Chapter 3

Effect of Nitrogen sources on the excision of *nifD* element of *Anabaena* PCC 7120

3.1 Introduction

The most abundant forms of nitrogen in both aquatic and terrestrial habitats are the inorganic species, dinitrogen (N_2) and to lesser extent nitrate and ammonium. Assimilation of nitrogen sources like ammonium, nitrate and nitrite are mediated by glutamine synthetase-Glutamine amide-2-oxoglutatrae amidotransferase (GS-GOGAT) system. GS (GlnA) activity is controlled by cumulative feedback inhibition, adenylation/deadenylation and gene expression (Rudnick *et al.*, 2002). Adenylyltransferase, encoded by *glnE* gene, catalyzes reversible adenylation (inactivation) of GS in response to nitrogen excess. Expression of *glnA* gene encoding GS protein is regulated by NtrBC two component system.

Nitrogen sensing in *Escherichia coli* and other enterobacteria involves concerted activities of the glutamine sensor, GlnD (Uridylyltransferase/ Uridylyl-removing enzyme) and one or more trimeric signal transduction components generally referred to as PII proteins (**Fig. 1**) (Maheswaran and Forchhammer, 2003). In *E. coli* nitrogen limitation sensed as an internal glutamine deficiency, results in the uridylylation of GlnB (PII) by GlnD and also in the expression and uridylylation of a second PII like protein, GlnK. Uridylylation of GlnB and GlnK prevents activation of the phosphatase activity of NtrB whereas unmodified GlnB brings about the phosphorylation of NtrC (Nitrogen regulator I- NR_I). Phosphorylated NtrC (NtrC~P) forms tetramers and activates a set of genes required for growth under these conditions (Merrick & Edwards, 1995). GlnB is allosterically regulated by 2-oxogulatarate, thus GlnB integrates signals of C/N status. In *E. coli* NtrB/NtrC two component regulatory

system and GlnE act as a receptor for GlnB signals. Ntr regulon activated by enhancer sequences bound to the transcriptional activator NtrC in *E. coli.* Activation of *glnA*, *glnK* and *nac* genes occur during the transition from growth on ammonium to nitrogen starvation in NtrC dependent manner (Atkinson *et al.,* 2002). When PII and GlnK are uridylylated, *glnA* gene expression gets activated by NtrC~P and GS is present in activated (deadenylated) form (Merrick & Edwards, 1995; Van Heeswijk *et al.,* 1996).

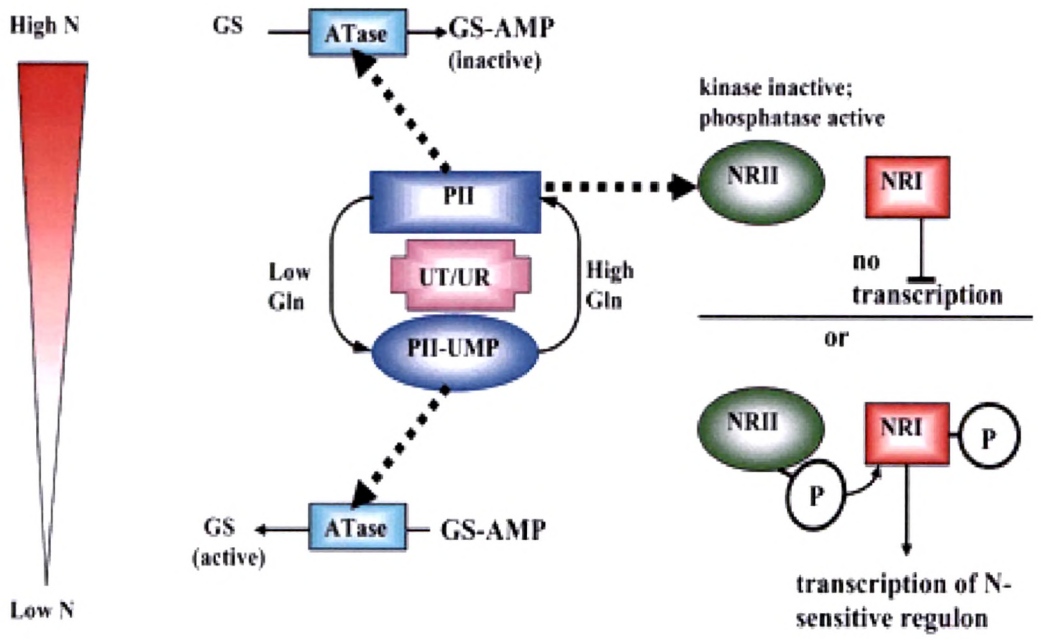

Fig. 1. Mechanisms of sensing N status in *E. coli* (Moorhead and Smith, 2003).

In response to nitrate, 51 operons were directly or indirectly activated by NarL and 41 operons were repressed (Harrod *et al.,* 2004). Limitation of nitrogen activates about 100 genes in *E. coli* by NtrC regulator. Phosphorylation of NtrC leads to transcription activation of promoters of many genes along with sigma 54 (σ54) dependent RNA polymerase.

Media components and growth conditions influence the expression of genes associated with catabolism. HtrD protein is involved in the regulation of intracellular cysteine levels as well as cysteine transport. *E. coli htrD* mutant had severe inhibition or

decreased survival at higher temperature in rich media (Delaney *et al.,* 1992). *lysS* and *lysU* genes code for lysly-tRNA synthetase. In rich media, *lysU* product in *E. coli* is present at very low and very high levels in aerobic and anaerobic conditions, respectively. However, in minimal medium expression of *lysU* was induced at low levels and it is very sensitive to anaerobiosis (Leveque *et al.,* 1991). Expression of *acrB*, *marA* and *soxS* genes were increased in early and late logarithmic growth phase in minimal medium compared to rich medium (Bailey *et al.,* 2006).

In logarithmic phase of rich media, genes encoding for proteins related to translation are expressed at very high rate in growth rate dependent manner. In minimal medium supplied with glucose as a sole carbon source during logarithmic phase, a significant increase in gene expression is seen in almost 50% of the RpoS dependent genes (Tao *et al.,* 1999). In rich medium, out of 409 genes involved in the carbon and energy metabolism only 8 genes were expressed at significantly higher level but in minimal medium 31 genes expressed at significantly higher level. These included genes involved in D-lactate utilization (*dld*), acetate formation (*poxB*), regulation of *poxB* expression (*rpoS*), acetate utilization (*aceA*, *aceB*, *gltA*, *icd*, and *mdh*), and coupling of glucose and acetate cometabolism (*uspA*). It suggests metabolism of D-lactate and acetate as prominent feature in glucose minimal medium. In this condition cell excretes acetate and low amount of D-lactate as overflow metabolites (Cronan & LaPorte, 1996; Neijssel *et al.,* 1996). Excretion of acetate leads to protection of the *E. coli* cells to the acetate stress in minimal medium (Tao *et al.,* 1999).

Effect of molar levels of ammonium on *E. coli* showed high resistance and cell growth due to high osmolarity or increase in ionic strength of the medium (Muller *et al.,* 2006). In *E. coli* K-12, γ-glutamyl-γ-aminobutyrate (GABA) hydrolase protein encoded by *puuD* gene expression gets reduced in the presence of NH_4Cl. PuuD is physiologically important for growth on putrescine as sole nitrogen source (Kurihara *et al.,* 2006).

In addition to the nutrients in the medium, aerobic and anaerobic growth conditions significantly alter gene expression. Microarray and bioinformatics tools revealed that the fumarate and nitrate reductase (FNR) regulon may include 104-115 operons (Overton *et al.,* 2006). During anaerobic growth 36 operons were repressed while 68 operons were activated.

Faster-growing cells contain proportionally more stable RNAs—rRNA and tRNA. The reason for this increased abundance of stable RNA could be very high protein synthesis requirement for fast growth. The growth rate of the bacterial cell increases in proportion to the quality of the growth medium (although not necessarily in proportion to its exact composition), and this increase in growth rate was accomplished by an increase in the number of ribosomes and the concentrations of translation accessory factors (Bremer & Denis, 1996). Seven *E. coli* rRNA operons are under the control of growth rate-dependent promoters and that expression of the ribosomal proteins, translation factors, and the transcription apparatus was all tied to the cellular concentration of rRNA (Bremer &Denis, 1996; Grunberg-Manago, 1996; Keener & Nomura, 1996). The rate of transcription initiation of the growth rate dependent *rrn* promoters is physiologically connected to the metabolic state of the cell by the concentration of nucleoside triphosphates—efficient transcription initiation from these promoters requires a high concentration of the initiating nucleotide (Gaal *et al.*, 1997).

The presence of high-quality nutrients in the growth medium results in high intracellular nucleoside triphosphate concentrations; hence, this model unifies the idea that the quality of the growth medium dictates the growth rate of the cell. Growth rate-dependent changes in cell composition are realized at the level of gene expression; for example, transcript levels corresponding to the protein components of the protein synthesis apparatus change in proportion to the growth rate as the rates of transcription or mRNA turnover are modulated (Grunberg-Manago, 1996; Keener & Nomura, 1996).

Other changes in cellular physiology can be more subtle, such as redirection of intermediary metabolism in response to changes in growth medium composition or the flow of carbon and electrons that is coupled to ATP generation, although many of these adjustments in metabolism are accompanied by changes in the concentrations of metabolic enzymes and electron transport chain components (Smith & Neidhardt, 1983a; Smith & Neidhardt, 1983b; Lynch & Lin, 1996; Magasanik, 1996; Saier, *et al.*, 1996).

Brusca *et al.*, (1990) overexpressed the *xisA* gene in *Anabaena* PCC 7120 vegetative cells and resulted in the excision of *nifD* element in the vegetative cells. Interestingly,

Razquin *et al.*, (1994) showed that under iron stress conditions the excision of the 11kb *nifD* element occurred in *Anabaena* PCC 7120 even in the presence ammonium. Thus, it appears that the frequency of excision of the *nifD* element in *E. coli* may be influenced by certain accessory proteins.

In order to determine such components, the excision of *nifD* element was monitored in *E. coli* strains which were grown in different media and culture conditions. Excision frequency was less in *recA* mutant which implicated the involvement of RecA protein in the rearrangement event. *E. coli* JM101 strain in LB and M9 minimal media showed 0.3% to 10% respectively (Karunakaran, 2000).

In order to understand the intracellular conditions prevailing during the excision events in the heterocysts, present study dealt with determining the effect of different growth media on the rearrangement of *nifD* element of *Anabaena* PCC 7120 in *E. coli*. Since earlier studies in our laboratory had demonstrated the excision efficiency in minimal medium than in Luria broth, the present excision studies were carried with (i) casamino acid as a nitrogen source (ii) casamino acid as both carbon and nitrogen source, and (iii) iron sufficient and deficient conditions.

3.2 Materials and Methods

Please refer chapter materials and methods.

3.3 Results and Discussion

3.3.1 Excision of *nifD* element in the presence of Casamino acids

Rearrangement of the *nifD* gene was monitored in *E. coli* JM101 (pMX25) strain in the presence of casamino acids as N source and as both C and N source (**Table 1**). Rearrangement frequency after 24 h of growth was around 2% in LB, 11% in M9 minimal medium containing 40mM NH_4Cl, 15% in M9 minimal medium containing 0.2% Casamino acid as nitrogen source and 23% in M9 minimal medium containing 1% Casamino acid as both carbon and nitrogen sources. In the absence of glucose, 1% Casamino acid was sufficient carbon as well as nitrogen source. In comparison to NH_4Cl, the rearrangement frequency was again increased up to 1.4 times and 2.3 times in 0.2% and 1% Casamino acid concentration, respectively. In Luria broth, rearrangement frequency is 5 times less than that on NH_4Cl while on Casamino acids it was less by 14 to 23 fold. High excision events on Casamino acid could be due to the stimulating factors for growth. Arginine, histidine and ammonia present in casmino acids were shown effective as stimulants (Kumagai *et al.*, 1968).

E. coli JM101 (pMX25/pMC71A) strain had positive effect of the *nifA* gene on the rearrangement under different medium conditions (**Table 2**). In rich medium like Luria broth, the excision of *nifD* element was increased by around 2 fold in the presence of NifA protein. In presence of NifA, 15 fold higher rearrangement a frequency was found in M9 minimal medium containing 40 mM NH_4Cl than that in LB. When glucose used as carbon source and 0.2% Casamino acid was used as nitrogen source, the excision increased by 10 fold higher than that on in medium with 40 mM NH_4Cl as nitrogen source. But as casamino acid concentration was increased form 0.2% to 1% and in absence of glucose as carbon source it has similar effect to that of 0.2% casamino acid with glucose. Excision of *nifD* element was found decreased in both conditions, when casamino acid was used at 0.2% concentration in presence and absence of glucose as carbon source in compare to the 40 mM NH_4Cl with glucose as nitrogen and carbon source respectively. While it is 5 fold higher in presence of 1% casmino acid along with glucose compare to Luria broth but 3 fold lower in compare to M9 minimal medium with 40 mM NH_4Cl. No significant increase in the excision was

observed with the amount of casmino acid. In presence of NifA, higher concentration of casmino acid has not that much effect in comparison to nitrogen source as 40 mM NH_4Cl.

NifA protein enhanced excision by 4 fold in M9 minimal medium and 0.2% casamino acid (**Table 1 & 2**). It was 25% less compared to M9 minimal medium where NH_4Cl used as nitrogen source (**Table 2**). Rearrangement frequency was around 50% in which carbon and nitrogen sources were glucose and 0.2% casamino acid and 1 % casamino acid alone as source, respectively. In M9 minimal medium with 1% casamino acid used as nitrogen source and glucose used as carbon source, rearrangement frequency was half in compare to absence of glucose and presence of 1% casamino acid (**Table 2**). These results demonstrate that the rearrangement of *nifD* gene facilitated by NifA protein is dependent on the nature of carbon and nitrogen sources. The reason for these variations could be due to the increased level of either *xisA* gene expression or other accessory factors.

3.3.2 *xisA* promoter assay

Promoter activity of the *xisA* gene was determined by β-galactosidase assay in logarithmic and stationary phases. In general, β-galactosidase activity was high in the stationary phase. In Luria broth β-galactosidase activity was about 256 U and 721 U in the logarithmic and stationary phases, respectively (**Table 3**). Similar pattern was found in M9 minimal medium but lesser than that in Luria broth. Hence, the rearrangement frequency of *nifD* gene was independent of *xisA* expression as high *xisA* expression in Luria broth than in M9 minimal medium whereas the excision pattern was different. Activity of *xisA* gene was not affected by the nitrogen source NH_4Cl or casamino acid. These results suggest that some other factor(s) may also be playing a role in the stationary phase especially in M9 minimal medium which could be present at lower amounts in the Luria broth. Previous studies have implicated a role for RecA protein (Karunakaran, 2000).

3.3.3 Effect of Fe status on the excision of *nifD* element

In both Fe-sufficient and Fe-limiting conditions, the rearrangement of *nifD* element was high and was similar extent to that brought about by NifA was present in the M9 minimal medium with 1% casamino acid in presence and absence of glucose (**Table 4**). These results are contrary with the previous results that iron status had no effect on the excision (Karunakaran, 2000). It is not clear the reasons for these variations in the excision frequency.

3.3.4 Excision in anaerobic and aerobic conditions

In *Anabaena* PCC 7120, excision of *nifD* element occurs in heterocysts which contain anaerobic condition. In order to understand the effect of anaerobic conditions, rearrangement was monitored in *E. coli* JM101 (pMX25) strain. Around 5% excision frequency was detected after 24 h of growth in anaerobic condition (**Table 5**). Thus anaerobic conditions may not contribute to the excision in the heterocysts.

In conclusion, rearrangement frequency showed dependency on nitrogen metabolism of the *E. coli*. Excision studies in *E. coli* could be an underestimate of the rearrangement events compared with that in *Anabaena* PCC 7120 as only one copy of *nifD* element per genome is present in the heterocysts while the substrate plasmid expected to be present in 15-25 copies in a bacterial cell and white colonies can only be formed when all copies undergo excision. Excision of *nifD* element is dependent on the accessory factors in addition to the amount of XisA protein.

Table 1 Excision of *nifD* element of *Anabaena* strain PCC 7120 in *E. coli* JM101 (pMX25) strain grown in the presence of casamino acids.

Condition/Time	No. of Blue	No. of White	Total	% Excision ±SD
0 h	2402	14(9)	2407	0.20 ± 0.15
24 h LB	4219	84	4303	1.95 ± 0.32
24h M9 min + 40 mM NH_4Cl + Glc	5399	661	6060	10.90 ± 7.48
24h M9 min + 0.2% CA + Glc	4006	652	4658	14.31 ± 2.67
24h M9 min + 1% CA – Glc	3689	1029	4718	23.14 ± 3.49
24h M9 min + 1% CA + Glc	3304	937	4241	23.61 ± 3.84

Table 2 Excision of *nifD* element of *Anabaena* strain PCC7120 in *E. coli* JM101 (pMX25/pMC71A) strain grown in the presence of casamino acids.

Condition	No. of Blue	No. of White	Total	% Excision ± SD
0 h	5018	21(7)	5032	0.27 ± 0.27
LB	1078	57	1135	5.02 ± 1.58
M9 min + 40 mM NH_4Cl + Glc	794	2392	3186	75.07 ± 8.99
M9 min + 0.2% CA + Glc	2812	3544	6356	56.04 ± 2.95
M9 min + 1% CA – Glc	4882	4889	9971	51.66 ± 3.79
M9 min + 1% CA + Glc	5901	2041	7942	26.33 ± 3.14

Table 3 Expression of PxisA::lacZ on different conditions in *E. coli* JM101 strain pMU575 and *E. coli* JM101 strain pKK1 at log and stationary phase grown cells.

Condition	pMU575		pKK1	
	Logarithmic phase	Stationary phase	Logarithmic phase	Stationary phase
LB	ND	23.5 ± 5.2	265..9 ± 49.5	721.8 ± 5.28
M9 medium with 40mM NH_4Cl	ND	13.5 ± 2.08	340.7 ± 35.6	734.7 ± 162.9
M9 medium with 0.2% CA	24.8 ± 9.4	27.1 ± 12.3	354.8 ± 95.8	401.9 ± 187.5
M9 medium with 1% CA –Glucose	21.6 ± 2.5	25.6 ± 2.7	526.5 ± 36.2	568.0 ± 55.3
M9 medium with 1% CA and Glucose	4.8 ± 2.0	18.1 ± 1.8	279.3 ± 45.3	474.6 ± 53.7

Table 4 Effect of Fe^{3+} and N source on the excision of *nifD* element of *Anabaena* PCC 7120 in *E. coli* JM101 (pMX25) strain.

Condition/ Time	No. of Blue	No. of White	Total	% Excision ± SD
0 h	3907	110	4017	2.68 ± 0.75
24h M9 minimal Fe limiting 0.9μM Ferric ammonium citrate	1067	961	2028	47.04 ± 7.064
24h M9 minimal Fe sufficient 30μM Ferric ammonium citrate	809	887	1696	54.27 ± 7.56

Table 5 Excision of *nifD* element of *Anabaena* PCC 7120 in *E. coli* Strain DH5α containing pMX25 under anaerobic conditions.

Condition/ Time	No. of Blue	No. of White	Total	% Excision ± SD
24h LB	2172	95	2267	4.6 ± 0.11
24h M9 minimal media	731	37	768	4.9 ± 0.42