A. GENETIC HOMOGENOTIZATION

Mutations in genes involved in nitrogen fixation (nif genes) in A. vinelandii are rather frequent and quite stable despite the presence of identical multiple chromosomes. This could be due to the presence of a very efficient homogenotization process carried out by some uncharacterized non-reciprocal recombination system. The cloning and molecular characterization of the recA and recF genes from A. vinelandii has been reported (Venkatesh et al., 1990, Badran et al., 1995). We therefore decided to study the role of these two genes, if any in the homogenotization process in A. vinelandii.

A. vinelandii strains, wild type (UW), recA mutant VK21 (Venkatesh et al., 1992) and recF mutant HB14 (Badran et al., 1999) were used for the present study. Recombination of a nifLA mutation was analyzed using these strains.

TO STUDY RECOMBINATION OF MUTATION AT THE nif LOCUS IN A. VINELANDII STRAINS

A. vinelandii UW as well as A. vinelandii VK21 and HB14 were transformed with pUK110::Ωkm (Bageshwar, 1995). This construct is a nifLA deletion mutant clone in pUC8, carrying a kanamycin resistance gene inserted at the nifLA locus. In all the strains used, about $10^{10}$ cells were plated to select for transformants on BNF plates containing ammonium acetate (1.1%) and kanamycin (5-15 μg/ml).

From the transformants obtained, about 200 colonies were patched on BNF plates containing ampicillin. The single point crossover recombinants (Amp resistant colonies) were not used for further work. The single point crossover recombinants
often turn out to be \textit{nif} positive and interfere as they would behave as false \textit{nif} positives.

All the double point cross over recombinants (Amp sensitive) obtained were tested for their \textit{nif} characteristics and all of them were found to be \textit{nif} minus. These double point cross over recombinants were further analyzed using Southern hybridization technique.

\textbf{SOUTHERN HYBRIDIZATION}

In order to understand the process of homogenotization in \textit{A. vinelandii} and to explore the involvement of \textit{recA} and \textit{recF} genes in this process, Southern hybridization of the genomic DNA isolated from double cross over recombinants obtained previously was carried out using wild type \textit{nifLA} fragment as a probe.

The logic for performing this experiment was as follows: If the homogenotization process was disrupted in a \textit{recA} minus or in a \textit{recF} minus strain of \textit{A.vinelandii}, then the inactivated \textit{nifLA} locus on \textit{pUK110::\Omega km} would not undergo recombination and hence would not be passed onto the other chromosomes. On the other hand, in the wild-type \textit{A.vinelandii} \textit{UW}, recombination with the active \textit{nifLA} locus on one of the wild type chromosomes would result in rescue of the \textit{nifLA::Kan} onto that chromosome and would then be passed onto all the copies of chromosomes. In that case signals obtained with \textit{nif} mutants in \textit{A.vinelandii} \textit{UW} would be of the mutant type band at 4.6 kb position. In case the recombination and transmission to other copies was disrupted if \textit{recA} or \textit{recF} were involved in this homogenotization process, then there would be a signal at the 3.2 kb position in case of \textit{A.vinelandii} \textit{HB14} and \textit{VK21} \textit{nif} mutants as this function would be disrupted. For this purpose, genomic DNA was isolated from the \textit{nif} mutants of each \textit{A.vinelandii} strain (\textit{UW, HB14, VK21}) that was cultured in presence and in absence of kanamycin. Genomic DNA was digested
with Sal I and the genomic digests were probed with labeled DNA fragment (3.2 kb) carrying wild-type nifLA gene derived from pUK121 (Raina et al., 1993).

The results of the Southern hybridization are shown in Fig 4.1. The signals were obtained at about 4.6 kb fragment position and no signal was obtained at 3.2 kb, where a signal was obtained in case of the genomic DNA sample from A. vinelandii UW. This confirmed that the nifLA mutation was transmitted to all copies of chromosomes in presence and in absence of selection pressure (kanamycin) even in a recA and a recF minus strain of A. vinelandii and that no wild type nifLA gene was left in any chromosome.

OTHER FACTORS INVOLVED IN HOMOGENOTIZATION FUNCTION

The above result indicated that the homogenotization process in A. vinelandii could possibly involve factors other than recA or recF. This could be due to presence of multiple chromosomes in A. vinelandii. It was observed that the mutations were obtained at a greater frequency than would be expected from a bacterium containing 80 copies of chromosomes. This was attributed to a very efficient non-reciprocal recombination system that catalyzed this “homogenotization” process and “transmits” a mutation to all the copies of chromosomes (Manna and Das, 1997). This pathway may involve other factors. Therefore, to look for such factor(s), it was decided to introduce random mutations into the A. vinelandii genome.
Fig 4.1: Southern Hybridization analysis of the Sal I digested genomic DNA of E.coli and different A. vinelandii strains. The \( ^{32} \)P-labeled probe used was the 3.2 kb Sal I fragment from pUK121 containing the whole \( nifL \) and partial \( nifA \) gene. Panel (B) Lane 1, E.coli DH5\( \alpha \); lane 2, A. vinelandii UW; lane 3, the 3.2 kb Sal I fragment from pUK121 containing the \( nifLA \) gene of A. vinelandii; lane 4, the 4.6 kb Sal I fragment from pUK110::\( \Omega \)km containing the \( nifLA \) gene of A. vinelandii with insertion of the 2.0 kb interposon \( \Omega \)km after the deletion in the \( nifLA \); lane 5, A. vinelandii UW with a genomic mutation in \( nifLA \) (0.6 kb deletion + insertion of the 2.0 kb interposon \( \Omega \)km), the strain was cultured in the presence of kanamycin (2 \( \mu \)g ml\(^{-1} \)); lane 6, the same as in lane 5 but the strain was cultured in absence of kanamycin; lane 7, A. vinelandii VK21 (recA deletion mutant) with a genomic mutation in \( nifLA \) as described for lane 5, the strain was cultured in absence of kanamycin (2 \( \mu \)g ml\(^{-1} \)); lane 8, the same as in lane 7 but the strain was cultured in absence of kanamycin; lane 9, A. vinelandii HB14 (recF deletion mutant) with a genomic mutation in \( nifLA \) as described for lane 5, the strain was cultured in the presence of kanamycin (2 \( \mu \)g ml\(^{-1} \)), lane 10, same as lane 9 but the strain was cultured in absence of kanamycin. (A) Agarose gel for the autoradiogram shown in (B).
CONSTRUCTION OF A PLASMID CLONE CARRYING AN IDENTIFIABLE DRUG RESISTANCE MARKER INSERTED IN A KNOWN GENE LOCUS FROM *A. VINELANDII*.

Detection of any disruption in the homogenotization process would require a construct comprising any known gene whose inactivation could be followed. The *nifHDK* genes from *A. vinelandii* were considered suitable for the purpose. A 3 kb *Sal I* fragment comprising a part of the *nifHDK* genes was purified by digesting plasmid pAM50 (Reddy, 1986). This fragment was cloned in pUC18 (Amp resistant) at the *Sal I* site (Fig 4.2b). A 2 kb DNA fragment carrying a tetracycline resistance gene (Interposon) was then cloned at the *Hind III* site within the *nifD* gene (Fig 4.2c). A recombination between inactivated *nifD* on the construct and the wild type *nifD* locus on the random mutant population of *A. vinelandii* would result in a *nif D* mutation on the chromosome that would then be transmitted to all the copies of chromosomes. However, we may also find a population of cells, which would be *nif* positive and at the same time ampicillin resistant (single point cross over recombinants). These colonies, even at a low frequency, would form a large number of transformants and could interfere with the actual experiment, as they would be false positives.

This problem was solved by cloning a conditionally lethal gene (Fig 4.2a), the levan sucrase gene (*sacRB*) of *Bacillus subtilis* (Gay et al., 1985) in the vector (pUC18). The *sacRB* gene encodes for levan sucrase (sucrose: 2,6-β-D-fructan 6-β-D-fructosyltransferase), a 50 kDa enzyme secreted in culture medium after induction by sucrose. The enzyme catalyzes transfructorylation from sucrose to various acceptors, which results in two main physiological reactions: (i) levan synthesis (ii) sucrose hydrolysis. The production of levan sucrase is lethal in the presence of 5% sucrose in agar medium in the case of *E.coli, Rhizobium melliloti*.
Fig. 4.2 (a) Schematic construction of pRS1 (not drawn to scale).
Fig. 4.2 (b) Schematic construction of pRS2 (not drawn to scale)
Fig. 4.2 (c) Schematic construction of pRS3 (not drawn to scale)
and *Agrobacterium*. This allows a positive selection for double point cross over recombinants by plating on a medium containing 5% sucrose. For this, pUCD800 plasmid DNA was digested with *BamHI* (end filled using Klenow fragment of DNA polymerase) and *Pst I* and the resultant fragment containing levan sucrase gene was cloned in pUC18 at the *Hind III* (end filled using Klenow fragment of DNA polymerase) and *Pst I* site. This would prevent survival of any single point cross over recombinants when 5% sucrose is added to the medium.

In order to test whether the levan sucrase gene cloned in pRS3 would function in *A. vinelandii UW*, pRS3 was transformed into *A. vinelandii UW* strain and the transformants obtained were checked for their sensitivity to 5% sucrose. It was found that the double point cross over recombinants (Tet resistant) were resistant to 5% sucrose whereas the single point cross over recombinants (Amp resistant, Tet resistant) where the entire construct (pRS3) was expected to be integrated into the chromosome were found to be sensitive to 5% sucrose. Thus, the *sacRB* gene was functional in *A. vinelandii UW*.

*A. vinelandii UW* was then transformed with pRS3 to determine whether the tetracycline resistance marker could be rescued onto the *A. vinelandii* chromosome by this procedure. The selection medium contained 5% sucrose. If there were any single point cross over recombinants where the complete construct was integrated into the chromosome, they would be Amp and Tet resistant [due to drug markers contributed by Tet interposon and the vector (pUC18) which is Amp resistant] and would be eliminated in presence of 5% sucrose. All the transformants were found to be *nif* minus and resistant to tetracycline and sensitive to ampicillin (double point crossover recombinants). Southern hybridization results further confirmed the recombination between inactive *nifHDK* on pRS3 and the *nifHDK* locus on the chromosome. The genomic DNA was digested with *Sal I* and hybridized with *nifHDK* fragment, which was used as a probe (Fig 4.3). In case of wild type *nif HDK* locus, the signal was obtained at 3
Fig 4.3: Southern Hybridization analysis of the Sal I digested genomic DNA of *E.coli* and *A. vinelandii* strains The $^{32}$P-labeled 3.0 kb Sal I fragment from pAM50 containing the whole *nifD* and partial *nifK* and *nifH* gene. Panel (B) Lane 1, *E.coli* DH5α; lane 2, *A.vineiandii* UW; lane 3, 4, 5, *A. vinelandii* UW with a genomic mutation in *nifHDK* (insertion of 2.0 kb interposon $\Omega$Tet); lane 6, *A.vineiandii* UW; lane 7, the 3.0 kb Sal I fragment from pAM50 containing the partial *nifHDK* gene; lane 8, a 2.8 kb and a 2.2 kb fragment from pRS3 containing the *nifHDK* gene from pAM50 and Tet interposon from pHP45$\Omega$ Tet (interposon $\Omega$Tet contains an additional Sal I site). (A) Agarose gel for the autoradiogram shown in (B).
kb whereas with the mutant nif HDK locus the signal was obtained at 2.2 kb and 2.8 kb, this was because of an additional Sal I site present in the tetracycline resistance gene got introduced into the nifD gene.

GENERATION OF RANDOM MUTANTS USING KAPA FRAGMENT

A partial digest of genomic DNA from A. vinelandii UW was generated using restriction endonuclease Sau3AI. The fragment size selected from the genomic digest was about 1-2 kb. The genomic DNA digest was then ligated to a purified 1.3 kb DNA BamHI fragment carrying the aminoglycoside phosphotransferase gene cartridge derived from pUC4-KAPA (Pharmacia Biotech.). The ligated mixture was then electroporated into A. vinelandii UW. The ligated molecules were expected to undergo a homologous recombination with A. vinelandii chromosome and the KAPA fragment would get transferred to the chromosome. Thus, transformants (Kan') carrying an aminoglycoside phosphotransferase (KAPA) gene cartridge on the chromosome at different gene loci were obtained in this way.

The random mutants generated using the KAPA fragment were transformed with pRS3. The transformants so obtained were repeatedly subcultured (about 10-15 times) in a BNF medium containing kanamycin, tetracycline and ammonium acetate to give sufficient time for homogenotization. These were plated on BNF plates containing kanamycin, tetracycline, 5% sucrose and molybdenum but lacking ammonium acetate. A. vinelandii UW was also transformed with pRS3 as a control.

There were some poorly growing colonies, which appeared on these plates about several days after plating. At about the same time, similar number of poorly growing colonies appeared on control plates. About 100 colonies appeared on
both the control and test plates. These colonies were patched again on plates containing Mo^{++} and lacking ammonium acetate to check if they could grow well on patching. The transformants obtained from both the control plates and KAPA mutant population were growing very poorly on plates lacking ammonium acetate. Even after a period of several days, they grew very poorly. Further attempts to maintain these colonies on plates lacking ammonium acetate were not successful. These colonies did not survive after being transferred to a fresh plate lacking ammonium acetate.

Southern hybridization was carried out to determine any disruption of *nifHDK* locus (Fig 4.4). The logic behind this experiment was: if there is a disruption of recombination between mutant *nifHDK* locus and the *nifHDK* (wild type) locus on the chromosome then the signal obtained would be mixed i.e. that of wild type and mutant both. Genomic DNA was digested with *Sal I* and probed with a 3 kb DNA fragment carrying *nifHDK* gene isolated from pAM50 (Reddy, 1986). The signals obtained for transformants from *A. vinelandii* UW as well as *A. vinelandii* (UW):: KAPA were at the same positions. Signals were obtained at 2.2 kb and 2.8 kb, no signal was obtained at 3 kb position. Possibly, the mutation had been 'transmitted' to all copies.

**GENERATION OF RANDOM MUTANTS USING TRANSPOSON (MINI Tn10)**

Random mutants were then generated using pNK862 (Way *et al.*, 1984). *A. vinelandii* UW was transformed with pNK862 that serves as a vehicle for transporting miniTn10 into cells. The removal of transposase gene to a position outside the transposing segment ensures that, once made, insertions are stable and there is no secondary transposition.
Fig 4.4: Southern Hybridization analysis of the Sal I digested genomic DNA of E.coli and KAPA mutants of A. vinelandii UW The $^{32}$P-labeled 3.0 kb Sal I fragment from pAM50 containing the whole nifD and partial nifK and nifH gene was used as a probe. Panel (B) Lane 1, A.vinelandii UW with a genomic mutation in nifHDK gene; lane 2, A. vinelandii KAPA mutant (Nif') with a genomic mutation in nifHDK gene; lane 3, 4, 5, 6, 7, 8, A.vinelandii KAPA mutants (poorly growing under Nif conditions) with a genomic mutation in nifHDK gene; lane 9, E.coli DH5α; lane 10, 3.0 kb Sal I fragment from pAM50 containing the partial nifHDK gene from A. vinelandii; lane 11, a 2.8 kb and a 2.2 kb fragment from pRS3 containing the nifHDK gene and the Tet interposon as well. (A) Agarose gel for the autoradiogram shown in (B).
Random mutants generated using pNK862 were also transformed using pRS3. The transformants so obtained were repeatedly subcultured (10 to 15 times) in BNF medium containing kanamycin, tetracycline and ammonium acetate. These were then plated on BNF plates containing kanamycin, tetracycline and molybdenum but no ammonium acetate. *A. vinelandii* UW was also transformed with pRS3 as a control.

After 5-6 days, some colonies appeared on plates. Except 4 colonies, all other colonies were growing very poorly. All the colonies obtained on control plates were also poorly growing. The 4 transformants would be referred to as *A. vinelandii* RS1, RS2, RS3, RS4. These transformants were taken up for further study.

These transformants were patched again on BNF plates containing kanamycin, tetracycline (5 μg/ml), and molybdenum (24 μg/100ml). The 4 transformants (which were growing well initially) were still growing well in media lacking a nitrogen source. These colonies were patched repeatedly on plates lacking nitrogen source (ammonium acetate) for about 30 days. They exhibited a stable *nif* positive character. The other transformants were poorly growing like those on the control plates and did not survive repeated patching on medium lacking ammonium acetate.

Southern hybridization results of genomic DNA obtained from these transformants are shown in Fig 4.5. In case of transformants (*nif* mutants) obtained with *A. vinelandii* UW, the signal appeared at 2.2 kb and 2.8 kb as expected in *nif* mutants. But in case of transformants obtained from miniTn10 mutants the signal appeared at 3 kb position like in *A. vinelandii* UW. No signal appeared at 2.2 kb and 2.8 kb. These cells were nevertheless, resistant to tetracycline.
Fig 4.5: Southern Hybridization analysis of the Sal I digested genomic DNA of E.coli and transposon mutants of A. vinelandii UW The $^{32}$P-labeled 3.0 kb Sal I fragment from pAM50 containing the whole nifD and partial nifK and nifH gene was used as a probe. Panel (B) Lane 1, A. vinelandii UW; lane 2, A. vinelandii UW with a genomic mutation in nifHDK (insertion of 2.0 kb interposon ΩTet); lane 3, Transposon mutant with a genomic mutation in nifHDK gene; lane 4, 5, 6, 7, transposon mutants which are resistant to tetracycline but no apparent genomic mutation in nifHDK gene (lane 7 in panel B); lane 8, E.coli DH5α; lane 9, the 3.0 kb Sal I fragment from pAM50 containing the partial nifHDK gene; lane 10, a 2.8 kb and a 2.2 kb fragment from pRS3 containing the nifHDK gene from pAM50 and Tet interposon from pH45ΩTet (interposon ΩTet contains an additional Sal I site). (A) Agarose gel for the autoradiogram shown in (B).
RESISTANCE TO ULTRA-VIOLET LIGHT

To determine the sensitivity of different strains of *A. vinelandii* towards UV rays, the *A. vinelandii* strains UW, VK21, HB14 and RS1 (one of the 4 transformants obtained from mini Tn10 random mutants) were grown in BNF supplemented with 0.11% ammonium acetate. They were then streaked on a BNF-agar plate and exposed to UV radiation for different lengths of time (0 second-40 seconds). These were subsequently grown in dark for 48 hrs. The results are shown in Fig 4.6. *A. vinelandii* UW and RS1 were not sensitive to ultra-violet light even up to 40 seconds of exposure unlike *A. vinelandii* HB14 and VK21, which were resistant to ultra-violet light only up to 30 and 10 seconds respectively.

GROWTH CHARACTERISTICS

Two cultures of *A. vinelandii* UW and RS1 inoculated simultaneously were allowed to grow in the presence and absence of ammonium acetate. There were two cultures inoculated simultaneously, for *A. vinelandii* UW and RS1. Samples were taken out at regular time intervals to determine the cell density. The O.D.<sub>600</sub> (optical density) for the cultures were taken at regular time intervals and a time versus ln O.D. graph was plotted. At the same time the cultures were also plated at regular time intervals to check for contamination. Results are shown in Fig 4.7. Both the strains showed very similar growth patterns. *A. vinelandii* UW and RS1 had very similar lag phases and these cultures also reached the log phase at almost the same time. In the absence of ammonium acetate also, *A. vinelandii* UW and RS1 showed very similar growth pattern.
Fig 4.6: Sensitivity of different strains of *A. vinelandii* towards UV rays
As described in the UV streak test (Materials and Methods). Topmost streak, *A. vinelandii* UW; streak second from the top, *A. vinelandii* HB14; streak third from top, *A. vinelandii* VK21; bottom streak *A. vinelandii* RS1. Exposure for UV ray was for 0, 10, 20, 30, 40 s.
Fig 4.7 Growth characteristics of strains *A. vinelandii* UW (■) and *A. vinelandii* RS1 (□). In presence of a nitrogen source (A) and in the absence of nitrogen source (B).
ATTEMPTS TO IDENTIFY THE HOMOGENOTIZATION FACTOR(S)

In order to identify the factor(s) involved in the homogenotization process, as a first step, an attempt was made to clone the genomic region where the transposon had got inserted. Genomic DNA was isolated from one of the four transformants (RS1) and digested with Sau3AI. A partial Sau3AI digest comprising DNA fragments about 8-12 kb in size was cloned into pBR322 digested with BamHI. There were no colonies obtained on a LB agar plate containing both ampicillin and kanamycin. However, 10 colonies were obtained on LB agar plates containing kanamycin only. These colonies were found to be sensitive to ampicillin.

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**Fig 4.8** A schematic map for pRS6. IS are the repeat elements of miniTn10. The solid lines represent the genomic DNA sequence from *A. vinelandii* and dotted lines are the vector sequences (pBR322). Arrows indicate the direction of primers (from transposon sequences) used for sequencing.

The plasmid DNAs were isolated from these colonies (pRS5-pRS14) and one of the DNA samples (pRS6) was subjected to DNA sequencing as a first step. The primers used for sequencing were complementary to Kan$^R$ gene of miniTn10 (Fig 4.8). The DNA sequence from this plasmid did not give much information because more than 50% of it could not be read, only bases up to the miniTn10

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Fig 4.9 a and b Nucleotide sequence data for pRS6
sequences could be read clearly (Fig 4.9 a and b). This happened every time the construct was subjected to sequencing. This could be because the genomic insert was probably unstable and rearrangements in the fragment had taken place.

B. NIF GENE REGULATION

In order to assess the role of HU and H-NS like proteins in nifLA gene regulation, a systematic study was initiated. As a first step, we purified these proteins from Klebsiella pneumoniae, and their binding with the sequences upstream of promoter region was analyzed. For this, gel mobility shift assays and DNA footprinting studies were carried out with the purified protein fractions from Klebsiella pneumoniae.

PURIFICATION OF HU AND H-NS LIKE PROTEINS FROM KLEBSIELLA PNEUMONIAE

Protein fractions were prepared from Klebsiella pneumoniae strain M5a1. It may be pointed out that the differences in the iso-electric pH of these two proteins enabled us to design the protocol for purification of these proteins.

Assuming that the proteins would not be very different from E.coli proteins, attempts were made to purify them on a cationic exchanger at neutral pH (pH 7.4). E.coli HU protein is a basic protein with a pl of 8.75 while E.coli H-NS protein has a pl of 7.4 making it a neutral DNA binding protein. Thus, H-NS protein was expected to elute at a lower salt concentration while HU protein
would be eluted at a higher salt concentration from a cation exchanger column. Crude extract in buffer A (50 mm Tris-Cl, 1 mM EDTA, 0.1 mM PMSF, 10% Glycerol, 0.014 M β-Mercaptoethanol, pH 7.4) was loaded onto a 1ml S-sepharose cartridge (Cation exchanger, Biorad USA) pre-equilibrated with buffer A at pH 7.4 containing 50 mM NaCl. The column was washed with 20 column volumes of buffer A containing 50 mM NaCl and bound proteins were eluted by applying a continuous salt gradient (50 mM-1M NaCl in buffer A). 20 fractions were collected and each of them was analyzed for DNA binding activity using the 137 bp, α²³[^P] labeled EcoRI – BamHI fragment derived from pAN 94 containing nifLA upstream regulatory sequences (from position –133 to –38 upstream of the transcription start site; Fig 4.10). Very low DNA binding activity was obtained by this method and the fractions were shown to contain several protein bands when subjected to denaturing gel electrophoretic analysis, although low molecular weight bands were visible. The protein profile for Klebsiella pneumoniae was very different from that of E.coli, HU protein and H-NS protein appeared to be associated with other proteins.

In order to improve separation, several protein purification techniques were employed. It is known that levels of HU and H-NS proteins vary with the growth phase (Claret and Rouviere-Yaniv et al., 1997; Dersch et al., 1993; Free and Dorman, 1995; Ueguchi et al., 1993). Therefore, the Klebsiella pneumoniae cultures were grown to different OD and the cells were harvested at different time points. Crude extracts were prepared from these cultures and were subjected to the S-sepharose column chromatography. However, the protein profile examined on SDS polyacrylamide gel did not show much improvement. Following this, attempts were made to purify these proteins using polyethyleneimine (PEI) to precipitate the proteins at various concentrations of PEI to determine if a particular concentration of PEI caused enrichment of HU and H-NS proteins. Urea was also used to separate these two proteins from other associated proteins.
Attempts were also made to purify proteins by pooling the low activity fractions and passing them through q-sepharose and then through heparin columns. These attempts did not give a pure protein although the DNA binding activity improved.

However, proteins were successfully purified using the ammonium sulphate fractionation method. For this the crude extract was fractionated using ammonium sulphate at 70-90% saturation. This ammonium sulphate precipitation was repeated twice (Aki and Adhya, 1997). The fraction was dialyzed against buffer A (pH 7.4) containing 50 mM NaCl. This fraction was loaded onto a 1ml S-sepharose cartridge (Cation exchanger, Biorad USA), pre-equilibrated with buffer A at pH 7.4 containing 50 mM NaCl. Twenty fractions were collected as described earlier. All the 20 fractions collected were assayed for DNA binding activity. Three different kinds of DNA binding activities were detected when the protein fractions were subjected to DNA binding assay. Early protein fractions (fraction number 4-6) produced a distinctively retarded species whereas the later fractions (fraction number 8-12) produced somewhat spreadout bands and beyond fraction number 13, fractions formed a complex, which did not enter the gel. The results are shown in Fig 4.11 A and B.
Fig 4.11 A and B: Purification of H-NS and HU like proteins from *Klebsiella pneumoniae* All the protein fractions were assayed for DNA binding activity. DNA protein complexes were resolved on 5% PAGE and autoradiographed. A. Odd number protein fractions (1-19), B. Even number protein fractions (2-20). Lane (C) No protein added. An α-32P end labeled 137bp EcoRI- BamHI fragment from pAN94 was used as template DNA.
Fig 4.10 Sequence of the nifLA promoter and upstream region of *Klebsiella pneumoniae*. The bases beneath the filled bars (■) represent the two NtrC binding sites (Buck *et al.*, 1986). The bases beneath the open bars (□) depict the -24 and -12 regions containing the nifLA promoter (Beynon *et al.*, 1983). The bases underlined represent the A and T stretches (Cheema *et al.*, 1999).

**THE PROTEINS PRESENT IN FRACTION NUMBER 6 AND 8 ARE CROSS REACTIVE WITH ANTIBODIES SPECIFIC FOR E.COLI H-NS AND HU PROTEINS, RESPECTIVELY.**

Since fraction 6 and 8 were the only fractions that gave single distinct complex, these were subjected to further analysis. It is known that the HU and H-NS like proteins from *E.coli* are of low molecular weight, we decided to analyze H-NS and HU like proteins present in fraction 6 and 8, respectively from *Klebsiella pneumoniae* to determine their molecular weight (Fig 4.12).
The DNA binding protein fractions purified from *Klebsiella pneumoniae* were subjected to electrophoresis on a 15% polyacrylamide gel under denaturing condition (0.1% SDS) along with the crude extract and 70-90% ammonium sulphate fraction and a molecular weight marker. The molecular weights determined for fraction 6 and 8 are quite comparable to the molecular weight of H-NS and HU from *E.coli* (Yaniv, 1979; Ussery *et al.*, 1994). The Western blot analysis of both DNA binding fractions 6 and 8 showed that these proteins from *Klebsiella pneumoniae* reacted with anti-H-NS and anti-HU antibodies raised against *E.coli* H-NS (Fig 4.12, lane 5) and HU (Fig 4.12, lane 6) proteins respectively.

**PROTEIN FRACTIONS NUMBER 6 AND 8 ISOLATED FROM *KLEBSIELLA PNEUMONIAE* ARE HEAT STABLE**

*E.coli* histone-like proteins HU and H-NS are known to be heat stable proteins (Yaniv and Gros, 1975; Ussery, 1994). To determine whether the proteins isolated from *Klebsiella pneumoniae* were also heat stable, the isolated protein fractions (number 6 and 8) were boiled in the presence of 50 μg/ml BSA for 10 minutes and then cooled. The DNA-protein complexes were formed with these denatured proteins using 3’ end labeled DNA fragment. DNA-protein complexes were also formed with native proteins used as a control. The complexes were resolved on 5% native polyacrylamide gel. The results are shown in Fig 4.13. The denatured proteins retained their DNA binding activity like the native proteins.
Fig 4.12: SDS-Polyacrylamide gel electrophoresis and Western blot of protein fraction number 6 and 8. The protein samples were resolved on 15% SDS-PAGE (acrylamide-bisacrylamide ratio was 29:1) and detected by silver staining. Lane (M) is molecular weight marker, lane (1) crude extract (5 μg), lane (2) ammonium sulphate precipitate (5 μg), lane (3) protein fraction 6 (0.5 μg) and lane (4) protein fraction 8 (0.7 μg). Lanes (5) and (6) same amount of protein fraction 6 and 8 as in SDS-PAGE were blotted and probed with anti-H-NS and anti-HU antibodies (raised against *E.coli* proteins) respectively.
| Fr.No.6(µg) | 2 | 2 | - | - |
| Fr.No.8(µg) | - | - | 4 | 4 |
| LANE       | C | 1 | 2 | 3 | 4 |

Fig 4.13: Protein fraction number 6 and 8 are heat stable Fraction 6 and 8 (about 10 µg each) proteins were subjected to boiling at 100°C for 10 minutes in presence of 50 µg/ml BSA. DNA fragment (137 bp EcoRI-BamHI fragment from pAN94) was end labeled and incubated with heat treated as well as untreated protein fractions. DNA protein complexes were resolved on 5% PAGE and autoradiographed. Lane (C) no protein added. Lane (1) heat treated protein fraction 6. Lane (2) fraction 6 protein (native). Lane (3) heat treated protein fraction 8. Lane (4) fraction 8 protein (native).
DNA-PROTEIN COMPLEX FORMATION WITH H-NS (FRACTION 6) AND HU (FRACTION 8) LIKE PROTEINS IS CONCENTRATION DEPENDENT

The DNA binding pattern of fraction 6 and fraction 8 proteins were examined (Fig 4.14). Fraction 6 and fraction 8 proteins form very distinct type of complexes with the intervening sequence upstream of the nifLA promoter. The proteins bound to DNA in a concentration dependent fashion. In case of complexes formed with protein in fraction 6, a clear shifted band appeared to be formed at lower concentrations of protein (lane 2 and 3). This specific band disappeared as the protein concentration was gradually increased and a highly retarded protein DNA complex formed (lane 4 and 5).

At lower concentrations of fraction 8 protein somewhat spread out but distinct band appeared. Similarly as with fraction 6 protein, DNA-protein complexes formed with increasing concentration of fraction 8 protein produced highly retarded complex(es). Both these proteins possibly exhibit non-specific DNA binding at higher concentrations.

EFFECT OF MONOVALENT AND DIVALENT CATIONS ON DNA BINDING ACTIVITY

Generally, monovalent and divalent cations are required for the activity of many DNA binding proteins. To test for the requirement of these proteins for monovalent (NaCl) and divalent (MgCl₂) cations, DNA binding reactions were performed in the presence of varying concentrations of NaCl and MgCl₂, keeping the protein concentration constant. The binding of fraction number 6 (H-NS like
Fig 4.14: Titration of protein fractions 6 and 8 DNA fragment (same as the one used earlier) was end labeled and incubated with protein fraction 6 and 8 (amounts indicated in photograph) under optimized conditions (see materials and methods). DNA protein complexes were resolved on 5% PAGE and autoradiographed.
protein, Fig 4.15A) and fraction number 8 (HU like protein, Fig 4.15B) to DNA was found to be sensitive to NaCl. Minimum Concentration of NaCl was found to be tolerable for the DNA binding activity of fraction 6 protein. As shown in Fig 4.15A at the minimum salt concentration of 75 mM there was a slight decrease in DNA binding of protein in fraction 6. At higher concentrations of NaCl (300 mM), the DNA binding activity of fraction 6 protein reduced drastically. Thus, higher NaCl concentrations were found to be deleterious for fraction 6 protein binding. However, in case of fraction 8, with increasing salt concentration, the complex migrated slightly faster upto 150 mM, after which the rate of migration of the complex remained unchanged.

The effect of Mg$^{2+}$ on the DNA complex formation with fraction 6 and 8, respectively is shown in Fig 4.16 A and B. Although, there was little change in the complex formation with increasing Mg$^{2+}$ concentration (lane 2-5), maximum binding was obtained at 7.5mM of MgCl$_2$ (lane 5). At 10 mM Mg$^{2+}$ concentration the binding seemed to be slightly reduced (lane 6). Fraction 8 protein binding to DNA was not affected by increasing concentrations of Mg$^{2+}$(Fig 4.16 B).

**PROTEIN FRACTION 6 ISOLATED FROM K. PNEUMONIAE HAS A HIGHER AFFINITY FOR CURVED DNA.**

H-NS protein has been shown to have a higher affinity towards curved DNA (Falconi et al., 1988; Yamada et al., 1991). We analyzed the protein fraction number 6 for the same. For this, a mixture of curved and non-curved 3' end labeled DNA fragments were mixed with varying amounts of fraction number 6 since we presumed that the protein fraction 6 is histone-like protein H-NS. The curved DNA fragment used was a 137 bp fragment from pAN94 (Cheema et al., 1999) and was mixed with a 190 bp EcoRV-BamHI fragment (a non-curved DNA
Fig 4.15 A and B: Effect of Na⁺ on binding activity of protein fractions 6 and 8 DNA protein complexes were formed (as described in materials and methods) in presence of indicated amounts of NaCl and fixed amount of proteins. The autoradiograms A and B show binding activity of protein fractions 6 and 8. Fraction 8 protein shows better binding than fraction 6 protein at higher Na⁺ concentrations.
Fig 4.16 A and B: Effect of Mg$^{2+}$ on binding activity of protein fractions 6 and 8 DNA protein complexes were formed (as described in materials and methods) in presence of indicated amounts of MgCl$_2$ and fixed amount of proteins. The autoradiograms A and B show binding activity of protein fractions 6 and 8. Fraction 8 protein shows better binding than fraction 6 protein at higher Mg$^{2+}$ concentrations.
fragment) from pBR322. The complexes thus formed were resolved on 5% native polyacrylamide gel. Fig 4.17A shows that fraction number 6 bound preferentially to curved DNA. Protein in fraction number 8 did not show any clear preference (data not shown).

For quantification, the autoradiogram of Fig 4.17A was scanned by densitometry. The percentage of unbound DNA was plotted against the amount of protein used. The plot also showed that Fraction number 6 indeed bound to curved DNA fragment preferentially as seen in Fig 4.17B. The scan showed a steady reduction in the amount of unbound DNA (curved) as fraction 6 protein concentration was increased, decrease in the amount of unbound DNA (non-curved) was not as significant.

**EFFECT OF POINT MUTATIONS IN INTERVENING SEQUENCE**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Site and Nature of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDJ22</td>
<td>Wild type</td>
</tr>
<tr>
<td>pAN242.2</td>
<td>TTTT at position –69 to –66 changed</td>
</tr>
<tr>
<td>pAN242.2D</td>
<td>AAAA at position –89 to –86 and TTTT at position –69 to -66 changed</td>
</tr>
</tbody>
</table>

Fig 4.18 Constructs with wild type *nifLA* sequence and mutated *nifLA* sequence
Fig 4.17 A and B: Protein fraction number 6 from *Klebsiella pneumoniae* has higher affinity for curved DNA than non-curved DNA fragments. A. Curved and non-curved DNA fragments [190bp EcoRI-BamHI fragment from pBR322 (non-curved) and 137bp EcoRI-BamHI fragment from pAN94 (curved)] were end labeled with Klenow fragment and equal amounts were preincubated simultaneously with the indicated amounts of protein fraction number 6. The DNA protein complexes were resolved on 5% PAGE and autoradiographed. Lane (6 and 7) DNA binding activity of protein fraction 6 with curved and non-curved DNA fragments individually. B. The amount of DNA unbound (from exp. in [A]) was quantified by densitometry and expressed as a percentage of the initial amount of DNA in the binding reaction.
Gel shift assays were also carried out using DNA fragment comprising the *nifLA* intervening sequence with mutations at specific A and T tracts present between -95 to -65. These stretches of A’s and T’s are known to be bent sequences (Cheema *et al.*, 1999) and mutations in these sequences are known to cause decrease in curvature for the DNA fragment. DNA binding reactions were performed using the labeled fragments with specific mutations shown in Fig 4.18. Both fraction 6 and fraction 8 proteins were used. Results are shown in Fig 4.19 and Fig 4.20.

Binding of fraction 8 protein to the intervening sequence did not seem to be affected by mutations at any of the bent sequences. However, fraction 6 binding did show a slight decrease in binding when out of the three bent loci, at least two had mutations in them that reduced the curvature (pAN242.2D).

**DNASE I PROTECTION STUDIES WITH HU AND H-NS LIKE PROTEINS**

DNase I protection studies were carried out to map HU and H-NS binding sites within the *nif* upstream sequences. To identify the protein binding sites in the intervening sequence upstream of the *nifLA* promoter, a DNA fragment digested with *EcoRI* and *PvuII* was end labeled for foot printing studies on one strand (top strand). For identifying protein-binding sites on the other strand (bottom strand) a DNA fragment digested with *HindIII* and *EcoRI* was end labeled and used. DNase I protection assay was performed with both the strands under binding reaction conditions optimized for gel retardation assay.

The results for DNase I protection are shown in Fig 4.21(A and B) and Fig 4.22(A and B). The proteins bound to DNA in a concentration dependent fashion.
Fig 4.19: Effect of mutations in *nifLA* upstream sequences on DNA binding activity of protein fraction 6 DNA fragments (*BamHI*-*HindIII* fragments) of the wild type (pDJ22) and mutant (pAN242.2 and pAN242.2D) *nifLA* upstream sequences were end labeled and incubated with protein fraction 6 (amounts indicated in picture). DNA-protein complexes were resolved on 5% PAGE and autoradiographed.
Fig 4.20: Effect of mutations in *nifLA* upstream sequences on DNA binding activity of protein fraction 8 DNA fragments (BamHI- HindIII fragments) of the wild type (pDJ22) and mutant (pAN242.2 and pAN242.2D) *nifLA* upstream sequences were end labeled and incubated with protein fraction 8 (amounts indicated in picture). DNA-protein complexes were resolved on 5% PAGE and autoradiographed.
At saturating concentrations of both fraction 8 and fraction 6 proteins, DNA appeared to be uniformly protected in the top strand (Fig 4.21A and 4.22A).

In case of top strand, fraction 8 protein at lower concentrations appears to protect two regions in the sequences upstream of nifLA promoter. The protected regions are from position -65 to -51 and from position -48 to -37 upstream of the transcription start site. Similarly for fraction 6 protein, the regions protected are at position -72 to -61 upstream of the transcription start site and from position -45 to -37 upstream of the transcription start site. Both the proteins show a preference in binding, they bind much better to the top strand.

Fraction 8 protein does not bind much to the bottom strand, on the other hand fraction 6 protein binds very weakly at position -56 to -44 upstream of the transcription start site.

Note- The footprint obtained in case of Fraction 8 protein with the bottom strand is due to the binding of Fraction 8 protein to vector sequence.

**EFFECTS OF HU AND HNS PROTEINS ON DNA BENDING**

The intrinsic bends and protein induced bends are shown to be present in many situations and influence transcription (Snyder et al., 1986; Zahn and Blattner, 1985; Santero et al., 1989). We wanted to explore the possibility of these proteins present in fraction 6 and 8 causing any change in DNA bending in the nifLA upstream sequences. For this, we used the 137 bp fragment from pAN94, which was cloned in vector pBend2 (pADH.1). Protein–induced DNA bending was measured by gel retardation assay using a set of permutated DNA fragments obtained by digesting the plasmid construct pADH.1 with different restriction enzymes.
Fig 4.21 A: DNase I footprinting pattern of the DNA regions protected by protein fraction 8 in top strand. End labeled DNA fragment was incubated with the protein. The complexes were probed with DNase I. Products were purified and resolved on a sequencing gel and autoradiographed. Lanes marked A, G, C and T represent dideoxy sequence markers.
Fig 4.21 B: DNase I footprinting pattern of the DNA regions protected by protein fraction 8 in bottom strand. End labeled DNA fragment was incubated with the protein. The complexes were probed with DNase I. Products were purified and resolved on a sequencing gel and autoradiographed. Lanes marked A, G, C and T represent dideoxy sequence markers.
Fig 4.22 A: DNase I footprinting pattern of the DNA regions protected by protein fraction 6 in top strand End labeled DNA fragment was incubated with the protein. The complexes were probed with DNase I. Products were purified and resolved on a sequencing gel and autoradiographed. Lanes marked A, G, C and T represent dideoxy sequence markers.
Fig 4.22 B: DNase I footprinting pattern of the DNA regions protected by protein fraction 6 in bottom strand. End labeled DNA fragment was incubated with the protein. The complexes were probed with DNase I. Products were purified and resolved on a sequencing gel and autoradiographed. Lanes marked A, G, C and T represent dideoxy sequence markers.
Since this DNA fragment already showed a natural curvature, we wanted to compare the extent of bending in absence and presence of proteins. The gel shift assays were performed as already discussed. The free DNA and DNA-protein complexes were loaded onto a 5% native polyacrylamide gel containing glycerol. The gel was run at 4°C. The results are shown in Fig 4.23 A and B and Fig 4.24 A and B. The binding of fraction 8 protein caused a change in the DNA bending pattern. Fraction 8 protein caused a shift in the overall DNA bending pattern. In case of DNA fragments that were unbound the most retarded DNA fragment was the EcoRV digested fragment while in the case of protein bound DNA fragments the most retarded species was the MluI digested fragment. Fraction 6 protein did not cause much change in bending.
Fig 4.23 A and B: Protein-induced DNA bending A. Permutated DNA fragments obtained by digesting pADH.1 with appropriate restriction enzymes, followed by end labeling with T4 polynucleotide kinase, were incubated with protein fraction 6 (2 μg). DNA protein complexes were resolved on 5% PAGE and autoradiographed. I denotes unbound DNA fragments and II denotes protein bound DNA fragments.

B. Graph of relative migrations of protein bound fragments (II). Abscissa is the distance of the fragment center from the middle of the bent sequence (five A's stretch). Ordinate is the ratio of the distances migrated by protein bound DNA fragments (II) and unbound DNA fragments (I).
Fig 4.24 A and B: Protein-Induced DNA bending: A. Permutated DNA fragments obtained by digesting pADH.1 with appropriate restriction enzymes, followed by end labeling with T4 polynucleotide kinase, were incubated with protein fraction 8 (1.2 μg). DNA protein complexes were resolved on 5% PAGE and autoradiographed. I denotes unbound DNA fragments and II denotes protein bound DNA fragments. B. Graph of relative migrations of protein bound fragments (II). Abcissa is the distance of the fragment center from the middle of the bent sequence (five A's stretch). Ordinate is the ratio of the distances migrated by protein bound DNA fragments (II) and unbound DNA fragments (I). The scales for plots for I and II have been separated to show the similar trend of both curves.