The Use of Cloned *nif* Regulatory Elements from *Klebsiella pneumoniae* to Examine *nif* Regulation in *Azotobacter vinelandii*

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Regulatory genes controlling *nif* expression and also *nif* promoters fused to *lacZ* from *Klebsiella pneumoniae* were cloned on wide-host-range plasmids and introduced into *Azotobacter vinelandii* to compare regulation of *nif* expression in the two organisms. A low-copy-number plasmid carrying *K. pneumoniae nifA* corrected an *A. vinelandii* Nif[−] regulatory mutation, whereas a plasmid carrying *ntrC* did not. A high-copy-number plasmid carrying *K. pneumoniae nifL* eliminated *nif* expression in *K. pneumoniae* but not in *A. vinelandii*. *K. pneumoniae nifL*– and *nifF*–*lacZ* fusions were expressed strongly in *A. vinelandii* and were not repressed by ammonium. A *nifH*–*lacZ* fusion was not expressed in any conditions in this background except very weakly when *K. pneumoniae nifA* was also present. The implications of these findings are discussed.

INTRODUCTION

The genetics of N₂ fixation has been analysed in greater detail in *Klebsiella pneumoniae* than in any other diazotroph, owing to the close relationship of this organism to *Escherichia coli*. The resulting model of *nif* gene structure, function and regulation has been referred to extensively in analyses of N₂ fixation in other species, and the availability of cloned *nif* genes from *K. pneumoniae* has been exploited to great advantage in comparative studies. Here we use this approach to explore the functional similarities between control of *nif* expression in the facultative anaerobe *K. pneumoniae* and in the obligate aerobe *Azotobacter vinelandii*.

The *nifLA* operon, within the 17 gene *nif* cluster of *Klebsiella*, encodes two regulatory proteins (Roberts & Brill, 1980; Kennedy *et al.*, 1981). The *nifA* product is an activator which is essential for full expression of each *nif* promoter except that of its own operon (Dixon *et al.*, 1980; Buchanan-Wollaston *et al.*, 1981*a*). The *nifL* product represses *nif* expression in response to oxygen, certain amino acids and low levels of ammonia (Hill *et al.*, 1981; Merrick *et al.*, 1982; Filser *et al.*, 1983) and appears to directly inactivate the *nifA* product, rather than itself binding to DNA (Buchanan-Wollaston & Cannon, 1984; Drummond & Kennedy, 1984). Transcription from the *nifLA* promoter is normally activated by the *ntrC* product which also controls the expression of other genes involved in the utilization of poor nitrogen sources such as arginine, proline and histidine (Drummond *et al.*, 1983; Merrick, 1983; Ow & Ausubel, 1983). *nifA* and *ntrC* show numerous similarities, including a common dependence on the *ntrA* product for their activity (Merrick, 1983; Ow & Ausubel, 1983). At high levels of ammonium, *ntrC* activation of the *nifLA* promoter is eliminated. Thus *nif* expression in *Klebsiella* is regulated at two levels, one general and one *nif*-specific.

The *Klebsiella nif* promoters have a structure unlike other promoters analysed in the enteric bacteria, and contain the consensus sequence CTGG–N₈–TTGCA instead of the usual –10 and –35 consensus sequences (Beynon *et al.*, 1983). The recognition properties of RNA polymerase must be specifically altered for these *nif* promoters, and it has been suggested that one of the proteins involved in activation, perhaps the *ntrA* product, is substituted for conventional sigma factor as a regulatory switch (de Bruijn & Ausubel, 1983). Promoters normally activated by *ntrC* in the enteric bacteria closely resemble the *nif* consensus promoter and can be activated to some extent by *nifA* (Ow *et al.*, 1983; Merrick, 1983; Ow & Ausubel,

1983). The converse, however, is not invariably true; in particular, the *nifH* promoter from *K. pneumoniae* is not positively regulated by *ntrC* (Ow *et al.*, 1983), although that from *Rhizobium meliloti* can be activated by either *nifA* or *ntrC* (Sundaresan *et al.*, 1983a, b).

Nucleic acid hybridization experiments and DNA sequence analysis show that *nif* genes are highly conserved between distantly related species (for examples see Ruvkun & Ausubel, 1980; Hase *et al.*, 1984). Such observations, in conjunction with the apparently random occurrence of diazotrophy within genera, suggest that modern diazotrophs have acquired their *nif* genes rather recently in evolutionary history (see Postgate, 1982). If so, one would suspect strong hybridization between DNA sequences immediately associated with nitrogenase function, but not necessarily between regulatory genes, since these are responsible for integrating the *nif* regulon into quite different metabolic backgrounds. This appears to hold for *K. pneumoniae* and *Azotobacter chroococcum* where hybridization was observed between the *nifKDH* and *V* genes of these two organisms but not between the *nifLA* genes (Jones *et al.*, 1984). However, in *R. meliloti* a region of DNA linked to the *nifHDK* operon hybridizes to *K. pneumoniae ntrC* and *nifA* DNA, and inserts of Tn5 in this region confer a regulatory Fix⁻ phenotype (Szeto *et al.*, 1984).

To investigate possible intergeneric homology at the functional level, we have introduced certain *Klebsiella* regulatory genes into *A. vinelandii* on wide-host-range plasmids and observed the effect on *nif* expression. We have also used *Klebsiella nif* promoters fused to *lacZ* on wide-host-range plasmids as probes for *Azotobacter* regulatory proteins functionally analogous to those in *Klebsiella*. Our findings suggest that the *nif* regulatory mechanism in *Azotobacter* includes an *ntrC*-like activator, but that *nif*-specific regulation is less like that of *K. pneumoniae* than was suggested by the earlier observation that the *Klebsiella nifA* product activates *nif* expression in *A. chroococcum* and *A. vinelandii* (Kennedy & Robson, 1983). These results may also explain why pRD1, which carries the entire *Klebsiella nif* cluster, fails to confer a Nif⁺ phenotype on *A. vinelandii* strains with mutations in the structural genes for nitrogenase (Cannon & Postgate, 1983).

METHODS

Bacteria. The strains used in this work are described in Table 1.

Plasmid construction. The plasmids used to construct various other recombinant plasmids are described in Table 2. Restriction enzymes, Bal31, T4 DNA ligase and polymerase were obtained from commercial sources and used in TARS buffer (33 mM-Tris/acetate pH 7.9, 66 mM-potassium acetate, 10 mM-magnesium acetate, 0.5 mM-dithiothreitol, 4 mM-spermidine, 10 µg pancreatic RNAase ml⁻¹) following standard procedures (Maniatis *et al.*, 1982). To make pMD114, a set of deletions was made in pMM12 (Espin *et al.*, 1982) by using Bal31 as an endonuclease on supercoiled DNA, followed by restriction, generation of blunt ends, and intra-molecular ligation. The structure of the recombinant plasmids was ascertained by restriction analysis of small scale DNA preparations (Holmes & Quigley, 1981).

Media. Burk's medium with 1% sucrose (BS; Newton *et al.*, 1953) was used for growing *A. vinelandii*; LA was used as rich medium and NFDM as nitrogen-free medium for growing *E. coli* and *K. pneumoniae* (Kennedy, 1977; Dixon *et al.*, 1977). Ammonium acetate (1.1 mg ml⁻¹) was added as nitrogen source. UW_r and UW_{1r} resisted rifampicin (25 µg ml⁻¹). Antibiotics were added for *E. coli* (and *K. pneumoniae*) and for *A. vinelandii*, respectively, as follows: streptomycin, 50 and 1 µg ml⁻¹; ampicillin, 50 and 15 µg ml⁻¹; kanamycin, 40 and 1 µg ml⁻¹; tetracycline, 10 and 2 µg ml⁻¹; sulfathiazole, 200 µg ml⁻¹ for both organisms. X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) was added to BS solid medium at 30 µg ml⁻¹.

Plasmid transfer. The Tra⁺ mobilizing plasmid pRK2013 (Figurski & Helinski, 1979) was used to mobilize other plasmids between *E. coli* strains or between *E. coli* and strains of *Azotobacter*. Tri-parental matings were done by mixing 25 µl exponential phase cultures on LB agar plates (Dixon *et al.*, 1977) (for transfer to *E. coli*) or on Burk's sucrose agar supplemented with 25% (w/v) LB agar, 0.2% glucose and 15 mM-ammonium acetate (for transfer to *A. vinelandii* or to *A. chroococcum*). Plates used for matings were incubated for 18 h at 30 °C after which the cells were scraped from the plates, resuspended in buffer and plated on selective medium. The various recombinant IncQ and IncP plasmids (Table 2) transferred to *A. vinelandii* at frequencies of 10⁻¹–10⁻² per recipient. Only pMD23 could be transferred to *A. chroococcum* MCD-1, and at a frequency of about 10⁻⁵. Since MCD-1 is resistant to streptomycin, there was no means of selecting for transfer of pMD61 to this strain. We were unable to transfer the IncP plasmid pCK3 to MCD-1 or its derivatives (see Table 1 and Robson *et al.*, 1984).

Although pRK2013 failed to replicate in *A. vinelandii*, it apparently could be transferred and transiently expressed to allow mobilization of plasmids to *E. coli*. Transfer frequency of pMD61 from *A. vinelandii* UW_r to HB101 after mixing these two cultures with JC5466(pRK2013) was about 10⁻⁴ to 10⁻⁵ per recipient. Similar

Table 1. *Bacterial strains*

Species	Strain no.	Relevant genotype/phenotype	Source or reference
<i>K. pneumoniae</i>	UNF931	<i>hisD2 recA56 srl-300::Tn10 hsdR1 Δ(lac)2002</i>	Drummond <i>et al.</i> (1983)
	UNF745	<i>nifA2787::Mud(Ap^r lac) recA56 srl-300::Tn10 hsdR1 Δ(lac)2002 Gal^r</i>	Merrick <i>et al.</i> (1982)
<i>E. coli</i>	ET8556	<i>ntrC1488 lacZ::IS1 gyrA hutC₈ Rbs⁻</i>	MacNeil <i>et al.</i> (1982)
	HB101	<i>hsdS recA13 proA lacY galK rpsL supE Xyl⁻ Mtl⁻ Ara⁻</i>	P. Nurse, ICRF, London, UK
	JCS466	<i>trp recA56 rpsE His⁻</i>	R. Dixon, AFRC Unit of Nitrogen Fixation, Sussex, UK
<i>A. vinelandii</i>	UW ^r	Nif ⁺ Rif ^r	Bishop & Brill (1977)
	UW1 ^r	<i>nif1</i> Rif ^r	Shah <i>et al.</i> (1973)
	UW2 ^r	<i>nif2</i> Rif ^r	Gordon & Brill (1972)
<i>A. chroococcum</i>	MCD-1	Nif ⁺	Robson <i>et al.</i> (1984)
	MCD-1008	Nif ⁻	Kennedy & Robson (1983)

Table 2. *Plasmid construction*

Designation ...	pMD21	pMD23	pMD61	pMD45	pMD132	pCK3
Promoter fusion or regulatory gene	<i>nifF-lacZ</i>	<i>nifL-lacZ</i>	<i>nifH-lacZ</i>	<i>ntrC</i>	<i>nifL</i>	<i>nifA</i>
Parent replicons*	RSF1010, pRD543	RSF1010, pRD512	RSF1010, pJB31	RSF1010, pMD114	RSF1010, pMD2	pRK290, pMC73A
Incompatibility group	IncQ	IncQ	IncQ	IncQ	IncQ	IncP
Site of cointegration	<i>EcoRI</i>	<i>EcoRI</i>	<i>PstI</i>	<i>PstI</i>	<i>EcoRI</i>	<i>BglII/BamHI</i>
Size	19.0 kb	19.6 kb	19.0 kb	14.8 kb	14.9 kb	27 kb
Drug resistances	Ap ^r Su ^r	Ap ^r Su ^r	Sm ^r	Sm ^r	Ap ^r Su ^r	Tc ^r
		<i>nifF-Sm^r</i>			<i>nifF-Sm^r</i>	<i>nifB-Km^r</i>

* References are as follows: RSF1010, Guerry *et al.* (1974); pRD543 and pRD512, Drummond *et al.* (1983); pJB31 and pMC73A, Buchanan-Wollaston *et al.* (1981a); pMD114 and pMD2, this paper; pRK290, Ditta *et al.* (1980). Plasmid pRD512 is analogous to pRD526 (Drummond *et al.*, 1983), except that the fusion junction is at codon 98 of *nifL* rather than codon 33.

frequencies were obtained for transfer of other IncQ plasmids from *A. vinelandii*, but pCK3, an IncP plasmid, could not be mobilized from UW1^r using pRK2013. Also, the presence of pCK3 inhibited transfer of pMD61 from UW^r; transfer of pMD61 to HB101 from UW^r(pMD61) (pCK3) was reduced by about 100-fold.

Enzyme assays. Cultures for measuring nitrogenase and/or β -galactosidase activities were prepared by first suspending organisms taken from 1-d-old selective agar plates in NH₄⁺-containing NFDM (for *K. pneumoniae*) or Burk's sucrose (for *A. vinelandii*) with appropriate antibiotics for plasmid selection. These liquid culture were grown with shaking for 6–8 h to a density of about 10⁹ cells ml⁻¹, and were then centrifuged, resuspended and diluted 10⁻¹ as follows: *K. pneumoniae* into 5 ml NFDM with and without added NH₄⁺ (15 mM) in 7 ml bijou bottles closed with a suba-seal; *A. vinelandii* into 5 ml Burk's sucrose with and without added NH₄⁺ (15 mM) in 25 ml cotton-stoppered flasks. NH₄⁺-free cultures of the Nif⁻ mutant UW1^r contained urea (2 mM) as a non-repressing nitrogen source. Culture vessels were incubated with shaking for 16 h at 30 °C. Acetylene reduction was measured for *K. pneumoniae* 15 min after injecting 0.5 ml acetylene directly into the closed bijou bottles. A sample (1 ml) of each *A. vinelandii* culture was transferred to a 7 ml bijou bottle for a similar 15 min assay. Two samples (0.1 ml) were removed from duplicate cultures for measuring β -galactosidase activity as described by Miller (1972).

RESULTS

Plasmid construction

Cointegrates were constructed *in vitro* between small plasmids carrying *K. pneumoniae* regulatory genes (*nifA*, *ntrC* and *nifL*) or *nif-lacZ* fusions and the wide-host-range plasmids pRK290 (IncP; Ditta *et al.*, 1980) or RSF1010 (IncQ; Guerry *et al.*, 1974). Their properties and mode of construction are summarized in Table 2, and the physical structures of the IncQ derivatives, as confirmed by restriction analysis, are depicted in Fig. 1. Plasmid pCK3 is a cointegrate of pMC73A and pRK290 formed by ligation between the respective *Bam*HI and

Fig. 1. Structure of IncQ plasmids carrying *K. pneumoniae* *nif* promoters and *nif*-*lac* fusions. Open boxes show the position and direction of transcription of structural genes. The dashed line in pMD45 designates sequences of unknown origin. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sma*I; Sa, *Sal*I; X, *Xma*III. Drug resistance genes: Ap, ampicillin; Sm, streptomycin; Su, sulphonamide; Tc, tetracycline. RSF1010; pBR322; pACYC177; *K. pneumoniae* sequences; *lac* sequences.

*Bgl*II sites. Rearrangements which occurred in the physical structure of this plasmid *in vivo* made it difficult to characterize fully by restriction analysis, but it retained all the relevant genetic markers of the component replicons, except for ampicillin resistance carried by pMC73A (Buchanan-Wollaston *et al.*, 1981 *a*). However, pCK3 did retain temperature-sensitive expression of the *Km*^r gene which in pMC3A is translationally fused to the *nifB* promoter and hence under the control of the thermolabile *nifA* activator (Kennedy & Robson, 1983).

The remaining cointegrates were all made with the IncQ plasmid, RSF1010. The *nifL* gene from *K. pneumoniae* was cloned into pBR322 on an *Eco*RI-*Xma*III fragment to give pMD2, which was joined to RSF1010 at the *Eco*RI site to give pMD132. The *ntrC* plasmid pMD114, used to make pMD45, was derived from pMM12 (Espin *et al.*, 1982) using *Bal*31 to delete 2.6 kb between the *Hind*III site in pACYC177 and the end of the regulatory gene, thereby removing *IS21* sequences present in this region. This was done in an attempt to stabilize the eventual cointegrate pMD45. The other small plasmids used in this study have all been described previously: pJB31, pMC73A, pRD512 and pRD543 used to make pMD61, pCK3, pMD23 and pMD21, respectively (see Table 2 for references).

During construction of these cointegrates we found that while pMD21, pMD23 and pMD132 were stable, similar constructs with the constituent replicons in the opposite orientation to that shown in Fig. 1 were not; the latter disintegrated in *E. coli* to give two plasmids indistinguishable in size from the parent plasmids. The reason for this instability is unclear.

Instability of a different kind was observed in pMD45. During prolonged growth with selection for streptomycin resistance in *E. coli* ET8556 (*NtrC*⁻), the original construct was gradually replaced in the population by a derivative which had spontaneously acquired an additional 960 bp of extraneous DNA between the *Km*^r promoter and the start of the *ntrC* gene. Nevertheless, the plasmid remained *ntrC*⁺ in *E. coli*.

The copy numbers of these cointegrates were not measured precisely in either *K. pneumoniae* or *A. vinelandii*, but rough estimates were possible based on comparisons of plasmid yields in small-scale DNA preparations. In *E. coli*, RSF1010 and its derivatives are maintained at 20–50 copies per cell (Nagahari & Sakaguchi, 1978) while IncP plasmids are present at about five copies per cell (Figurski *et al.*, 1979). The staining intensities on agarose gels of supercoiled plasmid DNA prepared from saturated cultures of all three organisms were similar, suggesting 5- to 10-fold higher copy number of IncQ than IncP replicons, whether or not these were present in the same cell. *A. vinelandii* has about 40 chromosomes per cell (Sadoff *et al.*, 1979) and therefore the plasmid to chromosome ratio is at least 2:1 for RSF1010 derivatives and 1:5 for the IncP plasmid pCK3.

Table 3. Nitrogenase activities [nmol ethylene produced min⁻¹ (mg protein)⁻¹] in *K. pneumoniae* and *A. vinelandii* transconjugants

Plasmid	<i>K. pneumoniae</i> UNF931*		<i>A. vinelandii</i> *			
			UWr		UW1r	
	-NH ₄ ⁺	+NH ₄ ⁺	-NH ₄ ⁺	+NH ₄ ⁺	-NH ₄ ⁺	+NH ₄ ⁺
None	58 (+)	0	65 (+)	0	0 (-)	ND
pMD132 (<i>nifL</i>)	0 (-)	0	61 (+)	0	0 (-)	ND
pCK3 (<i>nifA</i>)	65 (+)†	49†	48 (+)	52	60 (+)	9
pMD132 + pCK3 (<i>nifL</i> + <i>nifA</i>)	ND	ND	63 (+)	0	2.4 (-)	ND
pMD61 (<i>nifH-lacZ</i>)	0 (-)	0	58 (+)	ND	0 (-)	ND
pMD23 (<i>nifL-lacZ</i>)	55 (+)	0	55 (+)	ND	0 (-)	ND
pMD45 (<i>ntrC</i>)	(+)		60 (+)	0	0 (-)	0

ND, Not determined.

* (-) or (+) indicates extent of growth of transconjugant on nitrogen-free agar medium.

† In *nifLA* strain UNF745.*Effect of K. pneumoniae regulatory genes on N₂ fixation in A. vinelandii*

The IncP *nifA* plasmid pCK3 corrected the Nif⁻ phenotype in the *A. vinelandii* regulatory mutants UW1r (Table 3) and UW2r (not shown), restoring wild-type levels of nitrogenase activity. Like pCK1, an IncQ *nifA*-constitutive plasmid (Kennedy & Robson, 1983), pCK3 also activated *nif* expression in ammonium-grown cultures of UW1r or UWr, the Nif⁺ parent strain. Its compatibility with the IncQ cointegrates described above made possible the construction of several useful *A. vinelandii* transconjugants. A further advantage of pCK3 over pCK1 is that it did not inhibit growth of *A. vinelandii*, presumably a result of its lower copy number and hence lower dosage of the *nifA* gene.

The multicopy *nifL* plasmid, pMD132, completely repressed N₂ fixation in *K. pneumoniae*, but not in *A. vinelandii*. The *nifL* gene product did, however, prevent the *nifA* product cloned in pCK1 from restoring a Nif⁺ phenotype in the regulatory mutant UW1r (Table 3); nitrogenase activity in UW1r(pCK3)(pMD132) was only about 4% of that of UW1r(pCK3). A control experiment showed that the IncQ vector RSF1010, used to make pMD132, had no effect on activation of UW1r by pCK3 (data not shown).

To see whether multiple copies of the *K. pneumoniae nifH* promoter would inhibit N₂ fixation in *A. vinelandii*, pMD61 was introduced into the wild-type strain UWr. There was no apparent effect on nitrogenase activity in UWr, while, as expected, activity was not detected in *K. pneumoniae* carrying this plasmid (Table 3).

The *ntrC* plasmid pMD45 did not restore a Nif⁺ phenotype to the regulatory mutant *A. vinelandii* UW1r or prevent ammonium repression of N₂ fixation in the wild-type UWr (Table 3). This cointegrate was stable in *Azotobacter* as judged by sizing the plasmid DNA on agarose gels.

Expression of K. pneumoniae nif promoters in A. vinelandii

Azotobacter species are naturally Lac⁻ (Thompson & Skerman, 1979) and hence no mutant construction was necessary to measure expression from *nif-lacZ* fusions. Attempts were initially made to introduce *nif::Mudlac* transcriptional fusions derived from pRD1 (Dixon *et al.*, 1980) into *A. vinelandii*, but the resulting ampicillin resistant transconjugants were unstable, losing the plasmid during subculture. Thus it was necessary to integrate *nif-lacZ* translational fusions constructed *in vitro* into wide-host-range plasmids.

The β -galactosidase activity of various *K. pneumoniae* and *A. vinelandii* strains in ammonium-sufficient and -deficient conditions are shown in Table 4. The *nifL-lacZ* (pMD23) and *nifF-lacZ* (pMD21) plasmids were both expressed in *A. vinelandii* UWr and the mutant UW1r grown with or without ammonium, at levels higher than those observed in *K. pneumoniae* under any

Table 4. β -Galactosidase activity in *A. vinelandii* and *K. pneumoniae* transconjugants with wide-host-range *nif-lac* fusion plasmids

β -Galactosidase activity was determined according to Miller (1972). Values are expressed in Miller units and are the means of two to four independent measurements done in duplicate, and where possible are given $\pm$ SEM.

Plasmids	Fusion	<i>A. vinelandii</i>					
		<i>K. pneumoniae</i> UNF931 (Nif ⁺)					
		-NH ₄ ⁺	+NH ₄ ⁺	UWr (Nif ⁺)		UW1r (Nif ⁻)	
		-NH ₄ ⁺	+NH ₄ ⁺	-NH ₄ ⁺	+NH ₄ ⁺	-NH ₄ ⁺	+NH ₄ ⁺
pMD23	<i>nifL-lacZ</i>	808	27	1333 ($\pm$ 300)	2273 ($\pm$ 480)	1934 ($\pm$ 30)	1292 ($\pm$ 250)
pMD21	<i>nifF-lacZ</i>	ND	ND	850	750	850	1200
pMD61	<i>nifH-lacZ</i>	2344 ($\pm$ 400)	2	0	ND	0	0
pMD61 + pCK3	<i>nifH-lacZ</i> + <i>nifA</i> [*]	ND	ND	17 ($\pm$ 6)	20 ($\pm$ 2)	26 ($\pm$ 10)	18 ($\pm$ 2)

ND, Not determined.

* *nifA* is constitutively expressed.

conditions. *A. chroococcum* MCD-1 and the regulatory mutant derived from it, MCD-1008, gave similar levels of expression as judged by the chromogenic reaction on solid media containing X-Gal. In *K. pneumoniae*, the *nifH*, *nifL* and *nifF* promoters were expressed in anaerobic conditions in the absence of ammonium, but not in its presence. In contrast, *A. vinelandii* carrying pMD61 did not express the *nifH* promoter in Burk's sucrose medium, with or without ammonium, with 2 mM-urea (a non-repressive nitrogen source), with 5 mM-sodium acetate or without added molybdate, compounds known to affect *nif* expression in *Azotobacter* species (Ramos & Robson, 1985; Premakumar *et al.*, 1984). This lack of expression could not be attributed to plasmid instability, because when pMD61 was mobilized back into *K. pneumoniae*, *lacZ* was again expressed in a *nif*-specific manner. When *K. pneumoniae nifA* was introduced into *A. vinelandii* UWr on pCK3 along with pMD61, expression from the *K. pneumoniae nifH* promoter was activated slightly (Table 4). This β -galactosidase expression was obvious after 4–7 d incubation of the strain on medium containing X-Gal, while the transconjugant with pMD61 alone showed no chromogenic reaction.

DISCUSSION

Expression of *lacZ* from pMD21 (*nifF-lacZ*) and pMD23 (*nifL-lacZ*) but not from pMD61 (*nifH-lacZ*) in *A. vinelandii* suggests the presence in this organism of an activator protein with similar sequence recognition properties to that of *ntrC* from enteric organisms. This interpretation is supported by the recent isolation of a cosmid from an *A. vinelandii* library that restores an NtrC⁺ phenotype, including Nif⁺, in *ntrC* mutants of *K. pneumoniae* (A. Toukdarian, unpublished results). The failure of *nifL* and *nifF* fusions to be repressed by NH₄⁺ in *A. vinelandii* is surprising since nitrogenase synthesis and *nifHDK* mRNA synthesis is prevented by NH₄⁺ in this organism (Krol *et al.*, 1982). This could indicate that the *A. vinelandii* activator is synthesized in the presence of high levels of NH₄⁺ and repression occurs through a mechanism which fails to recognize the *K. pneumoniae* promoters. Alternatively, the *A. vinelandii* polymerase may not require a specific activator to recognize these foreign promoters, both of which show an appreciable NH₄⁺-constitutive *ntrA*-independent activity in *K. pneumoniae* which is not shared by the *nifH* promoter (Drummond *et al.*, 1983; Buchanan-Wollaston *et al.*, 1981a; Drummond, unpublished observations).

The question of *nif*-specific activation and repression is addressed by two sets of experiments presented here, utilizing the *K. pneumoniae nifH* promoter fused to *lacZ* and the *nifL* gene cloned on a wide-host-range vector. Repression of N₂ fixation by O₂ or by low levels of fixed nitrogen is

mediated by the *nifL* gene product in *K. pneumoniae*. A negatively acting regulatory gene may be present in *Azotobacter* because some Nif⁺ revertants of the Nif⁻ regulatory mutant UW2r are no longer repressed by NH₄⁺ (Gordon & Brill, 1972), a phenotype also shown by revertants of a Nif⁻ *nifL* mutant in *K. pneumoniae* (Merrick *et al.*, 1982; Filser *et al.*, 1983). Also, constitutive production of *nifA* prevents O₂ repression of *nif* genes in both organisms (Buchanan-Wollaston *et al.*, 1981a; Kennedy & Robson, 1983). Multiple copies of wild-type *nifL* prevent *nif* expression in *K. pneumoniae* in the absence of NH₄⁺ or O₂, presumably by titrating out a signal molecule that communicates derepressing conditions (Buchanan-Wollaston *et al.*, 1981b; Riedel *et al.*, 1983). Since the *nifL* product apparently prevents the *nifA* product from activating, rather than by binding directly to *nif* regulatory sequences (Buchanan-Wollaston & Cannon, 1984; Drummond & Kennedy, 1984), an excess of *nifL* in its repressive form will result in insufficient activator being present for effective expression of the other *nif* genes. If *A. vinelandii* has a gene functionally homologous to *K. pneumoniae nifL*, then one might observe an inhibitory effect of *nifL* on a multicopy plasmid introduced to *A. vinelandii*. We find, however, that pMD132 had no effect on *nif* expression in wild-type *A. vinelandii*, although the plasmid did prevent correction of the regulatory mutant by *K. pneumoniae nifA* product. The latter experiment showed that the *nifL* product is able to function in this foreign background. Expression from the *K. pneumoniae nifL* promoter is about twofold higher in *Azotobacter* than in its native background (Table 4), compensating in part for the lower plasmid to chromosome ratio. Although it is possible that the *A. vinelandii* activator is too abundant to be titrated out by *nifL* product from pMD132, the more likely interpretation of the negative result is that the *A. vinelandii nif*-specific activator is not affected by the *K. pneumoniae nifL* product. Also, the sequence recognition properties of the *nif*-specific *A. vinelandii* activator are probably different from those of the *K. pneumoniae nifA* product. This is indicated by the complete lack of expression of the *K. pneumoniae nifH* promoter on pMD61 in *A. vinelandii*. The low level of stimulation obtained by introducing the *K. pneumoniae nifA* gene is probably due to multiple copies of *nif* promoters on the *A. vinelandii* chromosome which bind most of the *nifA* product, leaving little to activate the *K. pneumoniae nifH* promoter. Multiple copies of the *A. chroococcum nifH* promoter prevent full expression of *nif* genes in *K. pneumoniae*, indicating a strong interaction with *nifA* product (Jones *et al.*, 1984). However, the converse is not true: the *K. pneumoniae nifH* promoter introduced on a multicopy plasmid had no effect on nitrogenase expression in *A. vinelandii*, while similar multicopy plasmids with the *A. chroococcum* or *A. vinelandii nifH* promoters greatly inhibited activity in the wild-type strain UW (Jones *et al.*, 1984; D. Dean, unpublished observations).

Thus, *nif*-specific regulatory proteins in *Azotobacter* are likely to show interesting differences from the *nifL* and *nifA* products found in *K. pneumoniae*. Perhaps they reflect differences in these two organisms such as the presence in azotobacters of a second *nifH* gene (Premakumar *et al.*, 1984; R. Robson, personal communication) and also the ability of *A. vinelandii* to fix N₂ on Mo-deficient medium (Bishop *et al.*, 1980). Elucidation of *nif*-specific control mechanisms in *Azotobacter* must await direct isolation of the relevant genes.

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