The Genetic Complexity of Nitrogen Fixation

The Ninth Fleming Lecture

By RAY A. DIXON

AFRC Unit of Nitrogen Fixation, University of Sussex, Brighton BN1 9RQ, UK

*(Delivered at the 100th Ordinary Meeting of the Society for General Microbiology,
11 April 1984)*

INTRODUCTION

The biological process of nitrogen fixation, which can only be rivalled chemically at high temperatures and pressures by the Haber-Bosch process, is a property which is found among a diverse number of non-related micro-organisms. In the late 1960s and early 1970s the nitrogen-fixing enzyme, called nitrogenase, was purified from a wide variety of organisms; it soon became apparent that the enzyme was a complex of two component proteins and that these proteins were extremely oxygen-sensitive, being irreversibly inactivated by oxygen in a matter of minutes. It was also known, from studies both *in vitro* and *in vivo*, that nitrogen fixation is a very energy-intensive process, requiring around 16 moles of ATP per mole of dinitrogen fixed (for a general review see Postgate, 1982). However, in 1970 nothing was known about the genes for nitrogen fixation and systems for genetic analysis of nitrogen-fixing bacteria had not been developed. It was therefore prudent to initiate genetic studies on the free-living nitrogen-fixing bacterium *Klebsiella pneumoniae*, a ubiquitous enteric organism which was expected to have similar genetic properties to the more widely studied *Escherichia coli* and *Salmonella typhimurium*.

Initially, it was envisaged that there would not be many genes required for nitrogen fixation. The biochemical work showed that nitrogenase consisted of two component proteins, so it was expected that only a few genes were involved. The early work established that nitrogen fixation (*nif*) genes mapped close to genes for histidine biosynthesis in *K. pneumoniae* (Streicher *et al.*, 1971; Dixon & Postgate, 1971) and that when the histidine (*his*) operon and adjacent DNA were transferred by conjugation into *E. coli*, the recipients gained the ability to fix nitrogen (Dixon & Postgate, 1972). This suggested that genes required for nitrogen fixation were located close to the histidine biosynthesis operon in *K. pneumoniae* and that they could be transferred into *E. coli* as a package which was regulated normally. However, when the *nif* region was analysed in more detail, the genetic organization was found to be far more complex than initially envisaged and we now know that 17 genes are required for the synthesis and activity of nitrogenase in *K. pneumoniae*. This finding was surprising in biochemical terms; it led to the discovery of a specific electron transport pathway to nitrogenase in *K. pneumoniae* as well as demonstrating that nitrogenase polypeptides require modification and/or processing for activity. It facilitated the discovery and assay of an iron- and molybdenum-containing cofactor, which has now been shown to contain the active site of nitrogenase. A further level of complexity was found in the genetic control of nitrogen fixation, since it was shown that the *nif* genes are regulated by a cascade mechanism, involving a series of transcriptional control circuits. In this article, I will review these developments, concentrating briefly on the organization of the *nif* genes and emphasizing the complex manner in which they are regulated.

ANALYSIS OF NIF GENES IN KLEBSIELLA

A combination of genetic mapping, complementation analysis, cloning and sequencing studies have established that 17 contiguous *nif* genes are clustered close to the *his* operon in

Fig. 1. Map of the *nif* gene cluster in *K. pneumoniae*, indicating the transcriptional organization and functions of the genes. The roles of the gene products are indicated schematically by the vertical arrows above the genetic map. The horizontal arrows indicate the extent and direction of each transcript, black dots representing the location of *nif* promoters.

K. pneumoniae, occupying 23 kb of the genome (Fig. 1). Most of the *nif* cistrons are transcribed in the same direction as the *his* operon but two unlinked genes, *nifF* and *nifJ*, are transcribed in the opposite orientation. This is of interest when the function of these two cistrons is taken into consideration since they both encode components of a specific electron transport pathway to nitrogenase (Roberts *et al.*, 1978; Hill & Kavanagh, 1980). The *nifF* product is a flavodoxin (Nieva-Gomez *et al.*, 1980) whereas the *nifJ* product derives reducing power from anaerobic metabolism as a pyruvate:flavodoxin oxidoreductase (Shah *et al.*, 1983).

Nitrogenase consists of two component proteins, Kp2, a dimeric iron-sulphur protein, and Kp1, a molybdenum- and iron-containing protein of two non-identical subunits which in its native form is an $\alpha_2\beta_2$ tetramer. Three genes, *nifH*, *nifD* and *nifK*, encode the three structural polypeptides of nitrogenase: *nifH* determines Kp2 while *nifD* and *nifK* code for the two subunit types of Kp1 (Dixon *et al.*, 1977; Roberts *et al.*, 1978). Although mutations in these three genes can clearly affect the structure of the nitrogenase proteins, other *nif* mutations were observed to impair nitrogenase function without altering the electrophoretic mobility of the component polypeptides. *nifM* mutants produce inactive Kp2 protein and the *nifM* product may therefore be required for processing this protein (Roberts *et al.*, 1978). Mutations in several different genes were shown to affect the activity of Kp1. Nitrogenase activity could be restored in some mutant crude extracts by adding an acid-treated extract of Kp1, which contains an iron-molybdenum cofactor known as FeMoco (Shah & Brill, 1977). Three genes, *nifB*, *nifN* and *nifE*, are now known to be involved in the synthesis of this cofactor. Mutations in a fourth gene, *nifV*, result in synthesis of Kp1 protein with an altered substrate specificity (McLean & Dixon, 1981). Since *NifV*⁻ strains produced a defective FeMoco, this result suggests that the cofactor contains or is part of the active site of nitrogenase (Hawkes *et al.*, 1984). The *nifQ* product may sequester molybdate required for FeMoco synthesis (Imperial *et al.*, 1984). The *nifS* and *nifU* gene products may also be involved in processing of Kp1 protein (Roberts *et al.*, 1978) but their precise function still remains elusive. The *nifX* and *nifY* genes were identified as polypeptide products synthesized from cloned *nif* fragments in *E. coli* minicells (Pühler & Klipp, 1981); their function has not yet been determined.

The *nifL* and *nifA* genes regulate transcription of each of the *nif* operons and their role will be discussed below.

REGULATION OF NITROGEN FIXATION

Synthesis of nitrogenase in *K. pneumoniae* occurs under anaerobic, nitrogen-limiting conditions and repression of transcription is observed in strains grown on ammonia (Tubb & Postgate, 1973) or in the presence of oxygen (Eady *et al.*, 1978). This pattern of regulation is entirely expected since the enzyme itself is irreversibly denatured in the presence of oxygen and nitrogenase synthesis is not required when fixed nitrogen is abundant. Enzyme synthesis was thought to be a derepression rather than an induction phenomenon since it occurred in an inert gas phase in the absence of dinitrogen (Parejko & Wilson, 1970). Hence the conclusion from the early data was that the *nif* genes were subject to negative control by one or more repressor genes. However, it soon became apparent that *nif* transcription was subject to positive control.

Activation of nif transcription

The first hint that regulation of *nif* transcription was not merely mediated by negative control came from studies of ammonia assimilation enzymes in enteric bacteria. Magasanik and his co-workers suggested that glutamine synthetase (GS), which is required for ammonia assimilation under nitrogen-limiting conditions, determines positive control of nitrogen-regulated operons (Magasanik, 1977). The evidence for the involvement of GS as a positive regulator came from studies of GS mutants; mutant strains lacking GS activity showed a pleiotropic defect in utilization of poor nitrogen sources whereas 'ammonia repression' of genes subject to nitrogen regulation did not occur in mutant strains which produced constitutive levels of GS. This correlation was also observed with the *nif* genes; nitrogenase synthesis was undetectable in strains deficient in GS, while *nif* transcription was not affected by ammonia in strains producing constitutive GS protein (Tubb, 1974; Streicher *et al.*, 1974). The concept of repression of *nif* transcription in response to ammonia was therefore abandoned, since the absence of nitrogenase in ammonia-grown cells could simply be explained on the basis of the lack of the activator, GS.

This simple model of *nif* regulation had only a short half-life however, since it became apparent that expression of the *glnA* gene, the structural gene for GS, was itself controlled by a combination of three regulatory genes and that two of these genes were located in the same operon as *glnA*. These three genes are termed *glnF*, *glnL* and *glnG* in some laboratories, but other workers prefer to designate them as *ntrA*, *ntrB* and *ntrC* respectively since they mediate 'general' nitrogen control rather than having a specific effect on GS itself; throughout this article I will use the *ntr* terminology. The *ntrB* and *ntrC* genes are linked to *glnA* in *K. pneumoniae* (de Bruijn & Ausubel, 1981; Espin *et al.*, 1982) and in other enteric bacteria (Pahel *et al.*, 1982; MacNeil *et al.*, 1982a; McFarland *et al.*, 1981; Rothman *et al.*, 1982). The *glnA ntrBC* operon is transcribed from a strong promoter upstream of *glnA* and from a relatively weak internal promoter located between *glnA* and *ntrB* (Pahel *et al.*, 1982; Alvarez-Morales *et al.*, 1984; Krajewska-Grynkiewicz & Kustu, 1984).

Studies in a number of laboratories have shown that the *ntrC* gene product is required for activation of transcription of nitrogen-regulated operons. *ntrC* mutations in *K. pneumoniae* result in a failure of strains to synthesize nitrogenase (Leonardo & Goldberg, 1980; de Bruijn & Ausubel, 1981; Espin *et al.*, 1981). There is now no evidence that GS protein has a direct role in positive control of nitrogen-regulated operons. The earlier evidence that mutations affecting GS synthesis had a pleiotropic effect on utilization of nitrogen sources could be explained either on the basis that such mutations were located in *ntrC* or that these mutations affected transcription of the downstream *ntrBC* genes. For example, strains carrying polar mutations in *glnA* are unable to activate expression of nitrogen-controlled genes since the level of the *ntrB* and *ntrC* gene products is considerably decreased (Guterman *et al.*, 1982; Krajewska-Grynkiewicz & Kustu, 1983). The *ntrC* gene product has been identified in a number of laboratories and NtrC protein from *E. coli* has been purified; it has a subunit M_r of approximately 54000 and behaves as a dimer on gel filtration (Reitzer & Magasanik, 1983).

The third nitrogen regulatory gene, *ntrA*, is unlinked to the *glnA ntrBC* operon in enteric bacteria, yet this gene has a vital role in nitrogen control since strains carrying *ntrA* mutations

are also unable to activate expression of nitrogen-regulated operons, including the *nif* genes of *K. pneumoniae* (Leonardo & Goldberg, 1980). Although expression of the *glnA ntrBC* operon is considerably decreased in strains carrying *ntrA* mutations, the pleiotropic effect of these mutations cannot be explained simply on the basis of reduced *ntrBC* transcription, since *ntrA* mutant strains carrying plasmids which express high levels of NtrC protein from a constitutive promoter are still unable to activate *nif* expression (Merrick, 1983; Ow & Ausubel, 1983). The role of the *ntrA* product is unknown; it does not apparently respond to the nitrogen status of the cell since constructed strains which produce constitutive levels of NtrC protein are able to activate nitrogenase synthesis in both the presence and absence of ammonia provided that the *ntrA* gene is functional. Moreover, the *ntrA* gene of *K. pneumoniae* does not appear to be transcriptionally regulated in response to nitrogenase source (de Bruijn & Ausubel, 1983; W. D. P. Stewart & M. J. Merrick, unpublished). The *ntrA* gene product has been identified in both *E. coli* and *K. pneumoniae* and has a subunit M_r of 75–85000 (Magasanik, 1982; de Bruijn & Ausubel, 1983).

Although activation of *nif* transcription requires the *ntrA* and *ntrC* gene products and occurs in the absence of the *ntrB* gene, activation of *glnA* transcription in *K. pneumoniae* requires the presence of all three regulatory genes, *ntrA*, *ntrB* and *ntrC* (Alvarez-Morales *et al.*, 1984). There is conflicting evidence regarding the role of the *ntrB* gene product in activation of *glnA* and other nitrogen-regulated operons in *E. coli* (MacNeil *et al.*, 1982; Backman *et al.*, 1983). It has been suggested that the *ntrBC* gene products form a regulatory protein complex (McFarland *et al.*, 1981; Wei & Kustu, 1981) and the possibility that both products are required for optimal transcription at nitrogen-regulated promoters cannot be ruled out.

A second line of evidence for positive control of *nif* transcription came from studies of the *nif* genes themselves. Dixon *et al.* (1977) observed that *nifA* mutations affect the synthesis of all three nitrogenase polypeptides, suggesting that the *nifA* product has a regulatory role as a positive activator for *nif* transcription. Expression from each of the *nif* promoters is decreased considerably in *nifA* mutant strains (Roberts *et al.*, 1978) and promoter activity can be restored by the presence of a *nifA* gene in *trans* (Dixon *et al.*, 1980). Furthermore, it is possible to by-pass the requirement for the *ntrC* gene as an activator of *nif* transcription by expressing the *nifA* gene product from a constitutive promoter. Hence the *ntrC* gene is not required for activation of *nif* transcription provided that the *nifA* gene product is expressed at a sufficient level for activation of the *nif* promoters (Buchanan-Wollaston *et al.*, 1981a; Sibold & Elmerich, 1982). Expression of the *nifA* gene does not normally occur in *ntrC* mutant strains since the *nifLA* promoter is the primary target for *ntrC*-mediated activation (Dixon *et al.*, 1980; Drummond *et al.*, 1983).

Positive control of *nif* transcription is therefore affected by a regulatory cascade mechanism in which *ntrC* activates transcription of the *nifLA* promoter and the *nifA* product in turn activates transcription of the remaining *nif* operons (Fig. 2). Why are the *nif* operons subject to a regulatory cascade mechanism? Nitrogenase is a relatively 'slow' enzyme (Thorneley & Lowe, 1983) and large amounts are apparently needed since nitrogenase can represent as much as 10% of the cellular protein in nitrogen-fixing cells. One attractive hypothesis is that the cascade regulation allows considerable amplification of *nif* gene expression without depleting the cell of NtrC protein which is required to activate a number of other operons under nitrogen-limiting conditions.

When sufficient levels of the *nifA* gene product are expressed, for example from a constitutive promoter, autogenous activation of the *nifLA* promoter can occur in the absence of the *ntrC* gene (Drummond *et al.*, 1983; Ow & Ausubel, 1983). The observation that the *nifA* product could substitute for the *ntrC* product at the *nifLA* promoter led to some surprising findings regarding the functional relationship between *ntrC* and *nifA*. Since *ntrA* was required for *ntrC*-mediated activation, the obvious question was to determine whether this gene was also required for activation mediated by *nifA* and indeed this was found to be the case at both the *nifL* and *nifH* promoters. In addition it was found that the *nifA* gene product could substitute for *ntrC* as an activator of a number of nitrogen-controlled genes including *glnA*, *hutUH* and genes for arginine and proline utilization (Sibold & Elmerich, 1982; Ow & Ausubel, 1983; Merrick, 1983). These two regulatory proteins have similar pI and M_r values. However, the *nifA* product does

Fig. 2. Current model for *nif* regulation in *K. pneumoniae*. The thin arrows indicate regulatory functions and the thick horizontal arrows represent transcripts.

retain some transcriptional specificity since the *K. pneumoniae nifH* promoter is not activated by *ntrC* (Merrick, 1983; Sundaresan *et al.*, 1983). A comparison of the structure of these two proteins may be extremely informative in view of their functional similarities.

Repression of *nif* transcription

Since *nif* expression is positively controlled by two transcriptional activators and requires the presence of the *ntrA* gene product, it is logical to assume that one or more components of the positive control system must be overridden in order to obtain 'repression' of nitrogenase synthesis in response to the nitrogen source or to oxygen. From a simplistic point of view, we can consider two mechanisms for preventing *nif* transcription. The first involves an anti-activation system in which one or more of the positive control elements are inactivated and the second assumes a more conventional negative control system in which repressor molecules directly interact with regulatory regions in order to inhibit transcription. As far as the *nif* genes are concerned, the former mechanism appears to be more prevalent than the latter. However, it should be emphasized that although we have identified some of the gene products involved, our knowledge of the pathway of repression by either fixed nitrogen or oxygen is very limited and is hampered by the complexities of metabolic regulation. We know that regulatory molecules respond to the presence of ammonia or oxygen in the growth medium, but the metabolic signals within the cell which trigger these responses have yet to be identified.

The initial studies on 'ammonium repression' of nitrogenase synthesis concentrated on the apparent role of glutamine synthetase in regulation. Mutants producing constitutive levels of GS could synthesize nitrogenase in the presence of ammonia (Tubb, 1974; Streicher *et al.*, 1974; Shanmugam *et al.*, 1975). However, it was not shown that these mutations were located in the *glnA* gene. Some of these mutants, which lacked GS catalytic activity but produced constitutive levels of GS protein, probably contained non-polar *glnA* mutations. Such *glnA* mutants are unable to show 'ammonia repression' of *glnA ntrBC* transcription; this suggests that repression is not mediated by ammonia itself but depends on the synthesis of glutamine or a metabolite derived from glutamine (Guterman *et al.*, 1982; Krajewska-Grynkiewicz & Kustu, 1983). Thus mutants deficient in GS activity are unable to generate the metabolic signal for repression in response to ammonia and non-polar *glnA* mutations give rise to constitutive *ntrBC* transcription. A number of laboratories have isolated mutants which give constitutive levels of catalytically active GS as well as being constitutive for utilization of nitrogen sources; many of these

mutations map in the *ntrB* gene (MacNeil *et al.*, 1982*b*; Chen *et al.*, 1982; McFarland *et al.*, 1981). The product of the *ntrB* gene therefore appears to respond to the nitrogen status of the cell and appears to have a negative function when ammonia is present in the growth medium.

When expressed from a constitutive promoter, the *ntrB* gene alone does not repress *glnA* transcription in *K. pneumoniae*, but repression is observed in the presence of both the *ntrB* and *ntrC* products. This suggests that NtrB does not function as a repressor molecule *per se* but can interact with NtrC protein in some way to modulate its activity (Alvarez-Morales *et al.*, 1984), the two proteins forming a regulatory protein complex. NtrB might therefore act as an anti-activator of *ntrC* in response to the nitrogen source. The lack of nitrogenase synthesis in ammonia-grown cells may therefore be due to lack of activation at the *nifLA* promoter which would consequently result in a lack of activation of the remaining *nif* operons.

There is a complication, however, since the *ntrC* gene product has recently been shown to be a bifunctional molecule, which has negative as well as a positive regulatory function. Although the *ntrC* gene product alone can activate *nifLA* transcription and apparently has no negative function at the *nif* promoters, it can autogenously repress transcription from the *glnA* and *ntrB* promoters *in vivo* (Wei & Kustu, 1981; Ueno-Nishio *et al.*, 1983; Alvarez-Morales *et al.*, 1984) and purified NtrC protein represses transcription from the *ntrB* promoter *in vitro* (Reitzer & Magasanik, 1983).

The different roles of the NtrC protein at various promoters can be explained on the basis of whether such promoters contain an activator site, a repressor site or both. We have postulated that *ntr*-regulated promoters can be divided into three classes: (a) those subject solely to negative control containing only a repressor-binding site for NtrC, e.g. *ntrBC* promoter; (b) promoters subject solely to positive control containing only a site for *ntr*-mediated activation, e.g. the *nif* promoters of *K. pneumoniae*; and (c) promoters subject to both positive and negative control which contain both repressor- and activator-binding sites, e.g. the *glnA* promoter (Alvarez-Morales *et al.*, 1984). Promoters which are subject to negative control by the *ntrC* gene product contain homologous sequences with dyad symmetry, indicative of repressor binding sites (R. A. Dixon, unpublished). We therefore envisage that *ntrC* product can bind to DNA and repress transcription, independently of its role as a transcriptional activator and that the latter role can be modulated by the *ntrB* product so that activation does not occur when 'repressive' levels of ammonia are present.

How is nitrogenase synthesis regulated in response to oxygen? The *ntrB* product does not apparently respond to oxygen and many of the nitrogen-regulated operons are expressed in aerobic growth conditions. The observation that oxygen repression occurs in mutant strains which express nitrogenase in the presence of ammonia clearly established that the pathways of oxygen and ammonia repression were different (Eady *et al.*, 1978). The presence of ammonia affects transcription of all the *nif* operons since it is mediated by the *ntr* genes at the *nifLA* promoter whereas transcription of the *nifLA* operon is relatively insensitive to the presence of oxygen (Dixon *et al.*, 1980).

The first evidence that 'oxygen repression' is mediated by a *nif*-specific gene came from an analysis of mutant strains in which transcription of the *nif* structural genes occurred in the presence of oxygen. Such strains carried mutations in the *nifL* gene (Hill *et al.*, 1981). Subsequently it was shown that the *nifL* product responds not only to oxygen but it also mediates 'repression' in response to fixed nitrogen (Merrick *et al.*, 1982). The *nifL* gene product is apparently more sensitive to nitrogen sources than the *ntrB* product since nitrogenase synthesis is repressed by Casamino acids in the growth medium (Shanmugam & Morandi, 1976), whereas other nitrogen-controlled operons are not affected. When present on a multicopy plasmid, the *nifL* gene inhibits *nif* transcription in the absence of fixed nitrogen or oxygen. This indicates that the *nifL* gene product is normally maintained in a non-inhibitory state in nitrogen-fixing cells and that factors required for this maintenance can be titrated out by several copies of *nifL* (Buchanan-Wollaston *et al.*, 1981*b*; Reidel *et al.*, 1983). As in the case of *ntrBC*, *nifL* may be an anti-activator of *nifA* and the two proteins may exist as a regulatory complex. When present in multiple copies, the *nifLA* genes autogenously repress transcription from the *nifLA* promoter in the absence of *ntrBC* but do not prevent activation in the presence of *ntrBC*, supporting the

Table 1. Conserved features of *ntr*-activatable promoters

Promoter sequences are aligned according to the invariant dinucleotides at -24 and -12 respectively, indicated by the vertical lines. The transcriptional start-sites (+1) are indicated by a dot above the relevant nucleotide. For promoters marked (a) the transcription start-site has not been reported and the promoter sequence is assumed on the basis of homology only. (b) The regulatory region of the *K. pneumoniae glnA* gene contains tandem promoters, one of which is repressed by the *ntrBC* gene products, the second being subject to *ntr*-dependent activation (R. A. Dixon, unpublished); only the activatable promoter is shown.

<i>Klebsiella pneumoniae</i>		-24		-12		+1	References																								
<i>nifJ</i> ^(a)	C	T	G	G	C	A	C	A	G	G	C	T	G	T	G	C	T	T	G	A	G	G	C	A	A	C	A	A		Shen <i>et al.</i> (1983)	
<i>nifH</i>	C	T	G	G	T	A	T	G	T	T	C	C	C	T	G	C	A	C	T	T	C	T	C	T	G	C	T	G			
<i>nifE</i>	C	T	G	G	A	G	C	G	C	G	A	A	T	T	G	C	A	T	C	T	T	C	C	C	C	T	C			Beynon <i>et al.</i> (1983)	
<i>nifU</i>	C	T	G	G	T	A	T	C	G	C	A	A	T	T	G	C	T	A	G	T	T	C	G	T	T	A	T	C			
<i>nifM</i>	C	T	G	G	C	C	G	G	A	A	A	T	T	T	G	C	A	A	T	A	C	A	G	G	A	T	A				
<i>nifF</i>	C	T	G	G	C	A	C	A	G	C	T	T	T	G	C	A	A	T	A	C	C	C	T	G	C	G					
<i>nifL</i>	A	G	G	G	C	G	C	A	C	G	T	T	T	G	C	A	T	G	G	T	T	A	T	C	A	C	C			Drummond <i>et al.</i> (1983)	
<i>nifB</i>	C	T	G	G	T	A	C	A	G	C	A	T	T	T	G	C	A	G	C	A	G	A	A	G	G	T	A			Beynon <i>et al.</i> (1983)	
<i>glnA</i> ^(b)	T	T	G	G	C	A	C	A	G	A	T	T	T	C	G	C	T	T	A	T	A	T	T	T	T	T	T			R. A. Dixon (unpublished)	
<i>Salmonella typhimurium</i>																															
<i>dhuA</i> ^(a)	A	T	G	G	C	A	C	G	A	T	A	G	T	C	G	C	A	T	C	G	G	A	T	C	T	G	T	A			
<i>argTr</i> ^(a)	A	T	G	G	C	A	T	A	A	G	A	C	T	T	G	C	A	T	G	A	A	A	A	A	G	T	C	T			Higgins & Ames (1982)
Overall consensus	5'-C	T	G	G	C	A	C	N	N	N	N	N	T	T	G	C	A	-3'													

hypothesis that *nifL* inactivates *nifA* product rather than directly repressing transcription at this promoter (Dixon *et al.*, 1983).

'Repression' of *nif* transcription, like activation, therefore occurs at two levels (Fig. 2). Specific *nif* repression results in an inhibition of *nifA*-mediated activation, thus preventing nitrogenase synthesis in the presence of oxygen or low levels of fixed nitrogen, without affecting *ntrC*-mediated activation of other nitrogen-controlled operons. The more general 'ammonia-repression' determined by the *ntrBC* genes ensures that transcription of nitrogen-regulated genes including the *nifLA* operon, is inhibited in nitrogen-enriched media. The precise role of *nifL* and *ntrB* in mediating *nif* 'repression' is difficult to determine since they show no apparent phenotype in the absence of the *nifA* and *ntrC* genes respectively. Further analysis will undoubtedly require purification of these regulatory proteins and the use of *in vitro* transcription systems to study their mode of action.

The structure of nitrogen-regulated promoters

The common requirement for the *ntrA* gene product in transcriptional activation and the functional homologies between the *ntrBC* and *nifLA* genes suggests that nitrogen-regulated promoters might share some sequence homology. All the *K. pneumoniae nif* promoters have now been sequenced, as well as the *glnA* promoter from two enteric bacteria and the promoters for the *Salmonella typhimurium* transport operons *argTr* and *dhuA*, which are also subject to nitrogen control. The transcription start-site for most of these promoters has been determined *in vivo* and a striking feature is that they lack the conserved '-10' and '-35' regions found in typical *E. coli* promoters (Rosenberg & Court, 1979; Hawley & McClure, 1983). Instead these promoters contain conserved regions of homology at -24 and -12 with respect to the transcription start-site (Table 1). The conserved region from -27 to -20 contains the consensus sequence CTGGCAC in which the purine dinucleotide GG is invariant, whereas the conserved sequence from -15 to -11, TTGCA, contains the invariant dinucleotide GC. It has been suggested that the 4 base pair sequence CTGG confers activator specificity (Beynon *et al.*, 1983). Indeed the C-residue around -27 is found in all seven *nif* promoters which are subject to *nifA* control whereas this residue is not conserved in the *nifL*, *glnA*, *dhuA* and *argTr* promoters, which are activated by *ntrC* (Table 1). In contrast Ow *et al.* (1983) proposed that the heptameric sequence TTTTGCA is a recognition site for *ntrC*-mediated activation, although this sequence is not always conserved in the -15 region of these promoters and in three promoters this sequence is not found close to the proposed transcription start-site.

When multiple copies of the *nifH* promoter are introduced into a Nif⁺ strain of *K. pneumoniae*, a Nif⁻ phenotype is observed since this promoter competes effectively for *nifA* gene product and titrates out this activator (Buchanan-Wollaston *et al.*, 1981*b*; Reidel *et al.*, 1983). This property has been utilized to select mutations in the *nifH* promoter which do not exhibit this effect. Two point mutations at -136 and two identical deletions from -184 to -72 were obtained. Another three mutants had single base pair changes at -14 and -12, emphasizing the importance of the conserved region for *nifA*-mediated activation (Brown & Ausubel, 1984). An identical C to T transition at -12 has also been isolated in the *nifL* promoter; this mutation acts as a strong promoter 'down' mutation and prevents activation of the *nifL* promoter by either *ntrC* or *nifA*, again indicating that the TTGCA sequence performs a vital role in promoter function (Dixon *et al.*, 1984). A mutation in the *nifH* promoter at -7 gives rise to partial constitutive expression of a *nifH-lacZ* fusion (Bitoun *et al.*, 1983) although it is not known whether this mutation results in the formation of a new transcription start-site.

Deletion analysis of the *nifL* promoter indicates that sequences as far as 150 nucleotides upstream from the transcriptional start-site are required for maximal activity. However, some promoter activity is still observed on cloned DNA fragments which carry only 28 nucleotides upstream from the transcription start, and in such deletions 'ammonia repression' and both *ntrC*- and *nifA*-mediated activation are still evident (Drummond *et al.*, 1983). These results show that the -35 region, which is normally required for RNA polymerase recognition of prokaryotic promoters, is probably not important and that any interaction between RNA polymerase, *ntrA* gene product and either the NifA or NtrC proteins must occur at the conserved sequence, although secondary binding sites for these regulatory molecules cannot be ruled out.

The unique sequence conservation among *ntr*-activatable promoters and their lack of homology with the canonical sequence for enteric promoters suggest that the transcriptional specificity of RNA polymerase has to be modified in order to recognize these atypical sequences. The *ntrA* gene product is the common factor in the activation system and therefore is a candidate for this role, perhaps acting as a specific sigma factor to allow RNA polymerase to recognize these atypical sequences. However, the roles of either NifA or NtrC proteins in activation are not understood and it is possible that these proteins can also interact with RNA polymerase. An analogy can be made here with bacteriophage T4 late promoters; these also contain atypical sequences and require three T4-encoded proteins which interact with host RNA polymerase to promote transcription (Geiduschek *et al.*, 1983).

CONCLUDING REMARKS

Genetic studies on nitrogen fixation in *K. pneumoniae* have produced some remarkable findings but many questions still remain unanswered. The roles of many of the *nif* gene products in nitrogenase synthesis are still relatively unexplored and the pathway of synthesis of the iron-molybdenum cofactor (FeMoco) is certainly worthy of investigation. Our knowledge of the other processing steps required for nitrogenase activity is still extremely primitive. What, for example, is the role of the *nifM* gene product in activating Kp2 protein? At the level of regulation, studies *in vivo* have allowed us to build up a complex model for transcriptional control, which will undoubtedly be analysed in detail using *in vitro* systems. The genetic approach, however, will still be useful for dissecting the interactions between regulatory molecules and the metabolic signals for 'repression'.

Finally, the reader will note that I have devoted this article entirely to studies of nitrogen fixation in *Klebsiella pneumoniae* and have chosen to omit the remarkable advances which have been made in analysing the *nif* genes of other diverse micro-organisms both free-living and symbiotic. Nevertheless as far as genetic studies are concerned, *K. pneumoniae* is still the '*E. coli*' of diazotrophs and will continue to provide a model system which can be extended to other organisms.

I would like to express my gratitude to all my colleagues in the Unit of Nitrogen Fixation who have provided both experimental and intellectual contributions to this work. I am particularly indebted to Mike Merrick, who

has been my scientific companion for the past eight years and has put forward many stimulating ideas. I would also like to thank the technical assistants and support staff in the Unit; without their contribution this work would have been impossible. Finally, I am grateful to Professor John Postgate for his comments on the manuscript and for encouraging the development of this research area.

REFERENCES

- ALVAREZ-MORALES, A., DIXON, R. & MERRICK, M. (1984). Positive and negative control of the *glnA ntrBC* regulon in *Klebsiella pneumoniae*. *EMBO Journal* **3**, 501-507.
- BACKMAN, K., CHEN, Y.-M., UENO-NISHIO, S. & MAGASANIK, B. (1983). The product of *glnL* is not essential for regulation of bacterial nitrogen assimilation. *Journal of Bacteriology* **154**, 516-519.
- BEYNON, J., CANNON, M., BUCHANAN-WOLLASTON, V. & CANNON, F. C. (1983). The *nif* promoters of *Klebsiella pneumoniae* have a characteristic primary structure. *Cell* **34**, 665-671.
- BITOUN, R., BERMAN, J., ZILBERSTEIN, A., HOLLAND, D., COHEN, J. B., GIVERL, D. & ZAMIR, A. (1983). Promoter mutations that allow *nifA* independent expression of the nitrogen fixation *nifHDKY* operon. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 5812-5816.
- BROWN, S. E. & AUSUBEL, F. M. (1984). Mutations affecting regulation of the *Klebsiella pneumoniae* (nitrogenase reductase) promoter. *Journal of Bacteriology* **157**, 143-147.
- DE BRUIJN, F. J. & AUSUBEL, F. M. (1981). The cloning and transposon mutagenesis of the *glnA* region of *Klebsiella pneumoniae*: identification of *glnR*, a gene involved in the regulation of the *nif* and *hut* operons. *Molecular and General Genetics* **183**, 289-297.
- DE BRUIJN, F. J. & AUSUBEL, F. M. (1983). The cloning and characterisation of the *glnF* (*ntrA*) gene of *Klebsiella pneumoniae*: role of *glnF* (*ntrA*) in the regulation of nitrogen fixation (*nif*) and other nitrogen assimilation genes. *Molecular and General Genetics* **192**, 342-353.
- BUCHANAN-WOLLASTON, V., CANNON, M. C., BEYNON, J. L. & CANNON, F. C. (1981a). Role of the *nifA* gene product in the regulation of *nif* expression in *Klebsiella pneumoniae*. *Nature, London* **294**, 776-778.
- BUCHANAN-WOLLASTON, V., CANNON, M. C., BEYNON, J. L. & CANNON, F. C. (1981b). Use of cloned *nif* (nitrogen fixation) DNA to investigate transcriptional regulation of *nif* expression in *Klebsiella pneumoniae*. *Molecular and General Genetics* **184**, 102-106.
- CHEN, Y.-M., BACKMAN, K. & MAGASANIK, B. (1983). Characterisation of a gene, *glnL*, the product of which is involved in the regulation of nitrogen utilisation in *Escherichia coli*. *Journal of Bacteriology* **150**, 214-220.
- DIXON, R. A. & POSTGATE, J. R. (1971). Transfer of nitrogen fixation genes by conjugation in *Klebsiella pneumoniae*. *Nature, London* **234**, 47-48.
- DIXON, R. A. & POSTGATE, J. R. (1972). Genetic transfer of nitrogen fixation from *Klebsiella pneumoniae* to *Escherichia coli*. *Nature, London* **237**, 102-103.
- DIXON, R. A., KENNEDY, C., KONDOROSI, A., KRISHNAPILLAI, V. & MERRICK, M. (1977). Complementation analysis of *Klebsiella pneumoniae* mutants defective in nitrogen fixation. *Molecular and General Genetics* **157**, 189-198.
- DIXON, R. A., EADY, R. R., ESPIN, G., HILL, S., IACCARINO, M., KAHN, D. & MERRICK, M. (1980). Analysis of regulation of the *Klebsiella pneumoniae* nitrogen fixation (*nif*) gene cluster with gene fusions. *Nature, London* **286**, 128-132.
- DIXON, R., ALVAREZ-MORALES, A., CLEMENTS, J., DRUMMOND, M., FILSER, M. & MERRICK, M. (1983). Regulation of transcription of the nitrogen fixation operons. In *Advances in Gene Technology: Molecular Genetics of Plants and Animals* (Miami Winter Symposia, Volume 20), pp. 223-232. Edited by K. Downey, R. W. Voellmy, F. Ahmad & J. Schultz. New York: Academic Press.
- DIXON, R., ALVAREZ-MORALES, A., CLEMENTS, J., DRUMMOND, M., MERRICK, M. & POSTGATE, J. R. (1984). Transcriptional control of the *nif* regulon in *Klebsiella pneumoniae*. In *Advances in Nitrogen Fixation Research*, pp. 635-642. Edited by C. Veeger, & W. E. Newton. The Hague: Nijhoff/Junk.
- Wageningen: Pudoc.
- DRUMMOND, M., CLEMENTS, J., MERRICK, M. & DIXON, R. (1983). Positive control and autogenous regulation of the *nifLA* promoter in *Klebsiella pneumoniae*. *Nature, London* **301**, 302-307.
- EADY, R. R., ISSACK, R., KENNEDY, C., POSTGATE, J. R. & RATCLIFFE, H. (1978). Nitrogenase synthesis in *Klebsiella pneumoniae*: comparison of ammonium and oxygen regulation. *Journal of General Microbiology* **104**, 277-285.
- ESPIN, G., ALVAREZ-MORALES, A. & MERRICK, M. (1981). Complementation analysis of *glnA*-linked mutations which affect nitrogen fixation in *Klebsiella pneumoniae*. *Molecular and General Genetics* **184**, 213-217.
- ESPIN, G., ALVAREZ-MORALES, A., CANNON, F., DIXON, R. & MERRICK, M. (1982). Cloning of the *glnA*, *ntrB* and *ntrC* genes of *Klebsiella pneumoniae* and studies of their role in regulation of the nitrogen fixation (*nif*) gene cluster. *Molecular and General Genetics* **186**, 518-524.
- GEIDUSCHEK, E. P., ELLIOTT, T. & KASSEVETIS, G. A. (1983). Regulation of late gene expression. In *The Bacteriophage T4*, pp. 189-192. Edited by P. Berget, E. Kutter, C. Matthews & G. Mozig. Washington, DC: American Society for Microbiology.
- GUTERMAN, S. K., ROBERTS, G. & TYLER, B. (1982). Polarity in the *glnA* operon: suppression of the Reg⁻ phenotype by *rho* mutations. *Journal of Bacteriology* **150**, 1314-1321.
- HAWKES, T., MCLEAN, P. & SMITH, B. E. (1984). Nitrogenase from *nifV* mutants of *K. pneumoniae* contains an altered form of the iron-molybdenum cofactor. *Biochemical Journal* **217**, 317-321.
- HAWLEY, D. K. & MCCLURE, W. K. (1983). Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucleic Acids Research* **11**, 2237-2255.

- HIGGINS, C. F. & AMES, G. F.-L. (1982). Regulatory regions of two transport operons under nitrogen control: nucleotide sequences. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 1083-1087.
- HILL, S. & KAVANAGH, E. (1980). Roles of the *nifF* and *nifJ* gene products in electron transport to nitrogenase in *Klebsiella pneumoniae*. *Journal of Bacteriology* **141**, 470-475.
- HILL, S., KENNEDY, C., KAVANAGH, E., GOLDBERG, R. & HANAU, R. (1981). Nitrogen fixation gene (*nifL*) involved in oxygen regulation of nitrogenase synthesis in *Klebsiella pneumoniae*. *Nature, London* **290**, 424-426.
- IMPERIAL, J., UGALDE, R. A., SHAH, V. K. & BRILL, W. J. (1984). Role of the *nifQ* gene product in the incorporation of molybdenum into nitrogenase in *Klebsiella pneumoniae*. *Journal of Bacteriology* **158**, 187-194.
- KRAJEWSKA-GRYNKIEWICZ, K. & KUSTU, S. (1983). Regulation of transcription of *glnA*, the structural gene encoding glutamine synthetase in *glnA::MudI* (Ap^Rlac) fusion strains of *Salmonella typhimurium*. *Molecular and General Genetics* **192**, 187-197.
- KRAJEWSKA-GRYNKIEWICZ, K. & KUSTU, S. (1984). Evidence that the nitrogen regulatory gene *ntrC* is transcribed from the *glnA* promoter as well as from a separate *ntr* promoter. *Molecular and General Genetics* **193**, 135-142.
- LEONARDO, J. M. & GOLDBERG, R. B. (1980). Regulation of nitrogen metabolism in glutamine auxotrophs of *Klebsiella pneumoniae*. *Journal of Bacteriology* **142**, 99-110.
- McFARLAND, N., McCARTER, L., ARTZ, S. & KUSTU, S. (1981). Nitrogen regulatory locus '*glnR*' of enteric bacteria is composed of cistrons *ntrB* and *ntrC*: identification of their protein products. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 2135-2139.
- MCLEAN, P. & DIXON, R. A. (1981). Requirement of *nifV* gene for production of wild-type nitrogenase enzyme in *Klebsiella pneumoniae*. *Nature, London* **292**, 655-656.
- MACNEIL, T., MACNEIL, D. & TYLER, B. (1982a). Fine-structure deletion map and complementation analysis of the *glnA*, *glnL*, *glnG* region in *Escherichia coli*. *Journal of Bacteriology* **150**, 1302-1313.
- MACNEIL, T., ROBERTS, G. P., MACNEIL, D. & TYLER, B. (1982b). The products of *glnL* and *glnG* are bifunctional regulatory proteins. *Molecular and General Genetics* **188**, 325-333.
- MAGASANIK, B. (1977). Regulation of bacterial nitrogen assimilation by glutamine synthetase. *Trends in Biochemical Sciences* **2**, 9-12.
- MAGASANIK, B. (1982). Genetic control of nitrogen assimilation in bacteria. *Annual Review of Genetics* **16**, 135-168.
- MERRICK, M. (1983). Nitrogen control of the *nif* regulon in *Klebsiella pneumoniae*: involvement of the *ntrA* gene and analogies between *ntrC* and *nifA*. *EMBO Journal* **2**, 39-44.
- MERRICK, M., HILL, S., HENNECKE, H., HAHN, M., DIXON, R. & KENNEDY, C. (1982). Repressor properties of the *nifL* gene product in *Klebsiella pneumoniae*. *Molecular and General Genetics* **185**, 75-81.
- NIEVA-GOMEZ, D., ROBERTS, G. P., KLEVICKIS, S. & BRILL, W. J. (1980). Electron transport to nitrogenase in *Klebsiella pneumoniae*. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 3346-3350.
- OW, D. W. & AUSUBEL, F. M. (1983). Regulation of nitrogen metabolism genes by the *nifA* gene product in *Klebsiella pneumoniae*. *Nature, London* **301**, 307-313.
- OW, D. W., SUNDARESAN, V., ROTHSTEIN, D. M., BROWN, S. E. & AUSUBEL, F. (1983). Promoters regulated by the *glnG* (*ntrC*) and *nifA* gene products share a heptameric consensus sequence in the -15 region. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 2524-2528.
- PAHEL, G., ROTHSTEIN, D. M. & MAGASANIK, B. (1982). Complex *glnA-glnL-glnG* operon of *Escherichia coli*. *Journal of Bacteriology* **150**, 202-203.
- PAJEJKO, R. A. & WILSON, P. W. (1970). Regulation of nitrogenase synthesis by *Klebsiella pneumoniae*. *Canadian Journal of Microbiology* **16**, 681-685.
- POSTGATE, J. R. (1982). *The Fundamentals of Nitrogen Fixation*. Cambridge: Cambridge University Press.
- PÜHLER, A. & KLIPP, W. (1981). Fine structure analysis of the gene region for N_2 -fixation (*nif*) of *Klebsiella pneumoniae*. In *Biology of Inorganic Nitrogen and Sulphur*, pp. 276-286. Edited by H. Bothe & A. Trebst. New York: Springer-Verlag.
- REIDEL, G. E., BROWN, S. E. & AUSUBEL, F. M. (1983). Nitrogen fixation by *Klebsiella pneumoniae* is inhibited by certain multicopy hybrid *nif* plasmids. *Journal of Bacteriology* **153**, 45-56.
- REITZER, L. J. & MAGASANIK, B. (1983). Isolation of the nitrogen assimilation regulator NR_1 , the product of the *glnG* gene of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 5554-5558.
- ROBERTS, G. P., MACNEIL, T., MACNEIL, D. & BRILL, W. J. (1978). Regulation and characterisation of protein products coded by the *nif* (nitrogen fixation) genes of *Klebsiella pneumoniae*. *Journal of Bacteriology* **136**, 267-279.
- ROSENBERG, M. & COURT, D. (1979). Regulatory sequences involved in the promotion and termination of RNA transcription. *Annual Review of Genetics* **13**, 319-353.
- ROTHMAN, N., ROTHSTEIN, D., FOOR, F. & MAGASANIK, B. (1982). Role of *glnA*-linked genes in regulation of glutamine synthetase and histidine formation in *Klebsiella aerogenes*. *Journal of Bacteriology* **150**, 221-230.
- SHAH, V. & BRILL, W. J. (1977). Isolation of an iron-molybdenum co-factor from nitrogenase. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 3249-3253.
- SHAH, V. K., STACEY, G. & BRILL, W. J. (1983). Electron transport to nitrogenase: purification and characterisation of pyruvate: flavin oxidoreductase, the *nifJ* product. *Journal of Biological Chemistry* **258**, 12064-12068.
- SHANMUGAM, K. T. & MORANDI, C. (1976). Amino acids as repressors of nitrogenase biosynthesis in *Klebsiella pneumoniae*. *Biochimica et biophysica acta* **437**, 322-332.
- SHANMUGAM, K. T., CHAN, I. & MORANDI, C. (1975).

- Regulation of nitrogen fixation. Nitrogenase-de-repressed mutants of *Klebsiella pneumoniae*. *Biochimica et biophysica acta* **408**, 101-111.
- SHEN, S.-C., XUE, Z.-T., KONG, Q.-T. & WU, Q.-L. (1983). An open-reading frame upstream from the *nifH* gene of *Klebsiella pneumoniae*. *Nucleic Acids Research* **11**, 4241-4250.
- SIBOLD, L. & ELMERICH, C. (1982). Constitutive expression of nitrogen fixation (*nif*) genes of *Klebsiella pneumoniae* due to a DNA duplication. *EMBO Journal* **1**, 1551-1558.
- STREICHER, S. L., GURNEY, E. & VALENTINE, R. C. (1971). Transduction of the nitrogen fixation genes in *Klebsiella pneumoniae*. *Proceedings of the National Academy of Sciences of the United States of America* **68**, 1174-1177.
- STREICHER, S. L., SHANMUGAM, K. T., AUSUBEL, F., MORANDI, C. & GOLDBERG, R. B. (1974). Regulation of nitrogen fixation in *Klebsiella pneumoniae*: evidence for a role of glutamine synthetase as a regulator of nitrogenase synthesis. *Journal of Bacteriology* **120**, 815-821.
- SUNDARESAN, V., JONES, J. D. G., OW, D. W. & AUSUBEL, F. M. (1983). *Klebsiella pneumoniae nifA* product activates the *Rhizobium meliloti nifH* promoter. *Nature, London* **301**, 728-732.
- THORNELEY, R. N. F. & LOWE, D. J. (1983). Nitrogenase of *Klebsiella pneumoniae*: kinetics of the dissociation of oxidised iron-protein from the molybdenum-iron protein: identification of the rate-limiting step for substrate reduction. *Biochemical Journal* **215**, 393-403.
- TUBB, R. S. (1974). Glutamine synthetase and ammonium regulation of nitrogenase synthesis in *Klebsiella*. *Nature, London* **251**, 481-485.
- TUBB, R. S. & POSTGATE, J. R. (1973). Control of nitrogenase synthesis in *Klebsiella pneumoniae*. *Journal of General Microbiology* **79**, 103-117.
- UENO-NISHIO, S., BACKMAN, K. C. & MAGASANIK, B. (1983). Regulation at the *glnL* operator-promoter of the complex *glnALG* operon of *Escherichia coli*. *Journal of Bacteriology* **153**, 1247-1251.
- WEI, G. R. & KUSTU, S. (1981). Glutamine auxotrophs with mutations in the nitrogen regulatory gene *ntnC*, that is near *glnA*. *Molecular and General Genetics* **183**, 392-399.