GENETIC HOMOGENOTIZATION

*A. vinelandii* cells contain more DNA than most other bacteria (Sadoff *et al.*, 1971; 1979; Punita *et al.*, 1989). Estimates from *A. vinelandii* indicate that there may be as many as 40 to 80 copies of chromosomes in vegetative cells. Since prokaryotes are normally haploid, these observations imply that *A. vinelandii* has unique genetics and an unusual regulation of its chromosome number. Besides *A. vinelandii*, few other prokaryotes are known to contain multiple copies of chromosomes; these include *A. chroococcum*, *Desulfovibrio gigas* and *D. vulgaris* (Postgate *et al.*, 1984; Robson *et al.*, 1984).

Mutants of *A. vinelandii* have been isolated with varying degrees of success. In a bacterium like *A. vinelandii*, containing about 40 to 80 copies of chromosomes per cell, mutants should be difficult to obtain. The long segregation period required for their formation would result in the loss of such mutations by dilution and recombination repair. However, stable *nif* mutants have been easy to obtain and in reasonable numbers but auxotrophic mutants, have been difficult to isolate. Manna and Das (1997) were successful in isolating some stable leucine auxotrophs. In case of some leucine prototrophs, Southern hybridization revealed the presence of a mutant band in addition to wild-type band. This heterozygosity was attributed to recombination. Manna and Das (1997) speculated that a recombination between inactivated *leu B* gene on a plasmid with wild-type *leu B* on a single chromosome took place and subsequently the mutation in single chromosome was then transmitted to all copies and they termed this process as 'homogenotization'.

If there are 80 copies of chromosomes the frequency of obtaining a mutant would be very less. However, it was observed that 80% of cells were leucine auxotrophs and there was no wild-type DNA visible in these auxotrophic cells. Also the ‘homogenotization’ of mutation in some genes (*nif* genes) is very fast.
compared to the amino acid biosynthetic genes. It was assumed that the homogenotization process may be carried out by some uncharacterized non-reciprocal recombination system (Manna and Das, 1997).

The cloning and molecular characterization of the recA and recF genes has been reported in A. vinelandii (Venkatesh et al., 1990, Badran et al., 1995). The recA gene is indispensable to a number of processes including DNA repair and recombination and SOS repair (Clark and Margulies, 1965; Clark and Volkert, 1978; Howard-Flanders and Theriot, 1966; Rupp and Howard-Flanders, 1968; Skarstad and Boye, 1988; Sweasy et al., 1990). A non-reciprocal transfer of sequence information between homologous DNA sequences has been reported in various organisms catalyzed by RecF protein (Yamamoto et al., 1988; 1992). We therefore decided to study the role of these two genes in the homogenotization process.

In order to test the role of recA and recF genes in homogenotization process, A. vinelandii strains, wild-type, and mutants in recA function (VK21) and recF function (HB14) were transformed with a nifLA deletion mutant clone in pUC8 containing an inserted Kan interposon (pUK110::ΩKm), which could undergo a double point cross-over with the chromosome rendering the wild-type strain a nifLA (nif negative) mutant. If either RecA or RecF were involved in ‘homogenotization’ process, only one or small number of copies of the chromosomes of transformants would have mutated nifLA gene while others would retain wild-type phenotype (nif positive). However, all the transformants obtained in this case exhibited a mutant (nif negative) phenotype. Southern hybridization also confirmed that no wild-type nifLA gene was left in any chromosome. All the transformants showed the presence of mutated nifLA.

Thus, nifLA mutation was ‘transmitted’ to all copies of chromosomes in A. vinelandii wild type and also in A. vinelandii strains deficient in recA and recF function. Therefore neither the recA nor the recF gene appears to be responsible
for 'homogenotization' of the mutation. Possibly the homogenotization process was more complicated due to presence of multiple chromosomes in *A. vinelandii* and may require a number of factors.

The best method to search for such gene(s) was to introduce random mutations in *A. vinelandii* genome and screen the random mutant population for such factor(s). For this screening two different random mutant populations were generated, one generated by inserting KAPA gene block in *A. vinelandii* genome and the other population contained miniTn10 insertions. A *nifHDK* mutant (insertion) clone was also constructed (pRS3), which could undergo a double point crossover with the chromosome and render the wild-type strain a *nifHDK* (Nif') mutant.

In case of KAPA mutant population, the transformants obtained were very poorly growing in absence of a nitrogen source. Southern hybridization further revealed that no wild-type *nifHDK* band was present in any of the poorly growing transformants, only mutant band was detected even after long exposure.

There were 4 transformants (RS1-RS4) obtained from miniTn10 mutant population that were growing very well even in absence of a nitrogen source. The *nif* positive characteristic of these transformants was stable even after repeated patching on plates lacking a nitrogen source. Southern hybridization revealed the presence of only wild-type *nifHDK* band. The presence of wild-type band indicated that possibly homogenotization function had been disrupted because only if 'homogenotization' of *nifHDK* mutation was disrupted, a wild-type band would get detected.

The transformants were then checked for UV sensitivity to determine the nature of mutation. Mostly disruptions in recombination functions render the cells sensitive to ultra-violet (UV) light. The transformants grew well even on exposure to UV light for 40 seconds.
The growth characteristics of transformants were compared to wild-type *A. vinelandii*. The growth curves of *A. vinelandii* wild type and the transformants were very similar both in the absence and presence of a nitrogen source.

Attempts were also made to identify the factor(s) responsible for homogenotization process. Genomic DNA was isolated from one of the transformants (RS1) and digested with *Sau3AI* to generate fragments 8-12 kb in size, which were then ligated to pBR322 digested with *BamHI* and transformed.

To characterize these recombinant colonies, the recombinant plasmid DNA from these colonies was subjected to sequencing. However, the DNA sequence did not give any conclusive result, as the sequences beyond the Tn10 sequences could not be read. Possibly some rearrangement had taken place in the genomic DNA cloned in pBR322 because earlier the resistance to ampicillin was lost in all these clones. Since the sequencing data was not informative, another attempt using an alternative technique may be required. The recombinant clone, which was defective in “homogenotization” function could be used as a probe to screen genomic library of *A. vinelandii* for a wild-type allele. Once the genomic DNA clone isolated this way is available, it would then be subcloned in an expression vector. Finally, the clone in expression vector would be used in complementation assay i.e. it should be able to compensate for the defect in homogenotization process.
NIF GENE REGULATION

The *nifLA* promoter of *Klebsiella pneumoniae* requires sigma-54 RNA polymerase holoenzyme and the phosphorylated form of NtrC for its transcriptional activation. The phosphorylated NtrC binds to sites located almost 100 bp upstream of the *nifLA* promoter. Transcriptional activation by NtrC is also face-of-the-helix-dependent (Minchin *et al.*, 1989).

Although it has been suggested that DNA loop formation may be involved in NtrC mediated activation of the *nifLA* promoter (Minchin *et al.*, 1989), the mechanism of interaction of NtrC with RNA polymerase bound downstream is not well understood. There are A and T stretches present in the intervening sequence i.e. the region between NtrC binding sites and the *nifLA* promoter sequences, that are known to impart an intrinsic curve to the DNA molecule. These A and T stretches have been shown to be required for the transcription from *nifLA* promoter (Cheema *et al.*, 1999). Sequence-induced DNA curvature is present in the replication origin of bacteriophage lambda (Zahn and Blattner, 1985) and an autonomously replicating sequence of yeast (Snyder *et al.*, 1986). Additionally, curved DNA regions have also been found in upstream regions of some *E.coli* promoters (Galas *et al.*, 1985). *In vitro* studies using such promoters revealed that addition of RNA polymerase sigma-70 holoenzyme itself was sometimes sufficient for transcription and that no upstream activator was necessary (Hsu *et al.*, 1991; Leirmo and Gourse, 1991). However, the relative curvature found in this system was less compared to other systems where presence of continuous AT stretches imparts a very high degree of curvature (Koo *et al.*, 1986). The bending angle for the wild type *nifLA* promoter has been estimated to be 79° (Cheema *et al.*, 1999). In case of *nifH* promoter, IHF protein is required to bend the DNA to promote transcription from *nifH* promoter (Santero *et al.*, 1989; Hoover *et al.*, 1985).
IHF protein is shown to bind DNA and cause sharp bend (>140°, Thompson and Landy, 1988).

However in case of the nifLA promoter, the mechanism for looping by which activator and the RNA polymerase enzyme would interact appears to be different. The in vivo experiments carried out earlier (Nichkawde, 1996) in hns', hupA' and hupB' background of E.coli suggested the involvement of H-NS and HU proteins (histone like proteins) in transcriptional regulation of the nifLA promoter of Klebsiella pneumoniae. Low molecular weight histone-like proteins are known to bind DNA in a non-specific manner and influence gene expression (Rouviere-Yaniv and Gros, 1975; Falconi et al., 1988; Ussery et al., 1994). Thus, the mechanism for transcriptional activation possibly involves loop formation and H-NS and HU may influence transcription from nifLA promoter.

HU plays a pleiotropic role, being involved in DNA repair, replication and recombination (Bramhill and Kornberg, 1988; Li and Waters, 1998; Boubrik and Rouviere-Yaniv, 1995). It has been shown that there is a cross talk between the relative quantity of HU in the cell and activity of topoisomerase I (Bensaid et al., 1996). It has also been shown that unbalanced synthesis of its two subunits induces SOS response (Rouviere-Yaniv et al., 1990). HU also participates in initiation of replication (Baker and Kornberg, 1988; Bramhill and Kornberg, 1988) and gene regulation (Table 5.2). Transcription repression from two overlapping gal promoters required HU in addition to the gal repressor (Aki and Adhya, 1997). HU together with Gal repressor was shown to form a specific nucleoprotein complex causing the formation of a DNA loop. In this case HU was also shown to bend DNA to enable a DNA loop formation.

HU has been shown to assist in transcription from promoter Ps of Pseudomonas putida (Martin and Lorenzo, 1995). Ps promoter sequence is endowed with a significant static bend found to be insufficient to generate a DNA
loop to facilitate transcription. HU protein is shown to be instrumental in exacerbating a preexisting curvature resulting in a loop formation in intervening sequence. Recently, a HU-like protein isolated from Rhizobium leguminosarum has been shown to bind to specific sites within nod promoters and influence transcription (Song-Tau Liu et al., 1998). Although the actual mechanism of its action is not known, it is thought to function by bending DNA molecules and through this promoting the formation of transcriptionally competent open complexes.

H-NS like protein is a small chromatin associated protein found in enterobacteria. H-NS is known to modulate the expression of a number of genes, mostly as a negative regulator (Table 5.1). The exact mechanism by which H-NS affects transcription remains unclear. In E.coli proU, rrnB, Shigella virB promoter regions, the H-NS binding sites overlap the promoter elements and it has been suggested that H-NS prevents the RNA polymerase from interacting productively with the promoter (Ueguchi and Mizuno, 1993). In E.coli hns and Salmonella typhimurium proU promoter regions, the major H-NS binding sites are located outside the promoter sequences. H-NS probably represses expression in this case by locally constraining negative supercoils. The finding supports this hypothesis that H-NS binding sites can be separated by more than 1 kb from promoter region without eliminating transcription repression. Many genes that are affected by H-NS are also regulated by environmental factors such as osmolarity, pH, temperature and anaerobiosis (Atlung and Ingmer, 1997).

Manna and Gowrishankar (1994) showed the stimulatory effect of HU and counter effect of H-NS on the expression of proU gene from Salmonella typhimurium. Expression from proU gene decreased significantly in a hupA hupB mutant and increased in a hns mutant.
### Table 5.1 H-NS targeted genes

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<thead>
<tr>
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<th>Activation</th>
<th>Repression</th>
<th>References:</th>
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<tr>
<td>proU</td>
<td></td>
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<tr>
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<td></td>
<td>✓</td>
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</tr>
<tr>
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<td></td>
<td>✓</td>
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<tr>
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<td></td>
<td>Higgins et al., 1988</td>
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<td>✓</td>
<td></td>
<td>Nichkawde, 1996</td>
</tr>
<tr>
<td>Gene(s)</td>
<td>HU</td>
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<tr>
<td></td>
<td>Activation</td>
<td>Repression</td>
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<tr>
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<td></td>
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</tr>
<tr>
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<tr>
<td>nifLA</td>
<td></td>
<td>✓</td>
<td>Nichkawde, 1996</td>
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Table 5.2 HU targeted genes
In order to assess the role of these two proteins in nifLA gene regulation, HU and H-NS like proteins were purified from *Klebsiella pneumoniae*.

When each of the twenty fractions obtained were assayed for their DNA binding activity, two distinct DNA binding activities were observed. The DNA binding fraction number 6 and 8 were then subjected to SDS-PAGE. Fraction number 6 revealed a protein band with a molecular weight of approximately 15 kDa whereas in case of fraction 8, the protein band was of about 9 kDa. Rouviere-Yaniv and Gros (1975) reported a subunit molecular weight of 9 kDa for HU protein while H-NS is a homodimer with a subunit molecular weight of 15.6 kDa (Ussery et al., 1994). Thus, the molecular weights of these protein fractions from *Klebsiella pneumoniae* were found to be comparable to *E.coli* HU and H-NS proteins, i.e. fraction 6 consists of H-NS like protein whereas HU-like protein is present in fraction 8.

The DNA binding fractions 6 and 8 from *Klebsiella pneumoniae* also reacted with anti-H-NS and anti-HU antibodies developed against *E.coli* H-NS and HU proteins, respectively further confirming the presence of these proteins in the respective fractions. Histone-like proteins are known to be highly conserved proteins and show a great deal of sequence homology between eubacteria (Drlica and Rouviere-Yaniv, 1987). Cross-reactivity occurs amongst HU-like proteins from a number of eubacteria. Functional homologies have also been reported. HU like protein from *Anabena sp.* will substitute for HU protein from *E.coli* in both initiation of DNA replication and transcription (Craigie et al., 1985; Dixon and Kornberg, 1984). Amino acid sequence analyses of HU like proteins from other eubacteria revealed significant homologies especially in DNA binding arms (Drlica and Rouviere-Yaniv, 1987).
H-NS and HU proteins are known to be heat stable (Yaniv and Gros, 1975; Ussery et al., 1994). They retain their DNA binding activity even when subjected to heating at 100°C. Fraction number 6 and fraction number 8 protein fractions from *Klebsiella pneumoniae* were also found to be heat stable.

H-NS protein interacts preferentially with curved DNA (Yamada et al., 1991). This preference for 'curved' DNA suggests that, although H-NS might interact with different types of DNA fragments, it would probably regulate only a subset of promoters (Hughes et al., 1992). Results from the present study are indeed in agreement with this as protein(s) present in fraction 6 was found to interact preferentially with curved DNA when a mixture of curved and non-curved DNA molecules were present in the solution. Fraction 6 protein was also found to be more sensitive to presence of monovalent and divalent cations in binding reaction than fraction 8 protein. All these properties of fraction 6 and fraction 8 proteins are very similar to H-NS and HU like proteins. These fractions possibly consist of H-NS and HU like proteins, respectively. Hence forth, these protein fractions will be called H-NS and HU proteins.

As discussed earlier, the A and T stretches present in the intervening sequence impart an intrinsic curvature to the DNA. Therefore, it was desirable to determine whether mutations in these stretches that caused a decrease in the intrinsic curvature of the DNA fragment influenced H-NS and HU binding. There are two A stretches and one T stretch present in the DNA fragment. H-NS binding to DNA was found to be slightly decreased when at least two of the three stretches were mutated. HU binding, on the other hand was not affected by mutations in the A and T stretches. *In vivo* studies indicated that expression from *nifLA* promoter was affected by mutations in A and T stretches. Mutations in these stretches caused decrease in expression from *nifLA* promoter. This decrease in expression is also accompanied by a decrease in relative curvature of the DNA fragment (Cheema et al., 1999). H-NS protein has a preference for curved or 'bent' DNA.
FIG 5.1 Protection pattern of nifLA upstream sequence with H-NS and HU like proteins: Protected regions are shown as solid lines and lines with arrows for H-NS and HU proteins respectively. Dotted lines show weakly protected regions in bottom strand by H-NS protein. 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).
sequences. Mutations in these bent sequences that caused a decrease in DNA curvature possibly caused decreased binding of H-NS to the mutated intervening sequence. HU shows no preference for curved sequences, which possibly explains why HU binding is not affected by these mutations.

We also carried out footprinting studies to locate protein-binding regions. H-NS and HU proteins bind to DNA in a somewhat non-specific manner. Although it has been shown that H-NS has some preference for curved DNA, HU on the other hand prefers distorted regions within DNA e.g. single stranded gaps (Castaing et al., 1995) and cruciforms (Bianchi et al., 1989; Bonnefoy et al., 1994). Recently, it has been reported that HU does bind to some specific sites in nod promoters of Rhizobium leguminosarum (Song-Tau Liu et al., 1998). Our results from footprinting studies illustrate for the DNA to be uniformly protected at higher concentrations of proteins, which is a characteristic of these proteins. However, at lower concentrations these two proteins protected regions primarily around the A and T stretches. H-NS is normally shown to protect extended regions and the protected areas are AT rich (Lucht, 1994). In case of HU binding sites in nod promoters, it was noticed that the A/T contents was always more than 50% and A/T’s usually appeared in clusters (Song-Tau Liu et al., 1998). The presence of such AT rich sequences might favour structural distortions in DNA which could be recognized by HU protein. Interestingly, binding of these two proteins is limited to only one strand (top strand) in this case (Fig 5.1). Both the proteins from Klebsiella pneumoniae do not show significant binding to the other strand (bottom strand). This strand preference could also be due to DNA insensitivity as it has been observed in our laboratory that certain DNA sequences could be highly resistant to DNase I.
As discussed earlier, intrinsic bends may lead to interaction of proteins bound at distant sites on the DNA thereby modulating gene expression (Martin and Lorenzo, 1995; Santero et al., 1992). There are several examples of protein induced DNA bending. HU promotes transcription from Ps promoter by assisting a loop formation to allow interaction between RNA polymerase and upstream activator. A HU like protein from *Rhizobium leguminosarum* also binds to nod promoter regions and bends it (Song-Tau Liu et al., 1998). The total bending angle for wild type nifLA promoter was estimated to be 79°. Additional proteins can help bring about a loop formation like in case of Ps promoter of *Pseudomonas putida* where estimated bending angle is about 70° and HU assists in DNA loop formation.

We, therefore, decided to study the effect of H-NS and HU like proteins from *Klebsiella pneumoniae* on the bending of the DNA fragment comprising nifLA intervening region. Protein-induced DNA bending can be measured using a set of permutated DNA fragments generated from the intervening sequence cloned in pBend2 (pADH.1). Vector pBend2 is designed to facilitate the DNA bending measurements (Kim et al., 1989). The binding of HU like protein (fraction 8) from *Klebsiella pneumoniae* causes a change in the bending pattern of DNA. In case of DNA not bound to protein the slowest migrating (most retarded) DNA fragment is an EcoRV digest whereas in case of DNA bound to protein, the slowest migrating species is a *MluI* digested fragment. H-NS like protein (Fraction 6) does not cause any change in bending pattern. This result is not surprising, as it has been shown earlier (Nichkawde, 1996) that HU plays a negative role in *Klebsiella pneumoniae* nifLA regulation. In absence of additional supporting data we can at least speculate that possibly by changing the bending locus HU interferes with NtrC- RNA polymerase interactions and hence the nifLA expression (Fig 5.2). This result also suggests that H-NS, which was shown to have a positive role in nifLA regulation most probably by assisting NtrC- RNA polymerase interaction has no
Fig 5.2 schematic model on the possible roles of HU and H-NS proteins in nifLA regulation. HU and H-NS are HU and H-NS proteins respectively. N and R are NtrC protein and RNA polymerase, respectively. b1, b2, b3 represent the A and T stretches. HU protein exerts an effect away from its initial binding site, which lies within the bent stretches (b1, b2, b3).
effect on preexisting DNA bend. This negative role of HU in nifLA regulation is contrary to what has been found in DNA transactions involving HU where it acts by assisting spatially separated sequence specific DNA bound proteins to associate by binding to and bending the intervening sequences (Craigie et al., 1985; Bramhill and Kornberg, 1988; Martin and Lorenzo, 1995; Song-Tau Liu et al., 1998).

We can, therefore, conclude from our findings that the DNA binding activities we have obtained with fraction number 6 and 8 from Klebsiella pneumoniae could in fact be H-NS and HU proteins, respectively. The footprinting studies showed that these two proteins bound preferentially around the AT rich region in the upstream sequence. H-NS appears to bind to the ‘bent’ stretches in the upstream sequence and mutations in the ‘bent’ stretches affect H-NS binding. HU on the other hand, affects the extent and pattern of bending. Further, these two proteins may influence each other in binding to the upstream sequence.