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The Genetic Analysis of Nitrogen Fixation, Oxygen Tolerance and Hydrogen Uptake in Azotobacters [and Discussion]

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Phil. Trans. R. Soc. Lond. B 1987 **317**, 159-171
doi: 10.1098/rstb.1987.0054

Phil. Trans. R. Soc. Lond. B **317**, 159–171 (1987)
Printed in Great Britain

The genetic analysis of nitrogen fixation, oxygen tolerance and hydrogen uptake in azotobacters

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Azotobacters are important in nitrogen-fixation research because of their ability to synthesize at least two alternative forms of nitrogenase and also because of their high tolerance to oxygen. Approaches to studying genes in azotobacters involved in these and related processes include the analysis of mutants, hybridization to genes of other organisms, and also complementation of *K. pneumoniae* and *E. coli* mutants by azotobacter DNA. Eight to ten different regions of the genome may contain DNA involved in nitrogen fixation in *A. chroococcum*. The largest of these is about 25 kilobases (kb) in length and resembles the *nif* cluster of *K. pneumoniae* to some extent. Other regions include those hybridizing to *fixABC* genes of rhizobia and those thought to be involved in the Va-based alternative nitrogenase. Regulation of expression of genes for Mo nitrogenase in *A. vinelandii* involves, as in *K. pneumoniae*, *ntrA* and *nifA* genes, but unlike *K. pneumoniae*, not *ntrC*. Another regulatory gene, called *nfrX*, has also been identified. Mutants of *A. chroococcum* with increased sensitivity to oxygen (Fos^-) have been isolated and their phenotypes related to mechanisms of oxygen tolerance. Two are characterized as being deficient in citrate synthase and PEP carboxylase, respectively; these indicate that efficient operation of the TCA cycle is important for respiratory protection of nitrogenase. Finally, genetic studies of hydrogen uptake in *A. chroococcum* include the characterization of 15 kb of *hup* DNA by hybridization and mutant-complementation experiments.

1. Introduction

Research on azotobacters began in 1901 (Beijerinck 1901) and so has occupied scientists for a large part of the century since nitrogen fixation was discovered. It was recognized early on that these organisms were particularly tolerant to oxygen (Prazmowski 1912) but it was only recently discovered that they have two enzyme systems for fixing nitrogen (Bishop *et al.* 1980), the conventional molybdenum nitrogenase and, in *Azotobacter chroococcum*, a vanadium nitrogenase (Robson *et al.* 1986). This paper presents a current view and recent results of the genetics of nitrogen fixation and oxygen tolerance. In addition to these features, azotobacters offer a bridge to understanding the genetics of nitrogen fixation and related aspects of metabolism in other organisms. Examples presented here include the structural relatedness of some genes in rhizobia, required for production of nitrogen-fixing nodules in legumes, to regions of the *A. chroococcum* genome and both structural and functional homologies of genes for hydro-

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genase (*hup*) in *Bradyrhizobium japonicum* and *A. chroococcum*. Also, the expression in azotobacters of several genes from *K. pneumoniae* or *E. coli*, and vice versa, has allowed mutations to be identified, azotobacter genes to be cloned, and insight to be gained into the regulatory mechanisms that govern gene expression.

2. Genetics of nitrogen fixation

The complexity in azotobacters imposed by having two nitrogenase enzymes and by their adaptation to fully aerobic growth on N_2 are expected to be reflected in a greater number of genes being involved in nitrogen fixation in these bacteria than in *K. pneumoniae* (see Dixon *et al.*, this symposium). This view is supported by the number of regions in the azotobacter genome which hybridize to known *nif* (or *fix*) genes from other organisms or which affect nitrogen fixation when mutated. These include a major group similar to a large portion of the *nif* cluster in *K. pneumoniae*, *nifFMVSU(X)NE(Y)KDH*, but without the flanking genes, nifQBAL...J. Two of these, *nifB* (*fixZ*) and *nifA*(*fixY*) have been located elsewhere, as have genes with homology to *Rhizobium fixABC* genes. At least one region has been identified which is involved in the vanadium-nitrogenase system, and another has a regulatory gene with no known counterpart in other diazotrophs. A difference in *nif* regulatory mechanisms is indicated by the presence in *A. vinelandii* of a *glnAntrBC* cluster, which is not involved in nitrogen fixation on Mo, in contrast to the requirement for *ntrC* in *K. pneumoniae*. These 6–8 regions are discussed in the following sections.

(*a*) *nif genes in azotobacters*

A cluster of genes in *A. chroococcum* spans 25–30 kilobases (kb) of DNA and contains the *nif* genes *F MVSU EN KDH* (summarized in figure 1) (Jones *et al.* 1984; Evans *et al.* 1985). There is some evidence that *A. vinelandii* has a similar cluster (Helfrich *et al.* 1985; Kennedy *et al.* 1986) and so the organization of *nif* genes in this region may well be common to all azotobacters. The best characterized of these genes in terms of structure and function are *nifHDK*, which encode the 3 nitrogenase polypeptides of molybdenum nitrogenase; the DNA coding sequences are very like those of the *nifHDK* cluster in *K. pneumoniae* (Brigle *et al.* 1985; Robson *et al.* 1985) and plasmids carrying *A. chroococcum nifHDK* can restore significant levels of acetylene reduction activity to *K. pneumoniae* strains mutated in any one of the three genes for nitrogenase (Jones *et al.* 1984).

A *nifEN* region located 2.3 kb from *nifK* is indicated by hybridization experiments. In *A. vinelandii*, these two genes have been sequenced and are significantly homologous to *nifDK*, with similarity between *nifE* and *nifD* and between *nifN* and *nifK* (Dean & Brigle 1985*a*, *b*). The

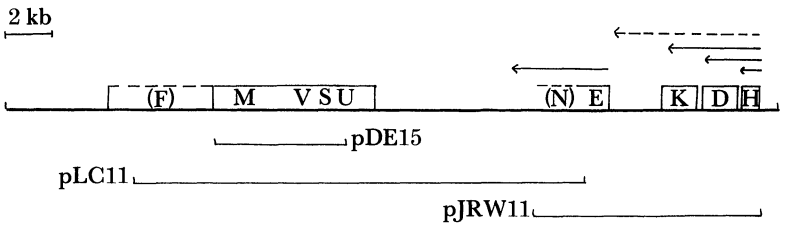

Figure 1. A cluster of *nif* genes in *Azotobacter chroococcum*. NH_4^+-repressible transcripts are indicated by arrows above the map. The plasmids shown beneath are able to complement the following mutants of *K. pneumoniae* for acetylene reduction: pJRW11, *nifH nifD nifK*; pLC11, *nifM nifV nifS*; pDE15, *nifM*. See text for further details.

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nifEN gene products are involved in Fe–Mo cofactor biosynthesis in *K. pneumoniae* and also in *A. vinelandii*. The function of *nifN* in the latter was demonstrated with a *nifN*::Tn5 mutant, identified genetically by complementation with plasmids carrying various mutated *nif* genes of *K. pneumoniae*. Nitrogenase activity, absent in the mutant, was restored to extracts by the addition of purified cofactor (Kennedy *et al.* 1986). Thus the homologous sectors of *nifE* and *nifD* and of *nifN* and *nifK* may encode portions of the proteins that interact with the cofactor and/or its biosynthetic precursors.

In *K. pneumoniae*, the *nifUSVM* operon is contiguous with *nifENX*; in *A. chroococcum*, a gap of 6–8 kb apparently separates the two regions. The *nifUSV* genes have been identified by hybridization experiments. Functional correspondence between *A. chroococcum* and *K. pneumoniae* genes is shown by the ability of a plasmid, pLC11 (shown in figure 1), carrying DNA from this region, to complement *nifM*, *nifV* and *nifS* mutants of *K. pneumoniae*. A subclone that complements *nifM* mutants carries a 5 kb KpnI fragment; therefore *nifM* must be entirely encoded within its limits. A Tn5-induced Nif$^-$ mutant of *A. vinelandii* has been characterized genetically as *nifM*$^-$ by complementation with *K. pneumoniae nif* plasmids and its biochemical phenotype is the same as *nifM*$^-$ *K. pneumoniae* mutants; the Fe protein (*nifH* product) of nitrogenase is synthesized in an inactive form (Kennedy *et al.* 1986). Thus, in both nitrogen-fixers, the *nifM* product activates nascent Fe protein.

One other *nif* gene identified in *K. pneumoniae* may be located within the 28 kb *nif* cluster of *A. chroococcum*. Hybridization to a *nifF* probe was observed (Evans *et al.* 1985), as shown in figure 1, but the region that hybridizes is rather large and awaits further characterization.

Other regions of the *A. chroococcum* genome that might be involved in nitrogen fixation are suggested by the hybridization of different gene probes from *Rhizobium leguminosarum* and *R. meliloti* to genomic digests. These probes include the *nifA* (*fixY*) genes from both rhizobia, the *fixZ* (*nifB*) gene of *R. leguminosarum*, which is contiguous with *nifA* (Rossen *et al.* 1984), and the *fixABC* genes of *R. meliloti*. The *fixABC* genes are necessary for nitrogen fixation in a number of rhizobium–legume nodules and also in free-living cultures of *Bradyrhizobium japonicum*; they may be involved in electron transfer pathways of aerobes (Gubler & Hennecke 1986).

The rhizobium *nifA* probes each hybridize to a number of restriction fragments in the *A. chroococcum* and *A. vinelandii* genomes; the number and size of fragments that hybridize to both suggests that there are two major regions with *nifA* homology. Genetic experiments indicate that at least one of these is involved in expression of other *nif* genes. Neither region appears to be the same as the one that hybridizes to the *fixABC* genes, although the *fixZ* and *nifA* probes both hybridize to *Bam*HI fragments of similar size.

Hybridization experiments with a *K. pneumoniae nifHDK* probe first indicated that genes identical with, or similar to, those encoding the nitrogenase structural proteins might be present in more than one copy in azotobacters (Ruvkun & Ausubel 1980; Medhora *et al.* 1983; Jones *et al.* 1984). Since then the presence of two *nifH*-like genes in *A. chroococcum* has been established by DNA sequencing (Robson *et al.* 1986*a*). The presence of three *nifH*-like genes in *A. vinelandii* is suggested by further hybridization analysis (Jacobson *et al.* 1986). Deletion mutants of both azotobacters, which lack the *nifHDK* genes described above, are capable of nitrogen fixation in the absence of molybdenum (Bishop *et al.* 1986*a*, *b*; Robson 1986). The second *nifH* gene of *A. chroococcum*, called *nifH**, is contiguous with a gene encoding a small ferredoxin-like gene. Transcription experiments, described below, indicate that these two genes are expressed in deletion mutants grown without molybdenum but not in its presence. Circumstantial evidence

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is therefore strong that products of *nifH** and the ferredoxin gene are involved in the activity of the second nitrogenase system, which is a vanadium-based enzyme in *A. chroococcum*. Amino-acid analysis of Ac2*, the Fe protein isolated from *nifHDK* deletion mutants fixing nitrogen in the absence of molybdenum, shows a composition in excellent agreement with that predicted from the DNA sequence of *nifH**. Further biochemical evidence (see Smith *et al.* this symposium) indicates that a Va–Fe protein, Ac1*, which is functionally analogous and structurally similar to the Mo–Fe protein, Ac1, of the conventional nitrogenase, is partner to Ac2* in the reduction of N_2. Although no genes encoding the structure of Ac1* have been unequivocally identified, a second region with homology to the *A. chroococcum nifK* gene has been observed in hybridization experiments. This homology is found on an *Eco*RI fragment of the same size, 8 kb, as one which hybridizes to the ferredoxin gene. Thus this region of *nifK* homology may contain a gene encoding one of the Va–Fe protein subunits which may lie within a few kilobases of the *nifH**–Fd genes. Another region of interest has been identified by hybridization to the *A. chroococcum nifEN* genes. Although no function has yet been assigned to genes located here, it is speculated that they might encode proteins involved in the formation of the FeVa cofactor which is associated with Ac1*.

In *A. vinelandii*, at least one gene in the major *nif* cluster is apparently required for the alternative system. The Tn5 NifM$^-$ mutant, MV21, is unable to grow on molybdenum-deficient agar medium (Kennedy *et al.* 1986). Although the Tn5 insert may affect the structure or expression of genes other than *nifM* in MV21, the only discernible biochemical phenotype of mutants grown in Mo-sufficient medium is that the nitrogenase Fe protein is inactive. It therefore seems likely that the *nifM* gene product activates nascent Fe proteins of both systems. The other Tn5 mutant, MV22, can grow on agar medium without molybdenum. Therefore the *nifN* gene product is apparently not involved in formation of a cofactor of the alternative nitrogenase of *A. vinelandii*.

(*b*) *Regulation of nitrogenase synthesis*

Environmental factors that influence nitrogenase synthesis in azotobacters, as in other diazotrophs, include the supply of NH_4^+, O_2, and molybdenum. In azotobacters the last of these apparently determines which nitrogenase enzyme will be available for nitrogen fixation. The genetic elements, including promoters and regulatory genes, involved in responses to the environment are discussed in the following paragraphs, along with the analysis of transcripts from various *nif* regions. Also, the DNA sequence of intergenic regions in the *nifHDK* operon suggests secondary structures that may influence the ratio of nitrogenase components.

(i) *nif promoters and regulatory genes*

The consensus DNA sequence found near the start of *nif* transcriptional units in *K. pneumoniae* and in various species of *Rhizobium*, CT*GG*-N_8-T*GC*A, is discussed by Dixon *et al.* (this symposium). Similar sequences are found at appropriate distances before the initiation codons of the *nifH* and *nifE* genes in both *A. chroococcum* and *A. vinelandii* (Robson *et al.* 1985; Dean & Brigle 1985*a*). Also, the 'upstream' motif of TGT-N_{10}-ACA, thought to be the binding site for the *nifA* activator, is found at appropriate distances in the *nifH* and *nifE* promoter regions. These promoter structures, indicative of the requirement for the *nifA* activator protein for their expression, are consistent with the following evidence for a *nifA* gene in azotobacters. Firstly, the *nifA* gene of *K. pneumoniae* cloned on pCK1 and pCK3 activates expression of nitrogen

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fixation in regulatory mutants of both *A. vinelandii* and *A. chroococcum* (Kennedy & Robson 1983; Kennedy & Drummond 1985). Secondly, the multicopy effect, whereby the *nifH* promoter on a high-copy-number plasmid abolishes nitrogenase activity in *K. pneumoniae*, occurs in both *A. vinelandii* and *K. pneumoniae* carrying many copies of the *A. chroococcum nifH* promoter. This effect is probably due to titration of *nifA* protein by its strong binding to the *nifH* promoter, leaving insufficient *nifA* to activate other *nif* promoters. It is relieved by providing additional copies of *nifA* in *trans* to the *A. chroococcum nifH* promoter (Jones *et al.* 1984). Thirdly, a Tn5-induced Nif^- mutant of *A. vinelandii* has been isolated that can be complemented by the *K. pneumoniae nifA* gene on pCK3, and DNA around the site of insertion hybridizes well to the two rhizobium *nifA* probes discussed previously. Other evidence comes from studies with a wide-host-range plasmid, pFAC11, carrying the *A. chroococcum nifH* promoter fused to *lacZ*, which is not expressed in *E. coli* unless the *K. pneumoniae nifA* gene is present. This promoter can also be activated by the *nifA* products of both *R. meliloti* (Weber *et al.* 1985) and *B. japonicum* (Fischer *et al.* 1986). Expression of pFAC11 is diminished to background levels in the presumptive *nifA* mutant of *A. vinelandii*.

Two other features relevant to *nifA*-dependent expression of *nif* genes in *K. pneumoniae*, are that the *ntrA* gene, identified in enteric organisms as an RNA polymerase recognition factor, is required for transcription of *nifA*-activated promoters, and, secondly, that *ntrA* and also *ntrC* products activate expression of the *nifLA* operon. Thus, in *K. pneumoniae*, synthesis of the *nifA* product requires *ntrC*. Both *A. vinelandii* and *A. chroococcum* have *ntrA* genes that can complement *ntrA* mutants of *K. pneumoniae* (or *E. coli*) and site-directed Tn5 mutagenesis of *ntrA* in *A. vinelandii* yielded mutants that are Nif^- (Toukdarian & Kennedy 1986) and fail to express the *nifH–lacZ* fusion on pFAC11.

In contrast, the *ntrC* gene of *A. vinelandii*, although able to restore an Ntr^+ phenotype to *E. coli* $ntrC^-$ mutants and Ntr^+Nif^+ to *K. pneumoniae* mutants, is apparently not involved in nitrogen fixation in Mo-containing medium. The *ntrC* gene of *A. vinelandii*, located in a *glnAntrBC* cluster just as in enteric organisms, was mutated throughout its length with Tn5 and reinserted into the genome. All mutants are able to fix nitrogen and have high acetylene-reducing activity. They do, however, have two distinguishing phenotypes: an inability to express the *ntrC*-dependent *K. pneumoniae nifL* promoter (see Postgate *et al.*, this symposium) and, like the *ntrA* mutants, the inability to induce nitrate reductase (Toukdarian & Kennedy 1986; Santero *et al.* 1986). Therefore, expression of the *nifA* gene of azotobacters does not apparently require the *ntrC* product. Also, nitrogen fixation was still repressed by NH_4^+ in the *A. vinelandii ntrC* mutants; this result indicates that the *ntrC* product is probably not involved in NH_4^+ repression of *nif* as it is for some non-*nif* promoters in *K. pneumoniae* and *E. coli* (e.g. *glnA*, *ntrB*; see Dixon *et al.*, this symposium).

Among the regulatory mutants isolated by random Tn5 mutagenesis of *A. vinelandii* were some in which the site of insertion was not in a region that hybridizes to the *Rhizobium nifA* probes. These mutants, called *nfrX*, were neither $ntrA^-$ nor $ntrC^-$. Cosmids with the wild-type *nfrX* gene were isolated from the pLAFR1 gene banks of both *A. vinelandii* and *A. chroococcum*. These cosmids do not complement the Ntr^- phenotype of *glnB* mutants of *K. pneumoniae*. Therefore *nfrX* does not appear to correspond to any gene known to affect expression of *nif* genes in other organisms. It may represent a regulatory gene unique to azotobacters and may activate *nifA* expression, as suggested by the fact that *nfrX* mutants are Nif^+ when pCK3 is introduced.

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Further insight into mechanisms controlling expression of *nif* genes in azotobacters is provided by experiments to examine both the expression and NH_4^+ repression of cloned *K. pneumoniae* (Kp) *nif* promoters fused to *lacZ* in *A. vinelandii* (Kennedy & Drummond 1985; Toukdarian & Kennedy 1986). The failure of pRD1 to correct *A. vinelandii nif* mutants, contrary to an early report, is mentioned by Postgate *et al.* (this symposium). In summary, the Kp *nifL* promoter is expressed in wild-type (in conditions of both NH_4^+-excess or -deficiency), and in *nifA* or *nfrX* mutants, but not in *ntrC* or *ntrA* mutants; the Kp *nifF* promoter is expressed in all backgrounds including a *nifAntrC* double mutant, but not in *ntrA*$^-$, and it is not repressed by NH_4^+; the Kp *nifH* promoter is expressed at only very low levels even if the Kp *nifA* product is present; and the Kp *nifE* and *nifU* promoters are expressed at high levels in wild-type and *ntrC* mutants, but not in the *nifA*, *ntrA* or *nfrX* mutants. As in *K. pneumoniae*, expression of the *nifE* and *nifU* promoters is repressed by NH_4^+ (15 mM) in *A. vinelandii*. Consistent with expression of these two *K. pneumoniae* promoters in *A. vinelandii* is the successful complementation of the Tn5 mutants, MV21 (NifM$^-$) and MV22 (*nifN*), described above, with pRD1 or its *nif*$^-$ mutant derivatives (Kennedy *et al.* 1986).

Thus, studies of Kp *nif* promoter expression in *A. vinelandii* reveals four types of regulatory response, the most explicable of which are the *nifA*-dependence and NH_4^+ repressibility of the *nifU* and *nifE* promoters and also the *ntrC*-dependence of the Kp *nifL* promoter. Less obvious is the expression of *nifF* in all mutant backgrounds (except *ntrA*$^-$) which may indicate another 'activator' gene not yet identified in azotobacters. However, the most intriguing of these is the failure of the *nifH* promoter to be expressed. The upstream and downstream promoter features discussed above are similar in *nifH* from both *K. pneumoniae* and *A. vinelandii*. Either the azotobacter *nifA* product activates only promoters with certain features that have not been determined or there is a negative control mechanism in *A. vinelandii* that overrides *nifA* activation of this promoter.

(ii) *Transcripts from the major nif gene cluster*

The *nifHDK* genes of azotobacters are apparently cotranscribed from a promoter adjacent to *nifH* because there is only a single *ntrA*, *nifA*-dependent consensus sequence located there from which transcription has been shown to be initiated in *A. chroococcum* (Robson *et al.* 1985). Promoter-like structures are not found adjacent to either *nifD* or *nifK*. Also, a *nifH*::Tn5 mutant of *A. vinelandii* fails to synthesize either *nifD* or *nifK* gene products, owing to the polar effect of Tn5 insertions (Kennedy *et al.* 1986). However, DNA sequences have been found between *nifH* and *nifD* and between *nifD* and *nifK*, in both azotobacter species, that have the potential to form stem and loop structures that could affect expression of these genes (Brigle *et al.* 1985; Robson *et al.* 1985). That such regulation does occur is inferred from the analysis of RNA transcripts from this region in both *A. vinelandii* and *A. chroococcum* (Krol *et al.* 1982; Jones *et al.* 1984). During the course of *nif* derepression after NH_4^+ is removed from the (+Mo) growth medium, three transcripts are synthesized, of 1.1, 2.6 and 4.3 kb legths, which in *A. chroococcum* exactly correspond to the sizes expected if transcription were sometimes interrupted at the intergenic sites. It can be speculated that levels of transcripts dictate amounts of nitrogenase components synthesized. Ratios ranging from 0.3 to 1 were reported for relative amounts of Ac1:Ac2 synthesized under different conditions of growth limitation in chemostat cultures of *A. chroococcum* (Walker *et al.* 1981). Electron flow through nitrogenase might be adjusted for different physiological conditions by post-transcriptional control of component synthesis.

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A fourth transcript from the *nifHDK* region is 6.4 kb and extends from *nifHDK* downward but not into *nifEN*. Its detection is difficult because it represents a very minor proportion (1–2%) of the total mRNAs transcribed from the *nifH* promoter. Preliminary sequencing data suggest that more than one and probably two open reading frames (ORFs) are encoded in this region, although it is not known if an equivalent of the *K. pneumoniae nifY* gene is present here. Deletions in this region do not apparently affect nitrogen fixation under conditions of either molybdenum sufficiency or deficiency.

NH_4^+-repressible transcripts from the *nifEN* and from the *nifMVS* regions detected by hybridization to probes from the major *nif* cluster; both were much less abundant than the 3 major *nifHDK* transcripts.

(iii) *Expression of alternative nitrogenase systems*

This issue is complicated by the fact that *A. vinelandii* may have two nitrogenase enzymes in addition to the conventional Mo nitrogenase. Indicators of this are that the *nifH* gene hybridizes to two other regions of the genome (Jacobson *et al.* 1986) and that during the course of derepression in Mo-free medium, *A. vinelandii* apparently synthesizes two different sets of what may be nitrogenase subunits, one during early stages (pattern B1) and another in late growth stages (pattern B2) (Bishop *et al.* 1982; Page & Collinson 1982). None of these is synthesized in either *nifHDK*-intact or -deletion strains if Mo concentration is 25 μM or more. Studies of transcripts show that a *nifH**–Fd region is present in *A. vinelandii*, as in *A. chroococcum*, and is only expressed in the absence of Mo. Whereas the *ntrA* mutants described above cannot grow on Mo-deficient agar (Toukdarian & Kennedy 1986), the *nifA*, *ntrC* and *nfrX* mutants can fix N_2 on this medium. It is possible that one or more of these genes is involved in regulation of one of the two alternative systems in *A. vinelandii*. If there are indeed two additional systems for nitrogen fixation, then they both depend on *ntrA* for their expression.

A. chroococcum appears to have only one alternative system, a vanadium-based nitrogenase which has been isolated from *nifHDK* deletion strains (Robson 1986; Robson *et al.* 1986*b*). As in *A. vinelandii*, the *nifH**–Fd region is only transcribed in the absence of Mo. A *nifH**–*lac* fusion is not activated by the Kp *nifA* product in an *E. coli* background (in contrast to the results with the *nifH–lac* fusion on pFAC11 mentioned previously). Although a ′-12,-24′ *nif*-like consensus sequence has been identified about 50 nucleotides before the translation start of *nifH**, no upstream activator motif is evident (Robson *et al.* 1986*a*). Thus it seems likely that another activator gene is needed in *A. chroococcum* for expression of the Va enzyme system. A candidate for this might be found in the second region that hybridizes to the rhizobium *nifA* probes.

(iv) NH_4^+ *repression*

In *K. pneumoniae*, NH_4^+ repression of *nif* gene expression occurs at two levels. Firstly, the *nifL* gene product responds quickly to the presence of excess fixed nitrogen and apparently inactivates the *nifA* protein, thus preventing further transcription of *nif* genes. Secondly, high levels of NH_4^+ prevent sufficient *ntrC* gene product from being made to activate expression at the *nifL* promoter so that *nifA* product is also not made. Although addition of NH_4^+ to nitrogen-fixing cultures of azotobacters results in rapid cessation of nitrogenase synthesis, it is not known whether a *nifL*-like negative effector is involved. However, the presence of the *K. pneumoniae nifA* gene expressed from a constitutive promoter is sufficient to prevent NH_4^+ repression in azotobacters (Kennedy & Robson 1983).

A number of *A. vinelandii* mutants have been reported that fix nitrogen in the presence of

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NH_4^+; some of these excrete excess NH_4^+ into the growth medium (Terzaghi 1983; Gordon & Jacobson 1983). One derepressed mutant was isolated by screening Nif^+ revertants of a Nif^- regulatory mutant (Gordon & Brill 1972). The nature of these mutations is not known.

3. Genetics of oxygen tolerance

Azotobacters have three ways of responding to oxygen stress that are relevant to nitrogen fixation. Two are defence mechanisms to protect nitrogenase from irreversible damage; the third stops synthesis of nitrogenase proteins (see Robson & Postgate 1980). The former include the ability of azotobacters to respire at high rates such that oxygen is consumed faster than it dissolves; this process is termed respiratory protection. If O_2 enters the solution more rapidly than it is removed by respiration, the second line of defence is important: this is the binding to nitrogenase of the 2Fe–2S protective protein to give an O_2-stable complex that is protected from O_2-damage but inert to nitrogenase substrates. Finally, nitrogenase synthesis stops during O_2-stress. The study of mutants has shed light on the molecular mechanisms underlying some aspects of these phenomena, especially those of respiratory protection.

Nearly 300 oxygen-sensitive mutants of *A. chroococcum* were isolated after chemical or transposon (Tn1) mutagenesis (Ramos & Robson 1985*a*). They share the phenotype of being unable to grow with N_2 as N source and a sugar as C source in air. Growth is restored to these Fos^- (fixation on sugars) mutants by decreasing the ambient O_2 concentration to between 0.2 and 1% or, in most, by the addition of acetate, 1 mM Ca^{2+}, or TCA-cycle intermediates to the growth medium. However, particular mutants showed differences in their correction by these added factors. Ten mutants were studied in more detail and fell into three classes:

(i) Group RI (2 mutants) have respiration rates similar to the parent strain but do not synthesize nitrogenase proteins. Introduction of the *nifA* plasmid pCK1, described above, corrected the Fos^- phenotype and restored synthesis of nitrogenase. This phenotype may be due to mutation of some O_2-sensing protein, similar to the *nifL* gene product in *K. pneumoniae*, which inactivates the *nifA* product in response to O_2; or the O_2-sensing molecule which is absent or mutated could be involved in one of a chain of coupled reactions leading to O_2 repression. If a *nifL*-like gene is involved in the response to O_2, then it must be somewhat different from that in *Klebsiella*, because the *Klebsiella nifL* product has no obvious effect on *nif* gene expression when introduced to *A. vinelandii* on a high-copy-number plasmid. It does, however, inactivate *K. pneumoniae nifA* product in this background (Kennedy & Drummond 1985).

(ii) Group RII (6 mutants) have respiration rates lower than wild type; their apparent affinity for O_2 is normal. They differ in their response to addition of TCA-cycle intermediates in terms of both correction to Fos^+ and restoration of normal rates of respiration. In general, both these and Group RIII mutants (described below) seem unable to metabolize sugars through to carboxylic acids as efficiently as wild-type individuals and so behave in a manner similar to organisms which are C-limited in chemostat cultures. In these conditions, nitrogen fixation is more sensitive to O_2 than in, for example, nitrogen-limited cultures. One RII mutant, Fos252, has very low citrate synthase activity and, as expected for such a mutant, it metabolizes both glucose and acetate quite slowly (Ramos & Robson 1985*b*). Growth on NH_4^+ is diminished but less drastically than on N_2. This mutant can be complemented by the *E. coli* citrate synthase gene cloned on wide-host-range plasmids, pJLR1 and pJLR5. All associated phenotypes are

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restored to normal in Fos252 carrying either plasmid. These include citrate synthase activity, growth and higher affinity for O_2 on acetate, normal rates of respiration, and wild-type growth rates with sugars on N_2 or NH_4^+.

(iii) The two RIII group mutants studied have normal respiratory rates but lower apparent affinity for O_2 than the wild type. Carboxylic acids that restore the Fos^+ phenotype also restore O_2 affinity. One of these mutants, Fos189, had been selected for failure to grow on pyruvate after the link with carboxylic acids had been made during characterization of earlier mutants. The phenotype of Fos189 is corrected by the TCA-cycle intermediates acetate, fumarate, oxoglutarate, malate and succinate. Its inability to grow on (or be corrected by) pyruvate has been correlated with a greatly diminished PEP carboxylase activity. This defect will result in respiration efficiency being limited during growth on sugars. The wild-type PEP carboxylase gene of *A. chroococcum* has been isolated from a pLAFR1 gene bank cosmid by its ability to complement carboxylase-deficient (*pcp*$^-$) mutants of *E. coli*. Subcloning indicates that 6.6 kb of DNA from the insert is necessary for complementation of both *E. coli pcp*$^-$ and *A. chroococcum* Fos189. PEP carboxylase is encoded by a 2.65 kb monocistronic gene in *E. coli*; this suggests that the *pcp* gene of *A. chroococcum* may be located within a polycistronic operon.

Whereas Fos^- mutant studies indicate that an active TCA cycle is necessary for respiratory protection in *A. chroococcum*, little is yet known about the nature of what must be a respiratory chain specially adapted to respond quickly to fluxes in p_{O_2}. Also, no mutants have yet been isolated that lack the protective protein. Because the synthesis of protective protein is not repressed by NH_4^+, it is apparently not *nif*-specific and so may have a physiological role in addition to that which allows these organisms to adapt to growth on N_2 after periods of O_2 stress.

4. Genetics of hydrogenase

The hydrogen-uptake activity of azotobacters has been characterized biochemically as a nickel-containing enzyme consisting of large and small subunits (see Smith *et al.* this symposium).

A genetic analysis of uptake hydrogenase (*hup*) in *A. chroococcum* began with the isolation of mutants defective in hydrogenase activity (Yates & Robson 1985). Physiological studies of mutant and wild-type organisms suggested that hydrogenase is beneficial during nitrogen fixation under C-limited conditions (Aguilar *et al.* 1985).

Hup^- mutants of *A. chroococcum* fall into four phenotypic classes. Type a mutants have very low H_2 uptake activity and do not evolve detectable H_2. Type b mutants also have very low H_2 uptake and low levels of H_2-evolving activity. Type c mutants have low but significant levels of both activities. The single type d mutant has high hydrogenase activity but unlike that of the wild type, the enzyme is not associated with the cell membrane.

A molecular analysis of *hup* genes in *A. chroococcum* was possible after the isolation of cosmids from a gene library by their ability to hybridize to *hup* DNA from *B. japonicum* (Tibelius *et al.* 1986; see also Evans *et al.* this symposium). The original *A. chroococcum* cosmids have now been characterized by a number of criteria, summarized in figure 2, which include defining the regions that hybridize to *hup* DNA of *B. japonicum*, those that complement or correct Hup^- mutants of *A. chroococcum* and, most recently, those that hybridize to synthetic oligonucleotides that encode the first several amino acids of the hydrogenase subunits in *A. vinelandii*. These studies indicate that 15 kb of DNA are involved in the formation of active hydrogenase in *A. chroococcum*.

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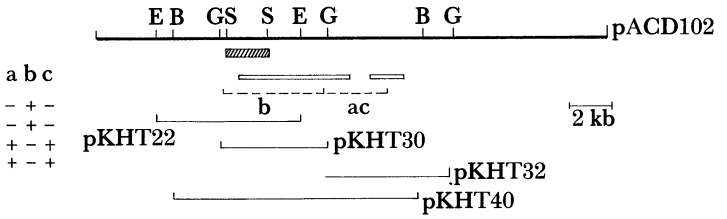

FIGURE 2. The *hup* region of *Azotobacter chroococcum* on pACD102. Regions hybridizing to *B. japonicum hup* DNA on pHU1 are indicated by open boxes; regions hybridizing to oligonucleotide probes, corresponding to the N-termini of *A. vinelandii* hydrogenase subunits, by the hatched box. The pKHT plasmids contain fragments subcloned into the wide host range vectors pLAFR1 (pKHT22), pRK290 (pKHT30 and pKHT32), and pRK404 (pKHT40). Their ability to complement 3 classes (a^-, b^-, c^-) of Hup^- mutants is shown on the left. Only some restriction sites on pACD102 are shown: E, *Eco*RI; B, *Bam*HI; G, *Bgl*II; S, *Sal*I.

Complementation of the type a, b and c mutants but not type d, has been achieved with DNA subcloned from the *hup* cosmids isolated from the *A. chroococcum* gene bank. The subclones used in complemetation tests were made with wide-host-range plasmids, such as pRK404, pLAFR1 and pRK290, P-type plasmids which can be mobilized into azotobacters. Type a and type c mutants are complemented by DNA regions separable from that which complements type b mutants. All three are complemented by a plasmid encompassing both regions. Type a and type c mutants are also complemented by pHU1 and pHU53 carrying *B. japonicum* DNA; b mutants can be weakly complemented by pHU2. Interestingly, the hydrogenase activity in the pHU2-complemented type b mutants has a pH optimum characteristic of a soluble hydrogenase. It is possible that the *B. japonicum* hydrogenase is unable to associate with the membrane of *A. chroococcum*.

Thus the hydrogenase structural genes of *A. chroococcum* may be located in the b region. Use of oligonucleotide probes confirms this view; D. Arp and colleagues (personal communication) have sequenced the N-terminal ends of *A. vinelandii* hydrogenase subunits. Labelled oligonucleotides synthesized according to sequences that might encode the first 6 amino acids of the 60 kDa subunit, hybridized to pACD101 and pACD102 within a 3.6 kb *Sal*I fragment in the b-complementing region. Similarly, oligonucleotides encoding the first 8 amino acids of the 30 kDa subunit hybridized to the same fragment.

We thank Dr Dan Arp (University of California, Riverside) for providing unpublished amino-acid sequences of *A. vinelandii* hydrogenase; J. R. Postgate, F.R.S., for critical reading of the manuscript; and Beryl Scutt for typing it.

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Note added in proof (13 *July* 1987). We have recently discovered that, contrary to our earlier conclusion, there is a role for the *ntrC* gene in nitrogen fixation in *Azotobacter vinelandii*. The *ntrC* gene is apparently required for expression of at least some genes necessary for the alternative vanadium nitrogenase system. This conclusion arises from the growth patterns observed on media containing Mo or V or neither metal (NIL medium) with mutants carrying single or double mutations in *nifA*, *nfrX* or *ntrC*. Mutations in *nifA* or *nfrX* prevent growth on NIL or NIL+Mo media while growth occurs on NIL+V. Therefore neither *nifA* nor *nfrX* are required for nitrogen fixation on Va (nitrogenase system II) but are involved in expression of genes for Mo nitrogenase, as reported in this paper, and for the putative third nitrogenase (system III, which requires neither metal) in *A. vinelandii*. However, double mutants that are *nifA*$^-$*ntrC*$^-$ fail to grow on all three media. Therefore *ntrC* is required for expression of V nitrogenase. Whether there is direct activation by the *ntrC* gene product of genes required for V nitrogenase, or whether it activates expression of an alternative *nifA*-like activator for V nitrogenase genes, remains to be determined.

Discussion

F. C. Cannon (*Biotechnica International Inc., Cambridge, Massachusetts, U.S.A.*). Dr Kennedy mentioned that *nifA* genes from *Bradyrhizobium japonicum* and *R. meliloti* could activate expression of the *A. chroococcum nifH* promoter in an *E. coli* background. In our laboratory we find that the expression of the *B. japonicum nifA* gene depends on the particular plasmid construction and can result in quite different levels of activation of *nif* promoters. Did Dr Kennedy's experiments and their interpretation take variable expression of *nifA* gene product into account?

C. Kennedy. Activation of the *A. chroococcum nifH* promoter on pFAC11 (p*H-lac*) in *E. coli* is observed with pMC71a (*K. pneumoniae nifA*), pRM541-10 (*R. meliloti nifA*), and with pRJ7523 (*B. japonicum nifA*). In all three, about 2000 Miller units of β-galactosidase are obtained, compared with none in the strain ET8000 (pFAC11) without a *nifA* plasmid. These levels are similar to those obtained with the same *nifA*s introduced into ET8000 (pMD61); this plasmid has a Kp *nifH* promoter – *lac* fusion (Kennedy & Drummond 1985). Although all three *nifA* plasmids are constructed to give constitutive expression of the *nifA* product from drug-resistance promoters, the details of their construction are different. pMC71a has *nifA* expressed

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from the Tc^r gene on pACYC184 (Buchanan-Wollaston *et al.* 1981). In pRMW541-10, the *nifA* gene is cloned on pACYC177 with expression from the promoter of the Km^r gene (Weber *et al.* 1985). The plasmid pRJ7523 is also based on pACYC177 with expression from the Cm^r gene cloned from pBR329 (Fischer *et al.* 1986). In the latter two, DNA had to be removed from the region between the promoter and the *nifA* gene to maximize expression. So whereas expression of the three *nifA*s is not subject to NH_4^+ or other types of control that might influence transcription from the natural promoters of these genes, expression could certainly vary owing to different strengths of the three drug-resistance gene promoters or because of varying efficiencies of translation initiation. The point to be made from the experiments, however, is that, on a qualitative basis, the *nifA* gene products from a variety of organisms can activate *nif* promoters which have certain features in common, the -12, -24 *nif* consensus and upstream TGT-N10-ACA promoter sequences.

Additional reference

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