INTRODUCTION
Nitrogen is an important bioelement, required for growth of plants. Although nitrogen is present in high amounts in atmosphere, plants cannot take it directly. They can only utilize nitrogen when present as ammonium, nitrate or nitrite ions. There are few prokaryotes known as diazotrophs, which can utilize this atmospheric nitrogen. These can be classified into several categories such as obligate and facultative anaerobes, obligate aerobes, photosynthetic bacteria and blue green algae. Among these, a few can fix nitrogen only in a symbiotic relation with plants while others can perform this function as free living organisms. The phenomenon of Biological nitrogen fixation (BNF) is a very high energy demanding process, therefore, very strictly controlled. BNF has been analyzed rather exhaustively in the free living diazotrophic bacterium, *K. pneumoniae*. Whereas it has been realised that under ideal condition, the reduction of one molecule of dinitrogen (catalysed by the nitrogenase enzyme complex) requires 16 ATP molecules (Brian and Maier, 1989).


### 1.1 Nitrogen fixing genes of *K. pneumoniae*

The principle enzyme of nitrogen fixation, nitrogenase can be divided into two oxygen sensitive proteins, the Fe-protein and Fe-Mo protein (Orne Johnson, 1985). It is encoded by three structural genes (*nifHDK*) and processed by product of several other genes. In *K. pneumoniae*, there are twenty one *nif* genes, clustered on the chromosome and are organised in eight different transcription units. (Cannon et al., 1985; Merrick, 1988), (Figure 1.1A). These twenty one genes have been broadly classified into five categories on the basis of their function, as follows:

(a) Genes involved in the synthesis of molybdenum iron protein (Kp1) and FeMoCo (cofactor).

(i) *nifK* and *nifD* are involved in synthesis of Kp1

(ii) *nifB, nifQ, nifE, nifN* and *nifU* are involved in iron molybdenum cofactor synthesis.

(b) Genes involved in the synthesis of iron protein (Kp2)

*nifH* codes for the iron protein (Kp2) while *nifM* is involved in its processing.

(c) Genes involved in the electron transport to nitrogenase: *nifF* and *nifJ*. 
(d) Genes involved in the regulation of other operons - *nifA* (positive regulatory element) and *nifL* (negative regulatory element).

(e) Genes with unknown functions - *nifS, nifV, nifX, nifY, nifT, nifZ* and *nifW.*

Of these only *nifF* and *nifJ* are transcribed in opposite orientation as compared to other *nif* operons (Arnold *et al.*, 1988).

A striking feature of all the *nif* operons is the absence of conserved -10 (TATA) and -35 (TTGACA) regions that are found in typical *E. coli* promoters which are regulated by σ70 (Rosenberg and Court, 1979; Hawley and Meclure, 1983). On the contrary, all these operons carry σ54 dependent promoters which have a characteristic consensus sequence CTGGN8TTGCA extending from -24 to -12 relative to the transcription initiation site (Beynon *et al.*, 1983; Ausubel, 1984). All these *nif* promoters except *nifM* and *nifLA* have a conserved upstream activating sequence (UAS) of consensus TGTN10ACA (Buck *et al.*, 1986) which usually lies more than 100 bp upstream of the transcription start site. Table 1.1 shows UAS (in full) and the consensus sequence of various *nif* promoters. Unlike other *nif* operons, the regulatory operon *nifLA*, carries NtrC binding site (Gussin *et al.*, 1986). The consensus NtrC binding sequence TGCACN7GTGCA is present in tandem repeats (Table 1.1).

1.2 Nitrogen fixing genes of *A. vinelandii*

*Azotobacter* is a free living diazotroph. Unlike *K. pneumoniae* it can fix nitrogen even in the presence of oxygen (Prazmowski, 1912). Notable aspect of nitrogen fixation in *A. vinelandii* is that it has more than one pathway for fixing nitrogen (Bishop *et al.*, 1980). Of these, one is the conventional nitrogen fixation pathway which is Fe²⁺ and Mo²⁺ dependent and involves *nif* genes (Brigle *et al.*, 1985). The first alternative pathway is vanadium dependent and works in the absence of molybdenum. It has *vnfH* structural gene for nitrogenase reductase (Raina *et al.*, 1988) and *vnfDGK* structural genes for nitrogenase (Joerger *et al.*, 1990) genes. The second alternative pathway functions in the absence of both molybdenum and vanadium and involves *anyHDKG* structural genes. (Joerger *et al.*, 1989). The genetic organization of the conventional pathway of nitrogen fixation shows considerable similarity with the *nif* cluster of *K. pneumoniae*, except for the fact that all the *nif* genes of the molybdenum dependent pathway of nitrogen fixation are not clustered together. About 30 potential ORFs have been identified and on the basis of sequence
Figure 1.1 Comparison of the physical organization of \textit{nif} and \textit{nif} associated genes from (A) \textit{K. pneumoniae} and (B) \textit{A. vinelandii}. Arrows indicate the position and direction for known or proposed transcription initiation sites. The numbering of the orfs (open reading frames) is arbitrary. Orf13 from \textit{A. vinelandii} is probably \textit{nifL} (Kennedy, Unpublished data) (From Dean and Jacobson, 1992).
Table 1.1. Promoter structures with putative NifA or NtrC binding sites in the *K. pneumoniae* nif gene cluster (from Arnold et al., 1988)

<table>
<thead>
<tr>
<th>NifA-binding site</th>
<th>Promoter sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>nifU: (a)</td>
<td>GGGCGCTTTTGCGCCCTGTCAACA -105 bp- CTGGCACACGGCTGTGCTTGAGG</td>
</tr>
<tr>
<td></td>
<td>GCACAGGCTTGTGCTTGAGGCAACA -404 bp- CTGGCGACCAATTCGCTGAATA</td>
</tr>
<tr>
<td></td>
<td>-94 bp- CTGGTATGTTCCCTGCAACTTCT</td>
</tr>
<tr>
<td>nifH:</td>
<td>GGCACATTGGTTCTGTTCTCCCACAA</td>
</tr>
<tr>
<td></td>
<td>nifE: (a)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>nifU: (a)</td>
<td>GCTAGCGCGTTCTGCTTTCTGACAA -83 bp- CTGGTATCAGCAATTGCTAGTTC</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>nifF:</td>
<td>AACTCCCTGTCGCGAGTTATGACAA -223 bp- CTGGCACACGGCTTCGAATC</td>
</tr>
<tr>
<td>nifB:</td>
<td>AAGCCTTATGTGAGATTCAGGACA -89 bp- CTGGTACAGCAATTGCTTAGG</td>
</tr>
<tr>
<td>N*</td>
<td>CGGCCTGTTGATTTGGCAAACA -134 bp- CTGGTGGCGAAGACTGCGAGG</td>
</tr>
</tbody>
</table>

Consensus sequences TGT -N_{10}-ACA

<table>
<thead>
<tr>
<th>NtrC-binding site</th>
<th>Promoter sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>nifL: (a)</td>
<td>GGTGCTTTTGCACCTACGCAGCGCCCA -127 bp- AGGGCGCACGGTTGCATGTT</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>nifZ:</td>
<td>GCTAGCGCGTTCTGCTTTCTGACAA -79 bp- CTGGCGCGAAGACTGCGAGG</td>
</tr>
</tbody>
</table>

*nifM**

Consensus sequences TGCACY-N_{3}-GGTGCA

* This structure is found in the coding region of *nifN*

** No putative binding site is found in front of the *nifM* promoter
analysis, 15 of these show similarity (except nifM) with *K. pneumoniae* (Figure 1.1B). These are nifHDKTYEMXUSVWZMN (Jacobson et al., 1989). Remaining ORFs also show potentiality of being nif genes. Here again nifH codes for iron protein or component II and nifD and nifK code for α and β subunits of Fe-Mo protein or component I, respectively (Dean and Jacobson, 1992). As in *K. pneumoniae*, nifE and nifN gene products are necessary for FeMo cofactor (Brigle et al., 1987). nifUSVWZM have been found to be organised in at least two overlapping transcriptional units. Where nifF is a flavodoxin and mediates electron transfer between nifJ encoded pyruvate oxidoreductase and oxidized nitrogenase Fe protein or component II. Unlike *K. pneumoniae* nifF is transcribed in the same direction as nifHDKTY, in *A. vinelandii* (Jacobson et al., 1989). In addition, nifB and nifQ have also been identified and exhibit similarity with corresponding genes of *K. pneumoniae*. Although nifA operon is also present, its regulation is not yet understood fully. Moreover products of some genes viz. nifB, nifQ, nifM, nifU, nifS, nifV, nfrX and ntrD are shared by all the three pathways (Kennedy and Dean, 1992).

As in enteric bacteria, genes ntrBC of *A. vinelandii* are also found to be linked to glnA and is transcribed by its own promoter (Toukdarian and Kennedy, 1986). ntrA gene (sigma factor, σ54) of *K. pneumoniae* and *A. vinelandii* show very high homology and the C-terminal of Ntr A has been found to contain DNA binding domain.

### 1.3 Regulation of Nitrogen fixation

Nitrogen fixation in microorganisms such as *K. pneumoniae* is repressed by fixed nitrogen or the atmospheric oxygen. The molecular mechanisms by which the nitrogen and oxygen status of the cell are relayed into nif gene expression or repression involve several trans- and cis-acting factors and is not yet fully understood. The role of these factors in the nif regulatory cascade has been shown to involve two levels of control. The first level of control involves the nitrogen sensing circuitry in which at least four proteins act in a cascade. Under nitrogen deficiency, genes involved in the second level of control are transcriptionally activated. These genes encode regulatory proteins that subsequently activate transcription of all other nif genes under anaerobic conditions.

#### 1.3.1 First level of control (Global regulation): The initial studies have revealed the involvement of glutamine synthase (GlnA) as a major controlling element of the nitrogen
fixation (nif) genes in K. pneumoniae. Later on another gene "glnR" was also found to affect expression of nitrogen fixation genes (Ausubel et al., 1979). Further, this ‘glnR’ gene was shown to give rise to two polypeptides of 36 KDa and 51 KDa which were redefined to be the products of ntrB and ntrC genes, respectively (Espin et al., 1982). Holtel and Merrick (1988) reported that PII protein, the sensor of cellular nitrogen status and a product of glnB gene, is involved in the same cascade. The uridylylated form of PII (PII-UMP) plays a significant role in response to changes in nitrogen status by counteracting the effect of PII on NtrB mediated dephosphorylation of NtrC. However, the PII protein is not involved in the nif specific response to changes in nitrogen status mediated by NifL (Holtel and Merrick, 1989). The glnB gene is regulated in various systems differently e.g. in case of Rhodobacter capsulatus, glnB is transcribed from two promoters - one of which requires NtrC but is rpoN independent and the other distal promoter is repressed by NtrC as it overlaps with the same site (Foster and Kranz, 1994). In case of Rhodospirillum rubrum, glnB is cotranscribed with glnA from a σ54 dependent promoter. However, an additional upstream σ70 putative promoter has also been identified (Johansson and Nordlund, 1996). In Azorhizobium caulinodans, glnB and glnA are organised in a single operon with a single start site, but they might be using different promoters, as glnB expression is high in both nitrogen excess and limiting conditions. PII is required only for symbiotic nitrogen fixation and is not necessary when the bacteria is growing as free living organism (Michel et al., 1997).

Another regulatory gene discovered in A. vinelandii, was defined and named nfrX (Santero et al., 1988). Later on, this gene was found to be highly homologous to that of the uridylyl transferase, the uridylyl removing enzyme encoded by glnD in E. coli. Contreras et al., (1991) suggested that nfrX modifies the activity of nifL product directly or indirectly. The UTase, a 90 KDa monomer, a product of glnD is considered to be the primary sensor of the cellular nitrogen status and responds to the intracellular ratio of α-ketoglutarate and glutamine pools. In nitrogen limiting condition, UTase mediates the uridylylation of PII by transfer of a uridylyl group onto a tyrosine residue on each of the four PII subunits (Figure 1.2). The K. pneumoniae glnD encodes for a 102.3 KDa polypeptide which is highly homologous to the predicted products of both E. coli glnD and A. vinelandii nfrX. glnD null mutant was unable to uridylylate PII involved in adenylation/deadenylation of glutamine synthetase. Uridylyl transferase is required for derepression of Ntr- regulated
Figure 1.2 The cascade of covalent modification that regulates the activity of the transcriptional activator NtrC in response to changes in intracellular nitrogen status. UR/UTase = uridylyl-removing enzyme, uridylyltransferase. +N, nitrogen excess; -N, nitrogen limitation.
promoters such as glnAp2 and nifL but is not involved in the nif specific response to changes in nitrogen status mediated by the NifL (Edwards and Merrick, 1995).

As already mentioned, NtrC is the next to be involved in this cascade. The uridylylated form of PII (PII UMP) promotes the phosphorylation of NtrC by NtrB. NtrB achieves the phosphorylation of NtrC in a two step process involving an autophosphorylation step (in which a histidine residue in NtrB is phosphorylated), followed by transfer of the phosphate to an aspartate residue in the N-terminus of NtrC (Keener and Kustu, 1988; Weiss and Magasanik, 1988).

NtrC has a DNA binding site, a phosphorylation site and an ATP binding site, each of which functions independently. A mutation at serine 170 to alanine in the proposed ATP binding site renders it nonfunctional to catalyse isomerization of the closed promoter complex between σ54 RNA polymerase and the nifL promoter to the open promoter complex. In some of the promoters the closed complex is also formed only in the presence of NtrC (Austin et al., 1991). Substitution of the serine residue 160 in the putative phosphoacceptor site of NtrC indicates that both the DNA binding and activator functions of NtrC are influenced by phosphorylation. Mutation at this site makes it less responsive to phosphorylation and dephosphorylation mediated by NtrB (Dixon et al., 1991). The whole cascade of regulation is summarized in Figure 1.3.

In other bacteria like Azospirillum brasilense also, ntrB and ntrC genes are present, arranged in a single open reading frame expressed from a promoter located upstream and negatively regulated by the ntrC gene product (Machado et al., 1995). The global (ntr) control of nif gene expression is mediated solely at the promoter level of the nifLA operon. The nifLA operon is expressed from a σ54 dependent promoter. Transcription from this promoter is positively regulated by the ntrC gene product (which coordinates the expression of many operons required for nitrogen assimilation) and also autogenously by the product of nifA gene (Drummond et al., 1983; Wong et al., 1987). In K. pneumoniae, two DNA binding sites for NtrC lie ~150 base pairs upstream of the NifLA promoter. The regulatory region upstream of NifLA promoter contains a NifA specific binding site at -59 and two NtrC binding sites at -142 and -163. The NifA binding site is required for the efficient transcription of nifF (present further upstream and divergently transcribed), whereas the NtrC binding site is necessary for nifLA expression. Phosphorylation of NtrC by NtrB is required for efficient binding of the former to these sites (Minchin et al., 1988).
Figure 1.3 Circuitry of the nif regulation in *K. pneumoniae* (From Gussin *et al.*, 1986)
Introduction

The NtrC assists in the formation of open complex from closed complex as it does in other $\sigma^{54}$, NtrC dependent gln promoters. Interestingly, it also stabilizes the interaction of $\sigma^{54}$ RNA polymerase with the -24, -12 region i.e. it catalyzes the closed complex formation (Minchin et al., 1989). Unlike the gln Ap2 promoter which is relatively insensitive to changes in DNA supercoiling, nifLA operon is supercoiling dependent. Open complexes formed on linear nifL promoter DNA are unstable and less extensive than those formed on supercoiled form. The nifLA promoter as a whole mediates the transcriptional response to DNA supercoiling. (Whitehall et al., 1992).

1.3.2 Second level of Control by nifLA gene product: In K. pneumoniae, the product of glnG gene activates transcription of nifLA operon in response to ammonia starvation. The nifA gene product is in turn required for transcription of all other nif operons, including the nifHDK operon. The nifHDK promoter has been mapped in K. pneumoniae and Rhizobium meliloti and the transcription start points have been determined. The lacZ fusion construct of both promoters in E.coli are activated by the product of K. pneumoniae nifA gene (Sundaresan et al., 1983). Similarly nifA gene product of K. pneumoniae can activate expression of genes in both A. vinelandii and A. chroococcum inspite of distinctly different physiology as compared to facultative anaerobic nature of K. pneumoniae (Kennedy and Robson, 1983). When K. pneumoniae nifH promoter was cloned in a multicopy plasmid, it inhibited chromosomal nif expression (Buchanan-Wollaston et al., 1981) which was proposed to be due to titration of the limiting regulatory factor, possibly NifA. When nifA gene was cloned in plasmid vectors and overproduced, it was found to be very insoluble and separated out with the cell debris. Localization of $\beta$-galactosidase activity from a nifA lacZ translational fusion confirmed the insoluble nature of nifA (Tuli and Merrick, 1988). Buck et al., (1986) later characterized the NifA binding domain present upstream of all nif promoters (except nifM and nifL) between 80 to 180 bp upstream of the transcription start site. Morett and Buck (1988) showed that the positive control protein NifA activates transcription of nif ($\sigma^{54}$ dependent) promoters by binding to these specific sites, known as upstream activator sequences (UAS). It was further shown that the UAS should be located on the correct face of the DNA helix with respect to the RNA polymerase recognition sequence in such a way that titration of nifA and efficient activation occur simultaneously (Buck and Cannon, 1989; Cannon et al., 1991).
Santero et al., (1989) have reported that the NifA protein was able to activate expression of a translational nifH-lacZ fusion in vitro in a coupled transcription translation system. Although NifA could stimulate the initiation of transcription by $\sigma^{54}$ holoenzyme from the nifHDK promoter in vitro but, it lost its activity very rapidly. It is now established that NifA actually activates transcription initiation by RNA polymerase containing $\sigma^{54}$ by catalysing the isomerization of closed promoter complexes to transcriptionally competent open complexes. The open complexes thus formed are comparatively more stable to heparin challenge in the presence of GTP than in the presence of ATP. This stability could be due to the fact that GTP is the initiating nucleotide (Eydmann et al., 1995). Functionally NifA possess two domains - a C-terminal domain (Carboxy terminus) which is the DNA binding, recognising domain and the second domain is the Central domain necessary for the positive control function. The function of the N-terminus is not yet known (Berger et al., 1994; Berger et al., 1995). Mutation in the carboxy terminus of NifA eliminates the DNA binding properties. However, it does not affect the positive activator function. The methylation protection assay also demonstrated that the UAS is recognised by the carboxy terminus of NifA (Bennett et al., 1988; Morett et al., 1988). The isomerization of closed complexes between $\sigma^{54}$ holoenzyme and a promoter to open complexes, catalysed by NifA is ATP dependent. The putative ATP-binding pocket in the central domain of NifA is necessary for the positive control function of NifA and is not required for DNA binding or recognition of NifA by NifL (Cannon and Buck, 1992; Ashraf et al., 1997). Osuna et al., (1997) using different secondary structure programme suggested that the central domain imparts an alpha/beta topology, a classical mononucleotide binding fold. The isomerization process is heat sensitive as NifA is heat labile. However, it retains the DNA binding ability indicating that the function of the helix-turn helix, DNA binding motif is not heat-labile (Morett and Buck, 1989; Lee et al., 1993).

In some of the organisms, it was found that another factor binds to the nifHDK promoter regulatory region that was later recognised to be integration host factor (IHF) binding site (Beynon et al., 1983). As this binding site lies between the upstream activator sequence (NifA binding site) and nifHDK promoter region, integration host factor could bend the DNA between these two sites to facilitate productive interactions between NifA and $\sigma^{54}$ holoenzyme (Figure 1.4; Buck and Cannon, 1989). NifA was not essential for formation of closed complex of nifH or nifU is case of K. pneumoniae. As in
Figure 1.4 Diagram depicting the role of IHF at the attachment site of phage and comparison with its postulated role in the *nifH* promoter regulatory region in *K. pneumoniae*. IHF is thought to bend the DNA at *attP* so that a monomer of integrase (Int) that is bound to the strong "arm-type" side can also contact a weak "core-type" site. In the same way IHF seems to be responsible for transcription activation of *nifH* promoter by protein-protein (NifA-σ^{54} holoenzyme) interaction (from Hoover *et al.*, 1990).
Introduction

*K. pneumoniae, nifH* and *nifU* promoter, the closed complex is formed even in the absence of NifA. NifA is essential for recruitment (Buck and Cannon, 1992). NifA solely activates (σ^54 dependent) under microaerobic conditions suggesting that oxygen sensitivity is an intrinsic property of the NifA protein (Shigematsu et al., 1997).

The *nifL* gene product of *K. pneumoniae* inhibits the activity of the positive activator protein NifA in response to increased levels of either fixed nitrogen or oxygen in the medium, both effectors being discrete. The NifL protein has been characterized in vitro. It has been shown that it dramatically reduces the activity of NifA in response to cellular Oxygen and Nitrogen status (Hill et al., 1981; Austin et al., 1990). NifL reduces the affinity of NifA for UAS, which involves interaction between NifL and NifA (Morett et al., 1990). The amino acid sequence of NifL exhibits partial or limited homology to the histidine protein kinases. Mutation in Gln273-Leu 317 region of NifL could facilitate interactions between NifL and NifA (Drummond and Wooton, 1987, Sidoti et al., 1993). In an in vitro transcription system, it was seen that NifL alone could inhibit transcription activation of NifA, and that no other factors were required for the same (Lee et al., 1993). As in case of *K. pneumoniae*, in *A. vinelandii* nifL was found just upstream of nifA. The C-terminal domain of NifL in *A. vinelandii* shows more homology to the histidine protein kinase than to *K. pneumoniae* NifL. The N-terminal region is more homologous to *bat* gene product. Unlike the *bat* gene product, it is not Oxygen sensitive and its expression is independent of RpoN or NtrC (Blanco et al., 1993, Raina et al., 1993). Contrary to other histidine autokinases in *Azotobacter vinelandii* NifL, no phosphorylation at His-304 (site of phosphorylation) is required for its activity.

NifL has been shown to interfere with contact between NifA and σ^54 holoenzyme (Berger et al., 1994). Edymann et al., (1995), have shown that Adenosine nucleosides (ADP) is specifically required for NifL to inhibit open complex formation by native NifA, in fact NifL modulates the activity of NifA via an adenosine nucleotide switch. It has also been shown that the inhibitory effect of NifL is localized to its carboxy terminal domain, which is more soluble than the intact protein itself. Any internal deletions in the amino terminal domain of NifL retained the inhibitory activity. Moreover, the maltose binding protein (MBP) fusion of the carboxy terminal domain alone exhibited the inhibitory effect which further confirmed the involvement of carboxy terminal domain in the inhibition of NifA activity (Narberhans et al., 1995). The *A. vinelandii* NifL protein comprises of a
relatively stable N-terminal domain and a C-terminal domain protected from trypsin
digestion in the presence of adenosine nucleotides. ATP imparts protection to the protein
from cleavage in the vicinity of potential nucleotide binding sites in the C-terminus
whereas ADP protects the entire C-terminal domain. The N-terminal domain is the flavin
binding domain. About 146 residues from N-terminal including a conserved s-motif of
NifL is found in large number of sensory proteins, wherein even the truncated protein is
competent enough to inhibit NifA activities in response to ADP. This confirms that redox
and nitrogen sensing functions of \textit{A. vinelandii} NifL are separable and discrete (Soderback
\textit{et al.}, 1998). Hill \textit{et al.}, (1996) showed that the purified NifL from \textit{A. vinelandii} is a flavo
protein with FAD as the prosthetic group which is reduced in the presence of sodium
dithionite. The oxidized form of NifL inhibits transcriptional activation by NifA. This
inhibition was reversed when NifL was in reduced form. Hence NifL is a redox-sensitive
regulatory protein and may represent a type of flavoprotein in which electron transfer is not
coupled to an obvious catalytic activity. It is also responsive to adenosine nucleotide
(particularly ADP) as reported earlier for \textit{K. pneumoniae} NifL. CD spectrum of purified
NifL indicated that FAD is bound to NifL in an asymmetric environment and the protein is
predominantly alpha-helical. The carboxy ferrohaem form of Hmp (flavohaem protein) was
competent to reduce NifL suggesting that electron donation to NifL is through flavin in
Hmp and not direct from the haem. Reoxidation occurs rapidly in air, hence the activation
of its inhibitory properties occur rapidly, in contrast to the switch from active to inactive
reduced form of NifL which occurs slowly (Macheroux \textit{et al.}, 1998). The purified NifL
carried FAD as cofactor irrespective of whether synthesized in the absence or presence of
nitrogen or oxygen. The redox potential of NifL-bound FAD remains more or less similar
both in the presence and absence of nitrogen source (Dixon R, 1998; Klopprogge and
Schmitz, 1999).

It is also very important to have a correct NifL-NifA stoichiometry for efficient
regulation of nitrogen fixation genes. In addition to transcriptional polarity there also is
translational coupling between \textit{nifL} and \textit{nifA}. Inspite of apparently efficient ribosome
binding site of \textit{nifA}, its rate of independent translation is very low. This is due to secondary
structure masking the Shine and Dalgarno sequence of \textit{nifA}, which is melted by ribosomes
translating \textit{nifL}. Translational coupling between the two cistrons is proposed as an efficient
mechanism to prevent production of an excess of NifA (Govantes \textit{et al.}, 1996).
Translational coupling in this case occurs by a reinitiation mechanism. Reinitiation at the \textit{nifA} can occur even in the absence of good Shine Dalgarno sequence. Positive translational enhancer sequences (downstream box) aid this (Govantes \textit{et al.}, 1998).

As has been said earlier, the inhibition of NifA by NifL requires stoichiometric amounts of the two proteins, implying that the mechanism of inhibition is by direct protein-protein interaction rather than by catalytic modification of the NifA protein. The formation of the inhibitory complex between NifL and NifA may be regulated by intracellular ATP/ADP ratio. The adenosine nucleotides (ADP) promotes complex formation and the removal of the nucleotide causes dissociation of the complex. The amino terminal domain of NifA and the C-terminal region of NifL are potential in the ADP dependent stimulation of NifL-NifA complex formation (Money \textit{et al.}, 1999).

Depletion of iron from the growth medium with the chelating agent o-phenanthroline mimics aerobiosis giving rise to inhibition of NifA activity even under anaerobic, nitrogen limiting conditions. Adding back Fe$^{++}$ in two fold excess restores NifA activity. However, there is no evidence that NifL contains Fe or Fe-S cluster (Schmitz \textit{et al.}, 1996). It is likely that an additional Fe-containing protein, not yet identified, is required to relieve NifL inhibition.

In \textit{Methanococcus maripaludis}, the genes of nitrogen metabolism are regulated coordinately at the transcriptional level via common repressor binding site or operator. This resembles the general bacterial paradigm for repression, but contrasts with well known mechanism of nitrogen regulation in bacteria. These also contained two \textit{glnB} genes involved in “ammonia switch off” the post transcriptional inhibition of nitrogen fixation upon addition of ammonia (Kessler and Leigh, 1999).

The binding activity of NifA to the UAS which resides in the C-terminal domain of NifA is not affected by the presence of oxygen in \textit{Azospirillum brasilense} (Passaglia \textit{et al.}, 1998). NifA from \textit{Herbaspirillum seropedicae} exhibits nitrogen control but no O$_2$ sensitivity or Fe dependence (Souza \textit{et al.}, 1999; Monteiro \textit{et al.}, 1999).

\textbf{1.4 Regulation of NifL activity}

The sensing of the cellular nitrogen status by UTase, the uridylylation of the PII protein in fixed nitrogen deficient condition, and the resulting phosphorylation of NtrC by NtrB which in turn acts as a transcriptional activator on the $\sigma^{54}$ dependent promoters, explains
the global regulation. NifL mediates the nif specific regulation. NifL is also regulated directly by fixed nitrogen and oxygen status of the cell, but is not related to the global regulation. There is evidence that NtrC is required for the control of K. pneumoniae NifL activity, as NtrC null alleles failed to release NifL inhibition, although NifL was no longer under Ntr control. It has been predicted that the transcriptional activation capacity of NtrC plays an important role. It activates transcription of a gene(s) whose product(s) in turn functions to relieve NifL inhibition under nitrogen limiting conditions. (He et al., 1997). A new gene glnK was discovered encoding a PII like allosteric effector in K. pneumoniae. Although the effector is structurally related to glnB, it cannot be substituted by GlnB. Moreover, product of glnK is involved in NifL regulation. Unlike GlnB, no uridylylation is required in case of GlnK for relieving NifL inhibition. NtrC acts as an activator for glnK transcription (He et al., 1998). Thus it can be concluded that control of NifL occurs at two levels: (i) Transcriptionally by global ntr system regulating the nifLA operon, and (ii) by regulation of NifL mediated inhibition of NifA by unknown factors as well as by GlnK. K. pneumoniae synthesizes two PII like signal transduction proteins GlnB and GlnK of which only GlnK is associated with NifL mediated inhibition of NifA. The GlnK aids NifL to detect nitrogen deficient condition and thereby relieves NifA inhibition directly or indirectly (Jack et al., 1999).

Similarly in A. vinelandii glnK has been identified whose translation product is highly similar to PII protein from other organisms. (Meletzus et al., 1998).

1.5 σ^{54} Dependent promoters

Bacterial sigma factors are a family of proteins that confer upon RNA polymerase the ability to recognize specific promoter sequences (Helmann and Chamberlin, 1988). Most of these sigma factors belong to a single family of proteins which appear to be related structurally and functionally to the major E. coli sigma factor, σ^{70}. However, many prokaryotes are now known to contain a novel sigma factor, σ^{54} (σ^{N}) which bears little or no resemblance to other members of the σ^{70} family, either structurally or in the mode of action. Thus, the enteric sigma factor σ^{54} and its relatives constitute a class of their own (Merrick, 1993). Level of σ^{54} is about 10% of the level of σ^{70} (Jishage et al., 1996) in E. coli. Genes expressed by RNA polymerase σ^{54} control diverse cellular functions and their promoters are characterized by the consensus sequence 5' TGGCAC-N5-TTGCA
located 11 to 12 bp from the transcription start site (Gussin et al., 1986). Generally, the polymerase $\sigma^{70}$ recognizes characteristic promoter sequences found upstream of the transcription start site. While some promoter sequences direct efficient transcription initiation in the absence of activators, others require activators for their activity. These activators work by recruitment i.e. the typical activator works by binding to specific sites on DNA and then contacts the RNA polymerase, essential for proper binding. On the contrary, $\sigma^{54}$ dependent polymerase binds stably and tightly to the promoter, and needs activators only for transcription initiation, which requires hydrolysis of ATP (Ptashne and Gann, 1997).

The nif genes generally carry $\sigma^{54}$ dependent promoters. Here the activator involved in most of the structural nif genes is NifA, the product of the nifLA operon. Whereas for nifLA operon, the positive activator is NtrC instead of NifA, which senses the fixed nitrogen and oxygen status through glnB gene product (Merrick, 1992; Gussin et al., 1986). The role of NifA/NtrC is in the formation of an open promoter complex from closed complex formed by binding of $\sigma^{54}$ holoenzyme to the promoter (Morett and Buck, 1989) leading to the initiation of transcription. The double stranded DNA is opened locally and transiently during the act of transcription (open complex formation). The opening up of DNA requires energy and is a slow and critical rate limiting step in transcription (Gralla, 1993). As mentioned earlier, $\sigma^{54}$ RNA polymerase holoenzyme has the distinctive property of binding to promoter to form a closed promoter complex, which isomerizes to the open complex only when acted on by an enhancer binding activator protein. When the holoenzyme binds to the promoter and makes contact at the -12 region, it promotes a local DNA distortion immediately adjacent to this promoter element. Only the complexes that contain the distortion are activated. The activator protein may promote a conformational change in the $\sigma^{54}$ holoenzyme to allow propagation of the altered DNA conformation, probably local unwinding, necessary for formation of melted DNA state (open promoter complex) (Morris et al., 1994). $\sigma^{54}$ polymerase complex begins transcription in abortive cycling mode i.e. after the first bond is made, approximately 75% of the time the short RNA is aborted and synthesis must be restarted. Polymerase is liable to abortive initiation until it reaches a position beyond +3 and before +7, at which stage polymerase is released from its promoter contacts and an elongation complex is formed (Tintut et al., 1995).
The interaction of $\sigma^{54}$ with promoter DNA is via DNA-protein contact. The DNA contacting surface lies inside the carboxyl terminal one-third of the $\sigma^{54}$ protein. This region is thought to be close to the surface of the protein and contacts DNA when either $\sigma^{54}$ or holoenzymes binds specifically to promoter DNA. (Cannon et al., 1994). Close contacts between the promoter and sigma factor is restricted predominantly to one face of the DNA helix extending from bases -31 to -5. The $\sigma^{54}$ protein makes multiple DNA backbone contacts across and beyond the -12, -24 consensus. Thymidine residues contributes towards important major groove contacts.

The $\sigma^{54}$ protein can be basically divided into 3 regions. The N-terminal (region I) which is essential for the correct interaction of holoenzyme with the promoter. Region III includes the C-terminal involved in specific DNA recognition as well as constitutes the core binding domain that resides in the adjacent peptide. Both of these are bipartite in function. Region II (acidic) is the middle region, influences both the above activities indirectly (Cannon et al., 1995). The secondary structure of $\sigma^{54}$ displays a two stage melting curve with a first melting temperature of 36°C and second melting temperature of 85°C. The second melting curve involves only C-terminal (Region III) that is important for structural integrity at high pH (Cannon et al., 1997).

$\sigma^{54}$ has a region homologous to $\sigma^{70}$, such residues are clustered closely and are involved in the binding to the core polymerase. Although $\sigma^{54}$ binds to the core polymerase through sequences homologous to $\sigma^{70}$, it uses unique motifs to modify this interaction (Tintut and Gralla, 1995). The C-terminal end of $\sigma^{54}$ is characterized by the presence of a highly conserved sequence of 10 amino acids (ARRTVAKYRE) termed as RpoN box that plays a significant role in recognition of -24 and -12 promoter consensus (Taylor et al., 1996). Amino acids 363 and 383 of $\sigma^{54}$ play a major role in binding to promoter as point mutation at either position destroys DNA binding activity (Guo and Gralla, 1997). The conserved phenylalanine residues in the DNA binding domain contribute to $\sigma^{54}$ function. However any mutation in these does not affect the binding but only affects the open complex formation (Oguiza and Buck, 1997).

 Majority of the $\sigma^{54}$ dependent promoters use transcriptional activators, the most common being those needed for nif gene regulation i.e. NifA and NtrC. The activator protein binds to the enhancer sequences around 100 to 150 bp upstream of the promoter.
region. NtrC generally binds to two sites. The binding at these sites is cooperative and facilitates the oligomerization of NtrC-phosphate endowing it with the ATPase activity required for its ability to serve as transcriptional activator (Brahms et al., 1995). It is interesting to note how an activator bound about 100 to 150 bp upstream reacts with the promoter that is bound to holoenzyme to form the open complex. Therefore, it was proposed that the DNA could bend due to intrinsic curvature or because of binding of DNA proteins like IHF which cause bending between the enhancer and promoter region. As a result, the activator and the holoenzyme are able to interact (Figure 1.5) (Merrick 1992). The transcriptional activation of *E. coli* RNA polymerase $\sigma^{54}$ at *glnA* promoter by NtrC was studied using scanning force microscopy. It was observed that different multimeric complexes of NtrC were bound to DNA template, such as closed complex of polymerase and $\sigma^{54}$, association of NtrC and promoter bound $\sigma^{54}$ and looping out of intervening DNA, the activated open promoter complex were also visualized (Rippe et al., 1997). The role of IHF in some $\sigma^{54}$ promoters is not only a structural aid for assembling a correct promoter geometry but also that of an active suppressor (restricter) of promiscuous activation by heterologous regulators for increased promoter specificity (Perez Martin and Lorenzo, 1995). Sometimes the IHF is replaced by HU which is a non-specific DNA binding protein that also causes bends and has a preference for stretch of A or T residues (Perez Martin and Lorenzo, 1995). Activation of transcription at $\sigma^{54}$ dependent promoters on linear template requires intrinsic or induced bending of the DNA (by IHF or HU) but the supercoiled DNA containing the $\sigma^{54}$ promoter may bypass this need (Carmona and Magasanik, 1996). It has also been reported that occasionally the coactivation effect of IHF can not only be mimicked by HU but also by HMG-1 (High mobility group) or intrinsically curved DNA (Perez Martin and Lorenzo, 1997; Carmona et al., 1997). The interaction of NifA (activator) with UAS is not strongly cooperative with holoenzyme and IHF, indicating that activation mechanism does not involve simple recruitment of factors to the promoter alone (Wang et al., 1997). The IHF not only activates the transcription but also restricts transcription from non-specific sequence. A precise promoter geometry is essential for IHF to positively regulate transcription and there by preventing activation from inappropriately spaced upstream sites (Dowrkin et al., 1997; 1998). The open complexes could be formed either on negatively supercoiled DNA or relaxed DNA, however, not on a positively
Figure 1.5 The interaction of NtrC and Eσ^{54} polymerase in transcriptional activation of the nifLA promoter of K. pneumoniae.
supercoiled DNA when NtrC and ATP are present, there by suggesting a presence of a thermodynamic barrier to the formation of open complex (Qureshi et al., 1997).

At times the activator of one promoter is inhibitory to other promoters. Like $\sigma^{54}$ transcriptional activator FlbD which activates specific transcription of a subset of late flagellar genes, it is capable of functioning as a specific repressor of the early flagellar flF operon thus accomplishing two regulatory mechanisms simultaneously (Wingrove and Gober, 1994). Similarly, in *Caulobacter crescentus* flagellar gene regulation flbD encodes a transcriptional activator for activation of $\sigma^{54}$ dependent promoter at levels III and IV and negative autoregulation of the level II flF promoter (Ramakrishnan et al., 1994). In *Rhizobium meliloti* dctA expression is mediated by activator DCTD and DCTB (DCTB is the sensor and DCTD is activator in phosphorylated form). NtrC can also act as an activator individually but not when DCTB or DCTD is present (Labes and Finan, 1993).

Certain mutants of $\sigma^{54}$ have been isolated which could bypass the need for enhancer protein. These mutations were located to four leucines in a small region between amino acids 25 and 31, indicating that stretch of leucines may be critical for keeping polymerase function in check (Syed and Gralla, 1997).

Further 3 pathways were found that bypass the enhancer requirement *in vitro*, these are either deletion of the $\sigma^{54}$ N terminus, or destruction of the DNA consensus -12 promoter recognition element or altering solution conditions to favour transient DNA melting (Wang et al., 1997).

In *K. pneumoniae* nifLA operon $\sigma^{54}$ dependent promoter has been identified and the transcription start site also has been mapped. Drummond et al., (1983) has further characterized two NtrC binding sites upstream of the promoter by footprinting analysis, leading to nearly complete understanding of nitrogen fixation and regulation of nifLA operon. On the other hand, *A. vinelandii* regulatory operon nifLA is not yet fully understood. Raina et al., (1993), who sequenced the nifLA upstream region indicated a putative $\sigma^{54}$ binding site. Blanco et al., (1993) mapped the transcription start site experimentally. However, the distance between the putative promoter and the start site does not match with the consensus sequence. Therefore, they had concluded that although there is a $\sigma^{54}$ like promoter region, it is nonfunctional and some alternative sigma factor may be.
involved. The nifLA promotor was observed to be active even in NtrA deficient strain supporting the above view (Raina et al., 1993).

Therefore, the present investigations were initiated with the aim to deduce the mechanism of regulation of the nifLA operon in A. vinelandii with respect to the following aspects.

(i) Identification and characterisation of functional promoter and sigma factor responsible for transcription initiation.

(ii) Identification of regulatory elements (activator and enhancers) and their binding sites affecting the expression of nifLA operon.

A brief account of the characteristic features of study material, the methods followed and experimental findings (supported by tables and figures wherever required) is prescribed in the subsequent pages which is followed by a discussion highlighting the salient features of the present investigation in light of contemporary literature. Although efforts have been made to consult all scientific literature available in the area of the present studies, it is likely that a few papers may have escaped any attention.