniae

*Klebsiella pneumoniae* cells were grown as batch cultures in 1.5 litres LB medium aerobically till OD₆₀₀ reached about 1.0. The cells were harvested by centrifuging in Beckman JA10 rotor at 5000 rpm for 10 minutes at 4°C. The cell pellet was washed with 0.8% saline and subsequently resuspended in pre-chilled lysis buffer (50 mM NaCl, 50 mM Tris-Cl (pH 7.4), 5 mM EDTA, 2 mM DTT, 0.1 mM PMSF and 10% sucrose). Lysozyme was added to the suspension at a concentration of 2mg/ml and the suspension was incubated on ice for another 20 minutes. Subsequently Brij-58 (0.6% v/v) was added and the cell suspension was allowed to remain on ice for 15 minutes. Further, cell lysis was carried out by rapid freeze thawing. For this, cell suspension was first frozen (by plunging) in
liquid nitrogen, followed by 10 minutes incubation at -70°C. The frozen cell suspension was subjected to a quick thawing in running water till it was half thawed. This half thawed suspension was then allowed to thaw completely on ice. This rapid freeze thawing procedure was repeated 3-4 times. The lysate so obtained was finally sonicated at an amplitude of 14-18 microns for 20 seconds per burst (with a gap of two minutes between each burst), till it was homogenous. The cell lysate was then centrifuged at 12000 rpm for 30 minutes at 4°C in a Beckman JA20 rotor. Supernatant thus obtained, referred to as crude extract was aliquoted and stored at -70°C for further use. Crude extract was fractionated by ammonium sulphate precipitation. Ammonium sulphate solution (100%) was added to the crude extract to a final concentration (v/v) of 70%. The solution was centrifuged at 8000 rpm for 10 minutes in a Beckman JA20 rotor and the pellet obtained was discarded. The supernatant was taken and ammonium sulphate solution (100%) was added to a final concentration (v/v) of 90%. The solution was recentrifuged at 10000 rpm for about 30 minutes in a Beckman JA20 rotor. The pellet obtained was very fragile and therefore the supernatant was removed carefully using a pasteur pipette. This 70-90% saturation step was repeated twice. The pellet was dissolved in buffer A (50 mm Tris-Cl (pH 7.4), 1 mM EDTA, 0.1 mM PMSF, 10% Glycerol, .014 M β-Mercaptoethanol) and then dialyzed against the same buffer containing 50 mM NaCl.

**Purification of HU and H-NS like proteins**

HU and H-NS like proteins were purified from *Klebsiella pneumoniae*. They were separated on the basis of their difference in pi (pi or isoelectric pH for *E.coli* HU is 8.75 and for H-NS it is 7.5). About 20ml of crude extract containing approximately 20 mg of total protein was loaded on a 1ml S-Sepharose cartridge.
(Biorad, USA) equilibrated in buffer A (50 mm Tris-Cl (pH 7.4), 1 mM EDTA, 0.1 mM PMSF, 10% Glycerol, .014 M β-Mercaptoethanol) containing 50 mM NaCl. The flow rate was maintained at 40 ml/hr. The column was washed with approximately 20 column volumes of buffer A containing 50 mM NaCl. The bound proteins were eluted by applying a linear gradient of NaCl (50 mM to 1000 mM) in buffer A (20 ml). A total of 20 fractions (1ml each) were collected and assayed for their DNA binding activity.

**ANALYSIS OF PROTEINS ON SDS-PAGE**

Protein samples were regularly separated on SDS-PAGE following the method of Laemmli (1970). Before pouring the gel, the glass plates were cleaned and assembled according to the manufacturer’s specifications (Atto, Japan). For separating gel, the appropriate amount of acrylamide stock solution was diluted in 1X separating gel buffer. Polymerization was initiated by adding ammonium persulphate (APS) and TEMED and the solution was poured slowly between the assembled plates to avoid formation of air bubbles. The gel was then layered with water-saturated butanol to hasten the polymerization. After polymerization butanol was carefully removed from the top and the stacking gel was overlaid on it.

The protein samples were boiled in 1X sample buffer (125 mM Tris-Cl, pH 7.4, 1% SDS, 5% β-Mercaptoethanol, and 10% glycerol with 0.001% w/v bromophenol blue). They were subjected to electrophoresis on the denaturing gel (composed of a 4% stacking gel and 15% separating gel both containing Tris-Cl and SDS as described below) at 120V, in Tris-glycine-SDS (0.6% Tris-base, 0.1% SDS, 2.88% glycine) buffer till the bromophenol blue dye reached the bottom of
the gel. The gel was stained with silver nitrate for visualization of the protein bands.

**Composition of separating gel:**

<table>
<thead>
<tr>
<th>Components</th>
<th>Volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide stock (30%)</td>
<td>15ml</td>
</tr>
<tr>
<td>4X separating gel buffer (1.5M Tris-Cl, 0.4% SDS pH adjusted to 8.8)</td>
<td>7.5ml</td>
</tr>
<tr>
<td>APS (10%)</td>
<td>300μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>20μl</td>
</tr>
<tr>
<td>Water</td>
<td>7.5ml</td>
</tr>
</tbody>
</table>

**Composition of stacking gel:**

<table>
<thead>
<tr>
<th>Components</th>
<th>Volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide stock (30%)</td>
<td>1.35ml</td>
</tr>
<tr>
<td>4X stacking gel buffer (0.5M Tris-Cl, 0.4% SDS at pH 6.8)</td>
<td>2.5ml</td>
</tr>
<tr>
<td>APS (10%)</td>
<td>100μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>20μl</td>
</tr>
<tr>
<td>Water</td>
<td>6.2ml</td>
</tr>
</tbody>
</table>
STAINING WITH SILVER NITRATE

Once the electrophoresis was over, the gel was soaked in 100 ml of fixing solution (50% methanol, 12% acetic acid, and 0.05% formic acid) overnight and left on a shaker. The gel was then subjected to dehydration in 50% ethanol solution for 30 minutes with one change in between. This was followed by sensitization with 0.012% sodium thiosulphate solution for one minute. The gel was rinsed briefly in water and soaked in 0.2% silver nitrate solution containing 0.03% formaldehyde for 20 minutes. The gel was again briefly washed in water and developed in a solution containing 3% sodium carbonate, 0.03% formaldehyde and 0.013% sodium thiosulphate and then fixed in a fixing solution as in the first step.

PROTEIN ESTIMATION

Quantification of proteins was carried out by Bradford’s method (1976). Protein samples were mixed with 5 ml of Bradford reagent (Coomassie brilliant blue G-250 in ethanol and phosphoric acid) and left untouched for 5 minutes. Change in the optical density was recorded at 595 nm. Standard curve was plotted using bovine serum albumin as the standard (Fig 3.1).

WESTERN BLOTTING

For Western blotting, purified protein was separated on 15% SDS-PAGE and electrophotted overnight on a nitrocellulose membrane using wet transfer apparatus. For wet transfers, one sheet of nitrocellulose membrane and three sheets of 3MM Whatman paper were cut to the same size as that of the gel. The
Fig 3.1 Standard curve was plotted using Bovine serum albumin as the standard. Known amounts of Bovine serum albumin were mixed with Bradford reagent and optical density was measured at 595nm.
nitrocellulose membrane was first soaked in distilled water followed by soaking in transfer buffer (1.4% glycine, 0.3% Tris-Cl, pH 7.4, 20% Methanol). The Whatman sheets were soaked in transfer buffer. The gel and nitrocellulose membrane sandwich was supported by Whatman sheets and the assembly was then transferred to a transfer apparatus placed in a tank which had platinum wire electrodes. The tank was filled with transfer buffer and electroblotted.

After the transfer was complete, the membrane was removed from transfer apparatus and soaked in TBS (Tris Buffered Saline; 150 mM NaCl and 10 mM Tris-Cl, pH 7.4) with 3% BSA for one hour at 37°C. The tray containing the TBS was placed on a rotary shaker all the time. The membrane was incubated with anti HU and anti H-NS antibodies at a dilution of 1:1000 in TBST (Tris buffered saline containing 1% BSA and 0.05% tween 20) for about 3-4 hours. The membrane was washed with TBST 3-4 times. Alkaline phosphatase linked to anti-rabbit IgG (sigma Chemical Company) was used as secondary antibody at a dilution of 1:30000 in TBS with 1% BSA and incubated for an hour. The blot was again washed with TBST. The blot was developed with BCIP/NBT (5-Bromo-4-chloro-3-indolyl-phosphate/4-nitroblue tetrazolium chloride) solution till a purple blue colour appeared. The blot was washed with water, dried and stored.

FORMATION OF DNA PROTEIN COMPLEX (S) AND ANALYSIS

In order to analyze DNA-protein interactions, DNA-protein binding reactions were carried out. A typical binding reaction contained 0.5 ng of radiolabelled DNA and varying amounts of protein fractions in 1X binding buffer (50 mM Tris-Cl, pH 7.4, 100 mM KCl, 1mM EDTA, 2 mM DTT, 5 mM MgCl₂, and 6% glycerol). The binding mixture was incubated at 37°C for 10 minutes. The DNA-protein complexes were then analyzed by electrophoresis in a 5% non-
denaturing polyacrylamide gel (acrylamide: bisacrylamide:: 29:1) in 1X TBE buffer for 1.5 hours at 10V/cm at room temperature in a vertical gel electrophoresis apparatus (Hoefer, USA). The gel was then transferred to a 3MM Whatman paper, dried under vacuum at 80°C for about an hour and exposed to Kodak XK-5 X-ray film at -70°C.

**DNA SEQUENCING (Sanger et al., 1977)**

DNA sequencing was done with M13 mp18, M13 mp19 or pUC plasmid derivatives according to the dideoxy chain termination reaction. The DNA samples were sequenced using Sequenase version 2.0 sequencing kit (United States Biochemicals).

Denaturation: In case of Double stranded plasmid, DNA (3μg) was taken in a volume of 20 μl and denatured with 2 μl of NaOH and 2 mM EDTA. After keeping for 5 minutes at room temperature, the denatured DNA was chilled in ice. The reaction mixture was neutralized with 8 μl of 1M Tris-Cl (pH 4.8) and the DNA precipitated with 9 μl of 1M sodium acetate (pH 5.5) and 95 μl of ethanol. The precipitated DNA was kept on ice for 15 minutes, followed by centrifugation at room temperature for 15 minutes. The DNA pellet was washed with 1 ml of 70% ethanol and centrifuged again at room temperature for 15 minutes and air dried for 5 minutes.

Annealing: The denatured DNA pellet was dissolved in 7 μl of sterile water and the reaction mixture was made up to 10 μl with 2 μl of sequencing buffer (200 mM Tris-Cl (pH 7.5), 100 mM MgCl₂ and 250 mM NaCl) and one pmole of end labeled primer (1 pmole/μl). Annealing was done in a 37°C water bath for 30 minutes. The DNA was then chilled on ice. For single stranded DNA, sample DNA was mixed with 0.5 pmole of primer and 5X annealing buffer in a
reaction volume of 10 µl and heated at 65°C for 5 minutes and cooled slowly to < 35°C over a period of 30-40 minutes.

Master-mixture preparation: Meanwhile, a master-mixture comprising 2 µl of GTP-labeling mixture (7.5µM dGTP, 7.5µM dCTP, 7.5µM dTTP), 1 µl of 0.1M DTT and 0.5 µl of Sequenase in 1.5 µl of enzyme dilution buffer (10 mM Tris-Cl pH 7.5, 5 mM DTT and 0.5 mg/ml BSA) was prepared.

5.5 µl of master mixture was added to the annealed DNA, centrifuged for a few seconds and DNA extension was allowed at room temperature for 10 minutes.

2.5 µl of each dideoxy nucleotide was dispensed into separate Eppendorf tubes and prewarmed at 37°C. 3.5 µl of the DNA extension reaction was added to each dideoxy nucleotide and the termination reaction incubated at 37°C for 5 minutes. 4 µl of stop solution (95% formamide, 20mM EDTA, 0.05% Bromophenol blue, 0.05% Xylene cyanol FF) was added to each reaction. The reaction mixture was stored at −70°C until further use.

**PREPARATION AND ELECTROPHORESIS OF SEQUENCING GELS**

The sequencing reaction products were resolved on a 6% denaturing polyacrylamide gel along with the DNA footprinting reactions.

The glass plates used for pouring the gel were washed thoroughly. The smaller of the two glass plates was siliconized with siliconizing solution (5% solution of dimethyldichlorosilane in toluene). The smaller plate was coated twice with it. Excess of the solution was wiped off with a tissue.

Spacers (0.4 mm) were placed between the plates (siliconized side facing inside) and the sides and bottom of the plates were sealed with an electrical tape. Binder clips were put on three sides of the plates.
A 40% acrylamide stock solution was prepared (38% of acrylamide and 2% of bis-acrylamide) and was deionized by stirring with 2% Amberlite monobed resin for 30 minutes in the dark. The solution was then filtered through Whatman No. 1 paper and stored at 4°C in a dark bottle.

100 ml of 6% acrylamide solution was prepared by mixing 50 g urea, 15 ml of 40% acrylamide solution, 10 ml 10X TBE buffer and 34 ml of sterile water. The solution was filtered through Whatman No. 1 paper. After the addition of 1 ml of 10% ammonium persulphate (APS) and 30 μl of TEMED, acrylamide solution was poured between the sealed plates. A 20 well flat tooth comb was then placed between the plates and clamped. After the gel polymerized, the open end carrying the comb was covered with wet tissue and Saran wrap if the gel was not used for electrophoresis immediately.

When the sequencing gel was to be electrophoresed, the clamps and tape were removed. The comb was withdrawn and the slots were washed well with distilled water. The plates were mounted onto a BRL sequencing gel electrophoresis apparatus model 52 and 1X TBE buffer was poured into the upper and lower tanks. The wells were cleaned thoroughly by flushing them with TBE buffer using a syringe. The gel was connected to a LKB 2197 power supply and pre-run at 2500 volts till the temperature of the plates was between 50-55°C. The temperature was monitored with the help of a PTC model 310C surface thermometer.

Meanwhile, the sequencing reactions and the footprinting reactions stored at -70°C were thawed out and boiled in a water bath for 10 minutes. They were chilled in ice and an aliquot of each was loaded onto the gel, after cleaning the
gel was fixed in a solution containing 10% methanol, 10% acetic acid in a tray and also sprayed very gently with the fixer using a syringe. The gel was then transferred to a 3MM Whatman paper and dried under vacuum for 2 hours at 80°C. It was then exposed to Kodak XK-5 X-ray film at -70°C.

**DNASE I TITRATION AND FOOTPRINTING ANALYSIS**

**DNase I titration**

To determine minimum concentration of DNase I required to produce a single hit per molecule, a titration reaction was carried out as follows. An appropriate one end labeled DNA probe (around 20000 cpm) in 50 μl of 1X binding buffer was probed with different concentrations of DNase I in presence of 1mM Ca++, 5 mM Mg++. The reaction was incubated at 37°C and was stopped by adding equal volume of stop buffer (20 mM EDTA and 1% SDS) at different time intervals. The digested DNA was then extracted once with phenol-chloroform-isoamyl alcohol mix and precipitated with ethanol and 0.3M Sodium acetate was also added. DNA pellet obtained upon centrifugation was washed once with 70% ethanol, vacuum dried and dissolved in minimum volume of formamide loading dye (98% deionized formamide, 10 mM EDTA, pH 8.0, 0.025% bromophenol blue). The loading mix was boiled for 90 seconds, chilled on ice and loaded on to a 6% urea-polyacrylamide sequencing gel. After completion of the electrophoresis, the gel was dried and autoradiographed. Minimum DNase I concentration required was determined from the pattern on the autoradiogram and was found to be 0.5 U/ml.
DNase I footprinting

DNA-protein complexes were formed as discussed earlier (only the reaction was scaled up to 50 μl) and probed with 0.5 U/ml of DNase I (Promega, USA) for 90 seconds at 37°C. The reaction was terminated by adding an equal volume of stop buffer (20 mM EDTA, 1% SDS). Reaction mixture was then extracted once with PCI mix followed by ethanol precipitation. The pellet obtained after centrifugation was washed with 70% ethanol 3-4 times and dried. It was then dissolved in 20 μl sterile water. For loading, equal counts was taken for each sample, dried under vacuum and dissolved in minimum volume of loading dye. The samples were then boiled and electrophoresed on a 6% urea-polyacrylamide sequencing gel. The gel was dried and autoradiographed.
### Bacterial Strains and Plasmids

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant Genotype or Phenotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia Coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F&lt;sup&gt;+&lt;/sup&gt; endA&lt;sup&gt;1&lt;/sup&gt; hsdR&lt;sup&gt;17&lt;/sup&gt; (r&lt;sup&gt;+&lt;/sup&gt; k m&lt;sup&gt;+&lt;/sup&gt; k) supE&lt;sup&gt;44&lt;/sup&gt; thia-1 recA&lt;sup&gt;1&lt;/sup&gt; gyrA (na1&lt;sup&gt;+&lt;/sup&gt;) relA&lt;sup&gt;1&lt;/sup&gt; Δ(lacIZYA-argF) U169 deoR</td>
<td>Woodcock et al. (1989)</td>
</tr>
<tr>
<td>HB101</td>
<td>F&lt;sup&gt;+&lt;/sup&gt;(gpt-proA) 62 leuB6 supE&lt;sup&gt;44&lt;/sup&gt; ara-14 galK2 lacY1 Δ (mcrC-mrr) rpsL20 (stt&lt;sup&gt;+&lt;/sup&gt;) xyl-5 mtl-1 recA&lt;sup&gt;13&lt;/sup&gt;</td>
<td>Boyer and Roulland Dussoix (1969)</td>
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<tr>
<td><strong>Azotobacter vinelandii</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UW</td>
<td>non-gummy derivative of wild-type strain OP</td>
<td>Bishop, and Brill, (1977)</td>
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<td>VK21</td>
<td>rif&lt;sup&gt;+&lt;/sup&gt;, recA deletion mutant of UW</td>
<td>Venkatesh et al. (1990)</td>
</tr>
<tr>
<td>UW (pNK862)</td>
<td>Transposon mini Tn10 is present at undetermined chromosomal locations, Kan&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>UW(pUC4-KAPA)</td>
<td>UW transformed with a Sau 3A I partial digest of UW DNA ligated to kanamycin resistance gene cartridge from pUC4 – KAPA, Kan&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
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<td>RS1 – RS4</td>
<td>UW with transposon mini Tn10 at an undetermined chromosomal location and also a Tetracycline interposon present at the nifHDK gene, Kan&lt;sup&gt;+&lt;/sup&gt;, Tet&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>HB14</td>
<td>recF deletion mutant of UW, Tet&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Badran et al. (1990)</td>
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</table>
**Klebsiella pneumoniae**

<table>
<thead>
<tr>
<th>M5a1</th>
<th>Wild type</th>
</tr>
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<tbody>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
</tr>
<tr>
<td>pUC18</td>
<td>Amp'</td>
</tr>
<tr>
<td>pBR322</td>
<td>Tet', Amp'</td>
</tr>
<tr>
<td>pUC4-KAPA</td>
<td>Amp', Kan'</td>
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<td>pUK110 ::Ωkm</td>
<td>Interposon Ωkm as a 2.0kb <em>BamHI</em> fragment from pH45ΩKan inserted into the <em>BglII</em> site of pUK110, Cm', Kan'</td>
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<td>pUK121</td>
<td>3.2kb <em>SalI</em> fragment from pUK100 containing part of <em>nifL</em> and part of <em>nifA</em> cloned in pUC 19, Cm'</td>
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<td>pH45Ω Tet</td>
<td>Tet' Amp'</td>
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<td>pADH.1</td>
<td>137 bp <em>EcoRI</em>-<em>BamHI</em> fragment from pAN94 cloned at the <em>XbaI</em> site of pBend2, Amp'</td>
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<td>pNK862</td>
<td>Kan'</td>
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<tr>
<td>pUCD800</td>
<td>Kan'</td>
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<tr>
<td>pRS1</td>
<td>3kb <em>Bam</em> HI –<em>Pst</em> I fragment carrying levan sucrase gene cloned in pUC18, Amp&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td>----------</td>
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<td>pRS2</td>
<td>3kb <em>Sal</em> I fragment carrying partial <em>nifHDK</em> gene of <em>A. vinelandii</em> cloned in pRS1 Amp&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>pRS3</td>
<td>2kb <em>Hind</em> III fragment carrying Tet interposon cloned in pRS2, Amp&lt;sup&gt;f&lt;/sup&gt;, Tet&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td>pRS5-pRS14</td>
<td>Partial Sau3AI genomic digest of <em>A. vinelandii</em> RS1 cloned in pBR322, Kan&lt;sup&gt;f&lt;/sup&gt;</td>
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