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Regulation of nitrogen metabolism in *Azotobacter vinelandii*: isolation of *ntr* and *glnA* genes and construction of *ntr* mutants

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The *ntrA*, *ntrB* and *ntrC* products are responsible for regulating the transcription of many genes involved in the assimilation of poor nitrogen sources in enteric bacteria. The presence of a similar system in the non-enteric bacterium *Azotobacter vinelandii* is reported here. Genes analogous to *ntrA* and *ntrC* were isolated from an *A. vinelandii* gene library by complementation of *Escherichia coli* mutants. The gene encoding glutamine synthetase, *glnA*, was also isolated and found to be adjacent to *ntrC* but distant from *ntrA*, as it is in enteric organisms. The cloned *Azotobacter* genes also complemented *Klebsiella pneumoniae* mutants and hybridized to *K. pneumoniae ntrA*, *ntrC* and *glnA* gene probes. The role of *ntrA* and *ntrC* in *A. vinelandii* was established by using Tn5 insertions in the cloned genes to construct mutants by marker exchange. These mutants show that both *ntrA* and *ntrC* are required for the utilization of nitrate as a nitrogen source. However, *ntrC* is not required for nitrogen fixation by *A. vinelandii*, in contrast with *K. pneumoniae* where both *ntrA* and *ntrC* are essential.

Key words: Azotobacter vinelandii/ntr genes/nitrogen fixation/nitrogen regulation/*glnA*

Introduction

The expression of many genes that allow enteric bacteria to grow on poor nitrogen sources is regulated such that their transcription is elevated when the concentration of supplied fixed nitrogen is low. This regulation is mediated by a central control system involving the products of three genes: *ntrA*, *ntrB* and *ntrC* (known as *glnF*, *glnL* and *glnG* in *Escherichia coli*) (reviewed by Magasanik, 1982). Positive control of nitrogen-regulated promoters requires both the *ntrA* and *ntrC* products (Garcia *et al.*, 1977; Kustu *et al.*, 1979; De Bruijn and Ausubel, 1981, 1983; Pahel *et al.*, 1982; MacNeil *et al.*, 1982; Merrick, 1983). The mechanism of this activation is not fully understood. However, a consensus sequence for *ntr*-activated promoters, different from the canonical *E. coli* promoter sequence, has been proposed based on the analysis of several such promoters (Dixon, 1984a) and it has been suggested that the *ntrA* product may be a sigma-like subunit of RNA polymerase that allows the recognition of a class of promoters expressed when fixed nitrogen is limiting (Beynon *et al.*, 1983; de Bruijn and Ausubel, 1983; Merrick and Stewart, 1985). The *ntrC* product can also repress transcription from certain promoters (see below). The apparent role of the *ntrB* product is to modulate both the activating and repressing functions of the *ntrC* product according to the nitrogen status of the cell (Alvarez-Morales *et al.*, 1984).

The *ntrB* and *ntrC* genes are adjacent to *glnA*, the structural gene for glutamine synthetase, in an operon where transcription of *ntrBC* is either from the same promoter as *glnA* or from a second promoter between *glnA* and *ntrB* (Pahel *et al.*, 1982; Alvarez-Morales *et al.*, 1984; Krajewska-Grynkiewicz and Kustu, 1984). This p*glnA*p*ntrBC* operon is subject to autogenous regulation. Under conditions of low fixed nitrogen, the *ntrC* product with the *ntrB* product activates expression at p*glnA*; with fixed N in excess, the *ntrC* product represses transcription from p*glnA* and p*ntrBC* (Pahel and Tyler, 1979; McFarland *et al.*, 1981; Pahel *et al.*, 1982; Alvarez-Morales *et al.*, 1984; Dixon, 1984b). The *ntrA* gene is unlinked to *glnAntrBC* and its expression is apparently not regulated by fixed N (de Bruijn and Ausubel, 1983; Castano and Bastarrachea, 1984; Merrick and Stewart, 1985).

Other genes shown to be under positive *ntr* control include those required for the assimilation of arginine, histidine or proline by *E. coli* and *Klebsiella pneumoniae*; for the transport of certain amino acids in *Salmonella typhimurium*; and for nitrogen fixation (*nif*) in *K. pneumoniae* (Magasanik, 1982, and references therein). In the complex 17-gene *nif* regulon of *K. pneumoniae*, the *ntrA* and *ntrC* products are required for activation of the *nifLA* operon. *nifA* encodes a *nif*-specific activator which acts with the *ntrA* product to effect the expression of the seven other *nif* operons (Ausubel, 1984; Dixon, 1984a).

The ability to fix nitrogen has been demonstrated in a number of other bacterial groups. Organisms in the genus *Azotobacter* have been particularly well studied because of their unique tolerance to oxygen when fixing nitrogen. In addition, *A. vinelandii* expresses genes for an 'alternative' nitrogenase when grown under conditions of molybdenum deprivation (Bishop *et al.*, 1980, 1982).

Recent studies have suggested similarities between the way *nif* is regulated in *K. pneumoniae* and Azotobacters. The *nif* genes of *A. vinelandii* and *A. chroococcum* can be activated by the *nifA* product of *K. pneumoniae* (Kennedy and Robson, 1983; Kennedy and Drummond, 1985) and regulatory mutants, at least superficially like *K. pneumoniae nifA*$^-$ strains, have been isolated in *A. vinelandii* (Shah *et al.*, 1973). Although the *nifH* promoter of *K. pneumoniae* is not expressed in *A. vinelandii*, the *nifL* promoter of *K. pneumoniae* is activated under conditions of both ammonium (NH_4^+) excess or limitation, suggesting that *A. vinelandii* has genes analogous to *ntrA* and *ntrC* (Kennedy and Drummond, 1985).

This paper reports the isolation of *ntrA*, *ntrC* and *glnA* genes from *A. vinelandii* by complementation of *E. coli* mutants. As in the enteric bacteria, *ntrC* is adjacent to *glnA* and distant from *ntrA* in *A. vinelandii*. The roles of these genes in regulating nitrogen metabolism were established by using Tn5 insertions in the cloned genes to construct *A. vinelandii ntrA* and *ntrC* mutants.

Results

Isolation of Azotobacter DNA complementing ntrA, ntrC and glnA mutations of E. coli

A library of *A. vinelandii* genomic DNA constructed in the

Table I. Glutamine synthetase and nitrogenase activities in complemented *E. coli* and *K. pneumoniae* [Δ(*glnAntrBC*)] mutants

Strain	Genotype	Glutamine synthetase[a]		Nitrogenase[b]
		pH 7.27	pH 7.9	
E. coli				
ET8000	*glnA*$^+$ *ntr*$^+$	1408	3664	
ET8894	Δ(*glnAntrBC*)	0	35	
ET8894 (pGE10)[c]		2217	7846	
ET8894 (pLV50)		36	161	
ET8894 (pAT512)		23	159	
K. pneumoniae				
KP5022	*glnA*$^+$ *ntr*$^+$	348	1533	100
UNF1848	Δ(*glnAntrBC*)	0	15	0
UNF1848 (pGE10)		916	4344	100
UNF1848 (pLV50)		149	320	20–80
UNF1848 (pAT512)		111	352	50–80
UNF1848 (pAT523)				50–80
UNF1848 (pAT508)				0

[a]Activity measured in cultures grown on limiting nitrogen using the Mn^{2+}-dependent γ-glutamyl transferase activity at pH 7.27 or pH 7.9. Activity reported as nmol/min/mg protein.
[b]Nitrogenase, acetylene reduction activity, measured in dilute 18 h derepression cultures. Activity reported as a percentage of KP5022 (0.32 nmol/min/OD_{540}).
[c]pGE10 is an IncP plasmid carrying *glnA, ntrB* and *ntrC* genes from *K. pneumoniae* (Espin *et al.*, 1981).

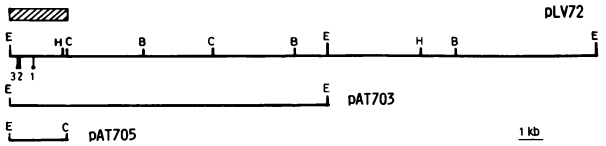

Fig. 1. The *ntrA* region of *A. vinelandii* DNA cloned in pLV72. The restriction enzymes used in mapping pLV72 were: *Eco*RI, E; *Hin*dIII, H; *Bgl*II, B; and *Cla*I, C. The subclones shown were constructed in pBR325, and are Ntr$^+$Nif$^+$ as determined by growth of the *E. coli ntrA* mutant ET8045 on arginine and the *K. pneumoniae ntrA* mutant CK273 on arginine or N_2. Tn*5* insertions which are NtrA$^-$ are in pLV72 (■) or pAT705 (●). ▨ shows the region of pLV72 which hybridized to the *K. pneumoniae ntrA* gene probe.

cosmid pLAFR1 was conjugally transferred into *E. coli ntr* mutants ET8045 (*ntrA*$^-$), ET8556 (*ntrC*$^-$) and ET8894 [Δ(*glnAntrBC*)]. Transconjugants corrected for the mutant phenotype were selected by growth on minimal glucose medium with tetracycline and either arginine (for Ntr$^+$ selection in ET8045 and ET8556) or ammonia (for Gln$^+$ selection in ET8894) as the nitrogen source. Several Ntr$^+$ or Gln$^+$ transconjugants of each strain were analysed further. To confirm their correcting ability and to test for *glnA*–*ntrC* linkage, the cosmids in these transconjugants were transferred into HB101 and then back into the three different mutants. In addition, plasmid DNA was purified and analysed on gels after restriction with *Eco*RI.

Three different cosmids were isolated from NtrA$^+$ ET8045 transconjugants. All three cosmids shared a 13-kb *Eco*RI fragment. When transferred back into ET8045 all three complemented it for growth on arginine but none corrected the *gln*A or *ntrC* mutations in ET8894 or ET8556. One of the three *ntrA*$^+$ cosmids, pLV72, was chosen for further studies.

Two cosmids, pLV50 and pLV51, which had 6-kb and 12-kb *Eco*RI fragments in common, were isolated from NtrC$^+$ ET8556 transconjugants. The GlnA$^+$ ET8894 transconjugants contained a cosmid apparently identical to pLV50. These ET8894 transconjugants were subsequently found to grow on arginine. This indicated that pLV50 provided both *glnA* and *ntrC* complementing genes. When pLV50 was transferred into HB101 and then back into ET8894 and ET8556 it corrected both the *glnA* and/or *ntrC* mutations in these strains.

When pLV51 was transferred back into ET8556 its ability to restore an NtrC$^+$ phenotype was confirmed. However, transfer of pLV51 into ET8894 gave only a low frequency of GlnA$^+$ transconjugants ($<10^{-7}$). Restriction analysis of the cosmids in these rare GlnA$^+$ transconjugants showed a pattern generally similar to pLV51 but certain fragments contained additional DNA. Neither pLV50 nor pLV51 could correct the *ntrA* mutation of ET8045.

It therefore appeared that *A. vinelandii* had genes funtionally equivalent to *glnA, ntrC* and *ntrA*, and that *ntrC* was close to *glnA* and distinct from *ntrA*.

Expression of A. vinelandii ntr-like genes in K. pneumoniae

Transfer of the *ntrA*-complementing cosmid pLV72 into the *K. pneumoniae ntrA* mutant CK273 restored its ability to utilize either arginine aerobically or N_2 anaerobically as nitrogen source. Acetylene reduction activity in derepressed cultures of CK273 (pLV72) ranged from 20 to 70% that of the wild-type strain KP5022.

The *glnA-ntrC* complementing cosmid pLV50 allowed *K. pneumoniae* strain UNF1848 [Δ(*glnAntrBC*)] to utilize arginine or N_2 as nitrogen source and also corrected the glutamine auxotrophy of this strain. Derepression of *nif* genes in UNF1848 (pLV50) resulted in acetylene reduction activity 20–80% that of the wild-type (Table I). pLV50 did not restore the ability of the *K. pneumoniae nifA*$^-$ strain CK2631 to grow on N_2 or to reduce acetylene.

These experiments indicated that the *ntrA* gene product of *A. vinelandii* could interact with both *ntrC*-activated and *nifA*-activated promoters of *K. pneumoniae*. Furthermore, the *ntrC* gene of *A. vinelandii* could substitute for the *K. pneumoniae ntrC* gene but not the *nifA* gene, at least under the conditions tested.

Identification of the ntrA gene

The region of DNA on pLV72 providing *ntrA* complementation was identified by subcloning fragments of this cosmid. A restriction map of pLV72 and of its subclones is shown in Figure 1. As expected, plasmid pAT703, which contained the 13-kb *Eco*RI fragment common to the three *ntrA*-correcting cosmids originally isolated, corrected the *ntrA* mutation of ET8045. pAT705, which contained a 2.4-kb *Eco*RI–*Cla*I fragment of *A. vinelandii* DNA,

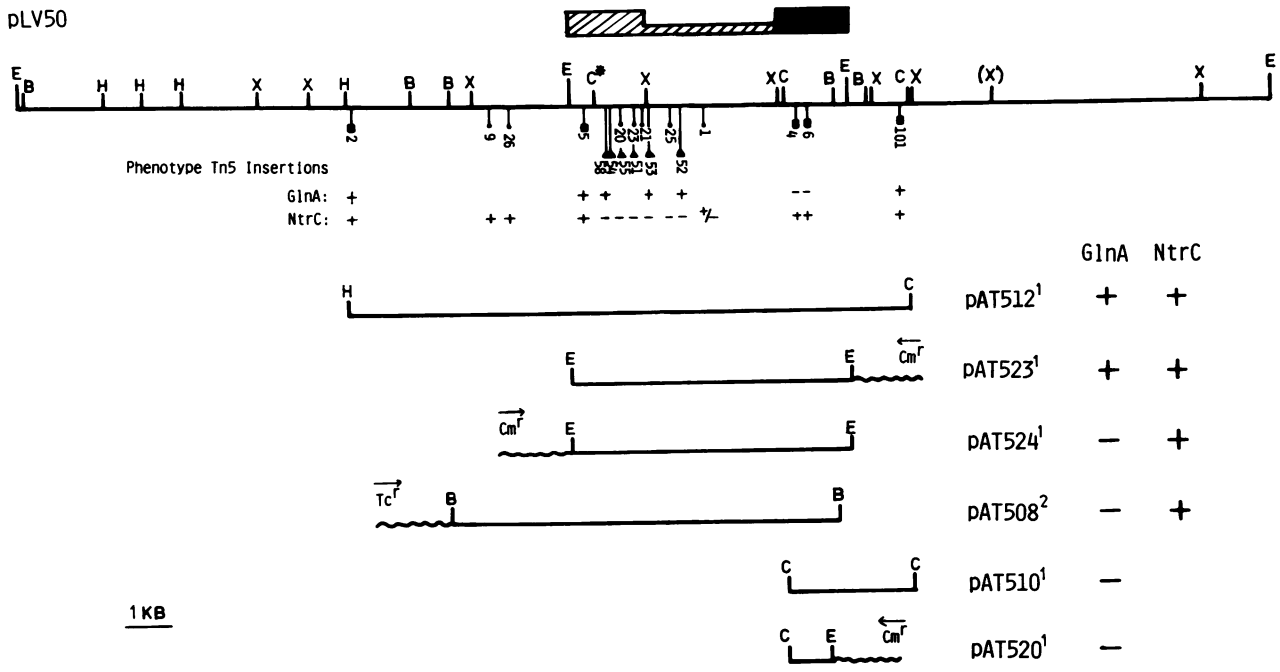

Fig. 2. The *glnA−ntrC* region of *A. vinelandii* DNA cloned in pLV50. Cosmid pLV50 was mapped using: *Eco*RI, E; *Bam*HI, B; *Hin*dIII, H; *Xho*I, X; and *Cla*I. C. (X) denotes an uncertain placement of an *Xho*I site and C* denotes one of the 6−7 *Cla*I sites detected in pLV50 isolated from *A. vinelandii* but not detected in pLV50 isolated from *dam*$^+$ *E. coli*. Vectors used in subcloning were pBR325 (1) or pACYC184 (2) and vector sequences, where important, are indicated by a wavy line. Tn5 insertions were in pLV50 (■), pAT512 (▲) or pAT508(●). The phenotype of subclones and of plasmids with Tn5 insertions was determined by growth of *E. coli* mutant strains carrying the plasmid: GlnA$^+$, growth of *E. coli* [Δ(*glnAntrBC*)] mutant ET8894 on ammonia; NtrC$^+$, growth of *E. coli ntrC*$^-$ mutant ET8556 on arginine. ■ indicates the region of pLV50 which hybridized to the *K. pneumoniae glnA* gene probe and ▨ the region which hybridized to the *ntrC* gene probe.

was constructed from pAT703 by deleting a *Cla*I fragment spanning insert and non-essential vector sequences. pAT705 corrected the *ntrA* mutations of ET8045 and CK273 in the same manner as pLV72 and therefore appeared to contain the entire *A. vinelandii ntrA* gene. Tn*5* mutagenesis of pLV72 and pAT705 yielded three insertions which were NtrA$^-$ in ET8045. These insertions, shown in Figure 1, mapped within the 2.4-kb *Eco*RI−*Cla*I fragment. When *Eco*RI + *Cla*I restricted pLV72 was probed with the *ntrA* gene of *K. pneumoniae*, only the 2.4-kb fragment hybridized to the probe (M.Merrick and A.Toukdarian, unpublished results).

Thus physiological, genetic and molecular evidence confirmed that a 2.4-kb *Eco*RI-*Cla*I fragment of *A. vinelandii* DNA contained the *ntrA* gene of this bacterium.

Identification of the glnA and ntrC genes

The locations of the *glnA* and *ntrC* genes on pLV50 were identified using a combination of subcloning, Tn*5* mutagenesis and Southern hybridization analysis. The cleavage sites in pLV50 for several restriction endonucleases were mapped (Figure 2). Various fragments of pLV50 were subcloned and their ability to complement *glnA* or *ntrC* mutants of *E. coli* was determined (Figure 2). The smallest fragment which complemented both *glnA* and *ntrC* was the 6-kb *Eco*RI fragment subcloned in pAT523. However, this fragment did not complement *glnA* when cloned in the opposite orientation (subclone pAT524) in the same vector (pBR325) nor when cloned in either orientation in the plasmid pRK2501 (data not shown). An interpretation of these results is that although the 6-kb *Eco*RI fragment encodes *glnA*, it lacks a complete promoter and so expression of *glnA* in pAT523 is from the constitutive Cm^r promoter of pBR325.

Subclone pAT512, which carries a 12-kb *Hin*dIII−*Cla*I fragment containing the 6-kb *Eco*RI fragment and flanking sequences, also complemented *glnA* and *ntrC* mutants (Figure 2). Expression of *glnA* from pAT512 is assumed to be from the Azotobacter *glnA* promoter as there are no known vector promoters reading through the insert in the correct orientation (Stüber and Bujard, 1981).

Besides complementing *E. coli glnA* and *ntrC* mutations, pAT512 and pAT523 also complemented both the *glnA* and *ntrC* mutations in *K. pneumoniae* UNF1848.

The glutamine synthetase (GS) activity expressed from pLV50 and from pAT512 in ET8894 and UNF1848 grown under nitrogen-limiting conditions was measured using the Mn^{2+}-dependent γ-glutamyl transferase assay. Activity was measured at both pH 7.27 and pH 7.9, the isoactivity points for the native *E. coli* and *K. pneumoniae* GS enzymes, respectively. The isoactivity point for the *A. vinelandii* enzyme, pH 8.5, was not used as both the adenylylated and unadenylylated forms of the enzyme have low activity at this pH (Kleinschmidt and Kleiner, 1978; Siedel and Shelton, 1979). As shown in Table I, similar levels of transferase activity were obtained with both plasmids in either background. Though this activity was much higher than that in the mutants, it was significantly lower than that found in either of the *glnA*$^+$ parental strains or in either mutant carrying a plasmid, pGE10 (Espin *et al.*, 1981), containing the *glnAntrBC* region of *K. pneumoniae*. The low activity from pLV50 and pAT512 may reflect a lowered level of *glnA* expression; however, the transferase assay is known to underestimate the activity of unadenylylated GS in *A. vinelandii* (Kleinschmidt and Kleiner, 1978; Siedel and Shelton, 1979).

A number of subclones complemented ET8556 for growth on arginine (Figure 2). Since *ntrC* but not *glnA* is expressed from plasmids pAT524 or pAT508 there must be a promoter in the *A. vinelandii* DNA from which *ntrC* expression can occur independently of the *glnA* promoter.

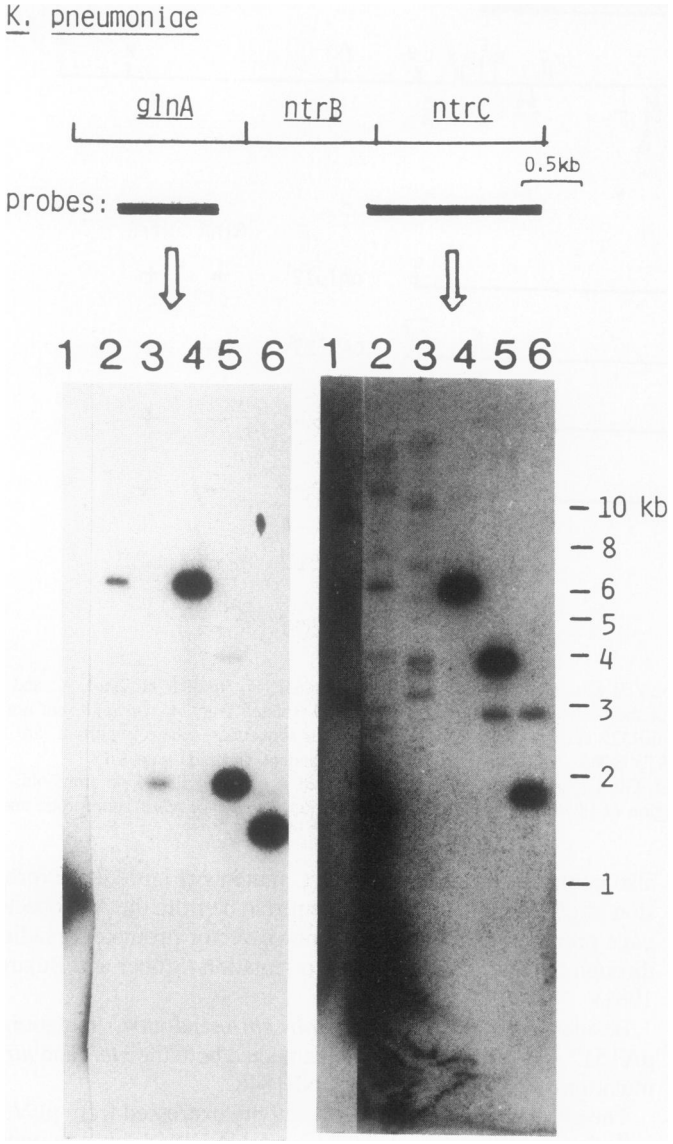

Fig. 3. Hybridization of *K. pneumoniae glnA* and *ntrC* genes to *A. vinelandii* genomic DNA and to pLV50. The *glnA* and *ntrC* probes used in the hybridization are indicated above the appropriate autoradiograph and are described in Materials and methods. The probes were hybridized to: **lanes 1**, *K. pneumoniae* DNA, *Eco*RI digested; **lanes 2**, *A. vinelandii* DNA, *Eco*RI digested; **lanes 3**, *A. vinelandii* DNA, *Xho*I digested; **lanes 4**, pLV50, *Eco*RI digested; **lanes 5**, pLV50, *Xho*I digested and **lanes 6**, pLV50, *Eco*RI + *Xho*I digested. The fragments of pLV50 hybridizing to *glnA* or *ntrC* are indicated above the map of pLV50 in Figure 2.

Not all $NtrC^+$ subclones could activate *nif* expression in UNF1848. Only those plasmids which were $GlnA^+$ as well as $NtrC^+$ had detectable levels of acetylene reduction activity (Table I) or allowed anaerobic growth of UNF1848 on NH_4^+-free agar plates containing 0.1 mg/ml glutamine. To examine this, subclone pAT508 was transferred into the *K. pneumoniae* $ntrC^-$ point mutant UNF1828. UNF1828 (pAT508) could grow aerobically on arginine or anaerobically on N_2 and had approximately wild-type levels of acetylene reduction activity.

The localization of the *glnA* and *ntrC* coding regions on pLV50 was confirmed by Southern hybridization analysis. Restriction digests of pLV50 and of *A. vinelandii* genomic DNA were probed with *glnA* or *ntrC* from *K. pneumoniae*. As shown in Figure 3, specific fragments of pLV50 DNA hybridized to both probes. Fragments of *A. vinelandii* genomic digests which hybridized to *glnA* were the same size as those which hybridized from pLV50. However, with the *ntrC* probes, 5–6 fragments in addition to those corresponding to pLV50 fragments were detected in the genomic digests. Those restriction fragments of pLV50 which showed homology to the *ntrC* and *glnA* probes are indicated in Figure 2.

Tn*5* mutagenesis of pLV50, pAT512 and pAT508 resulted in a number of insertions which were $NtrC^-$ or $GlnA^-$ according to their inability to complement *E. coli* mutants (Figure 2). These insertions were in the regions identified by subcloning and Southern hybridizations as containing *ntrC* or *glnA* of *A. vinelandii*. The Tn*5* insertions in *glnA* (plasmids pLV50::Tn*5*-4 and pLV50::Tn*5*-6) did not apparently affect *ntrC* expression since ET8556 containing these plasmids grew on arginine. The Tn*5* insertions in pAT512 which prevented the utilization of arginine by ET8556 or ET8894 (*ntrC*::Tn*5*-52, -53, -58) had no apparent effect on *glnA* expression in ET8894 or UNF1848. Tn*5* insertion no. 1, in pAT508, resulted in slower growth of ET8556 on arginine and was found to map in the region between *glnA* and *ntrC* (Figure 2). This insertion may be in an Azotobacter gene analogous to *ntrB* since that gene lies between *glnA* and *ntrC* in enteric organisms.

The results reported above show that the coding regions of *A. vinelandii glnA* and *ntrC* are located on a 6.0-kb *Eco*RI fragment. The *glnA* gene extends beyond the unmethylated *Cla*I site in this fragment (see Figure 2) since neither pAT510 nor pAT520 complement *glnA* mutations. The *glnA* promoter is located within a 1.2-kb *Eco*RI–*Cla*I fragment adjacent to the 6.0-kb *Eco*RI fragment. The *ntrC* gene lies within 2.6 kb of DNA defined by Tn*5* insertions 1 and 5 (Figure 2). Since *ntrC* but not *glnA* is expressed in pAT508 and pAT524 there must be a promoter from which *ntrC* expression can occur independently of p*glnA*.

Construction of A. vinelandii ntr mutants

ntr mutants were constructed by transforming competent wild-type *A. vinelandii* strain UW with plasmids containing Tn*5* insertions in either *ntrA* or *ntrC* and selecting transformants that were Km^r (the drug resistance encoded by Tn5). Those isolates which were Km^r as a result of integration of the transforming plasmid by a single cross-over recombination event or as a result of maintenance of the transforming plasmid as an autonomous replicon were eliminated by screening for the antibiotic resistance determinant(s) encoded by the parent replicon. Less than 1% of all isolates had the parent replicon drug resistance marker, suggesting that a double cross-over recombination event resulting in marker exchange was the predominant event.

One Km^r transformant from each of the Tn*5* insert plasmids used was characterized. These isolates were presumptive $ntrC^-$ strains MV511, MV512, MV513 and MV516 derived, respectively, from *A. vinelandii* transformed with pAT512::Tn*5*-51, 52, 54 and 58; and the presumptive $ntrA^-$ strain MV700 isolated from *A. vinelandii* transformed with pLV72::Tn*5*-1. To confirm that these mutants were the result of homologous recombination leading to marker exchange, DNA was isolated and restriction digests were hybridized to various probes. The results, shown in Figure 4, confirmed that Tn*5* was inserted in these strains in the same position in the genome as they were in the plasmid used for their construction and that no wild-type sequences remained. Furthermore, neither intact plasmids nor additional sites of Tn*5* insertion were detected.

The ability of the mutants to use various nitrogen compounds for growth was determined. All strains grew as well as the wild-

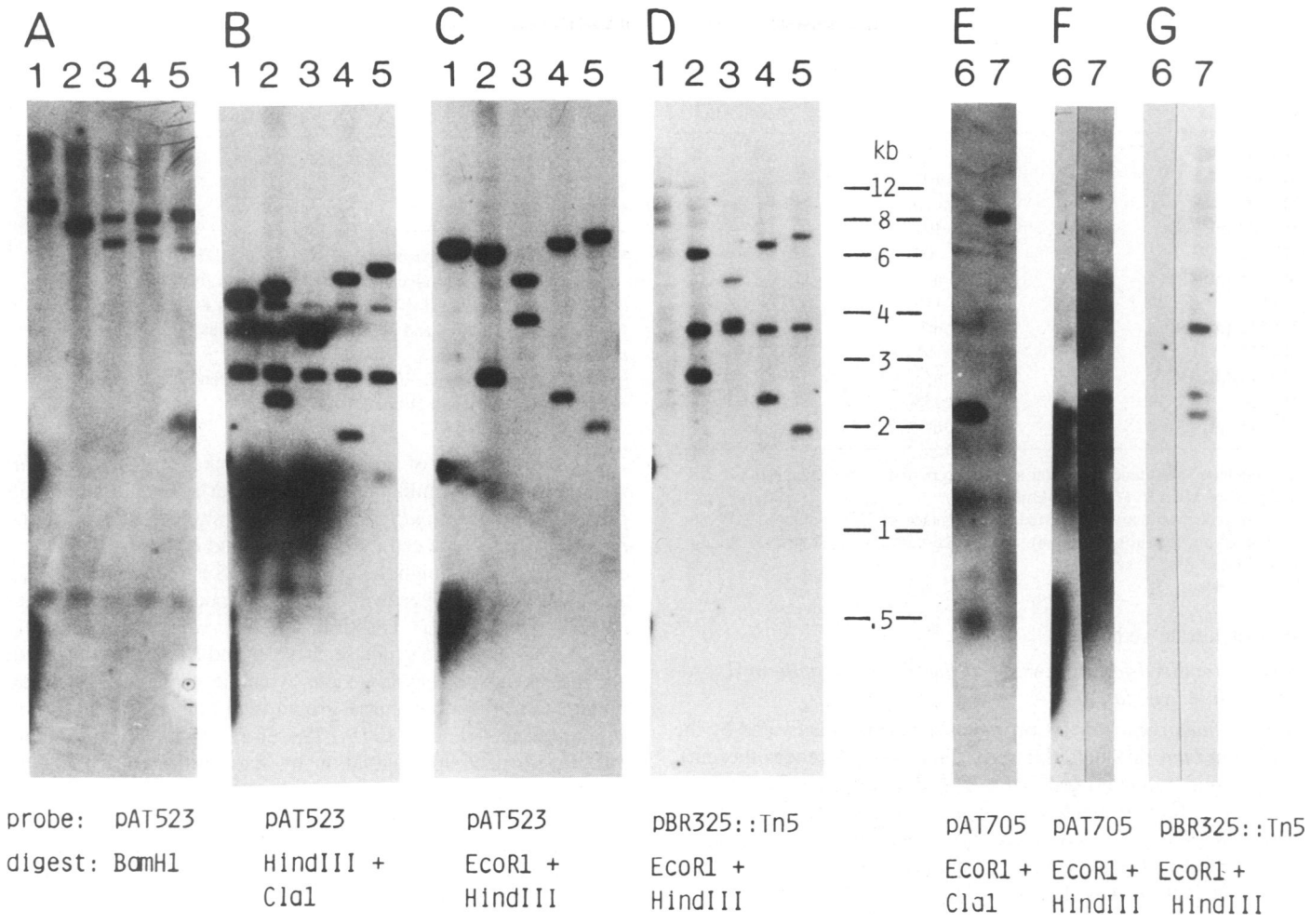

Fig. 4. Hybridization analysis of genomic DNA from various *A. vinelandii ntr* mutant strains. *A. vinelandii* DNA probed was isolated from strains: UW (wild-type parent) **lanes 1** and **6**; MV511, **lanes 2**; MV512, **lanes 3**; MV513, **lanes 4**; MV516, **lanes 5** and MV700, **lanes 7**. Strains MV511, MV512, MV513 and MV516 are *ntrC* mutants; MV700 is an *ntrA* mutant. Restriction enzymes used to digest the DNA and the plasmids used as probes are as shown in the figure.

type on ammonia and none required glutamine. The *ntrA* mutant MV700 could not grow on N_2 or nitrate but could grow on urea as N source. *ntrC* mutants MV511, MV512, MV513 and MV516 grew well on N_2.

Nitrogenase and nitrate reductase were assayed in these mutant strains. As shown in Table II, derepressed cultures of all *ntrC* mutants had approximately the wild-type level of nitrogenase (acetylene reduction) activity. The *ntrA* mutant MV700 had no detectable acetylene reduction activity, but transfer of pLV72 into MV700 restored activity to the wild-type level.

Nitrate reductase activity in NO_3^--induced cultures of both the *ntrC* and the *ntrA* mutants was 3−11% that of the wild-type. Transfer of pLV50 into the *ntrC* mutants or pLV72 into the *ntrA* mutant restored activity to 30−80% of the wild-type activity in induced cultures (Table II).

Nitrate reductase activity was also restored in *ntrC* mutants MV511 and MV516 by pLV50::Tn*5*-5 and by pGE10 (a plasmid carrying the *glnA−ntrC* genes of *K. pneumoniae*). The Tn*5* insertion in pLV50::Tn*5*-5 does not affect *ntrC* function in *E. coli* (Figure 2) and is located 0.5 kb downstream of the *ntrC*$^-$ insertion no. 58 (the insertion used to construct MV516). The distance between these two inserts (0.5 kb) makes it unlikely that the inability to induce nitrate reductase activity in MV516 (and the other *A. vinelandii ntrC* mutants) is a result of polarity by Tn*5* on the transcription of a downstream gene(s) involved specifically in nitrate utilisation. These results support the conclusion that *ntrC* function is necessary for the induction of nitrate reductase in *A. vinelandii*.

The ability of mutants to utilize N_2 and to reduce acetylene when grown on molybdenum-deficient medium was tested. The *ntrC* mutants but not the *ntrA* mutant grew on Mo-deficient N-free medium. The *ntrC* mutants also reduced acetylene under these conditions. This indicated that the 'alternative' system for fixing N_2 found in *A. vinelandii* required *ntrA* but not *ntrC*.

That these effects on nitrogen metabolism were due to the specific Tn*5* insertion and not just the presence of Tn*5* in the cell was supported by the construction of an *ntr*$^+$ strain, MV600, by transformation of *A. vinelandii* with pAT508::Tn*5*-26, a plasmid in which Tn*5* is inserted downstream of *ntrC*. MV600 had wild-type levels of nitrogenase activity in derepressed cultures and nitrate reductase activity in NO_3^--induced cultures (Table II).

Thus the construction and study of *ntr* mutants in *A. vinelandii* has demonstrated that *ntrA* but not *ntrC* is essential for nitrogen fixation in *A. vinelandii* both with Mo-sufficient and Mo-deficient conditions; and both *ntrA* and *ntrC* are essential for the induc-

Table II. Nitrogenase and nitrate reductase activities of *A. vinelandii ntr* mutants

Strain	Phenotype	Nitrogenase[a]	Nitrate reductase[b]
UW136	Ntr^+	100	100
MV600	Ntr^+	103	110
MV700	$NtrA^-$	0	3
MV700 (pLV72)	Ntr^+	101	34
MV511	$NtrC^-$	100	5
MV511 (pLV50)	Ntr^+	nt[c]	87
MV512	$NtrC^-$	119	4
MV512 (pLV50)	Ntr^+	nt[c]	69
MV513	$NtrC^-$	95	9
MV513 (pLV50)	Ntr^+	nt[c]	82
MV516	$NtrC^-$	138	11
MV516 (pLV50)	Ntr^+	nt[c]	38

[a]Nitrogenase, acetylene reduction activity, reported as percentage of derepressed UW136 (10.6 nmol/min/OD_{600}).
[b]Nitrate reductase activity reported as percentage of NO_3^--induced UW136 (2.9 nmol nitrite/min/mg protein). Uninduced cultures of all strains including UW136 had 2–7% activity.
[c]nt, not tested.

tion of nitrate reductase.

ntrC is required for expression of the K. pneumoniae nifL promoter in A. vinelandii

The *K. pneumoniae nifLA* promoter is normally activated by the *ntrC* and *ntrA* products (Dixon, 1984a and references therein). A previous study showed that a *K. pneumoniae* p*nifL–lacZ* fusion could be expressed in *A. vinelandii* (Kennedy and Drummond, 1985). The *A. vinelandii ntrC* and *ntrA* mutants constructed in the present study made it possible to determine if this activation was dependent on *ntrC* and/or *ntrA*.

As shown in Table III, the p*nifL–lacZ* fusion carried on plasmid pMD23 was highly expressed in UW136 both in the presence and absence of NH_4^+. The activities obtained were comparable with those reported previously for this strain (Kennedy and Drummond, 1985). Expression of the fusion was completely abolished in the *ntrA* mutant MV700; only 2–10% the activity of UW136 (pMD23) was obtained with the *ntrC* mutant MV511 carrying pMD23.

These results demonstrate that the expression in Azotobacter of *K. pneumoniae* p*nifL* is dependent upon the *A. vinelandii ntrA* and *ntrC* genes described in this paper. The results also re-confirm that this activation is independent of the N status of the cell.

Discussion

Genes involved in the nitrogen regulatory system (*ntr*) found in enteric bacteria have been identified in the non-enteric *A. vinelandii*. The isolation of *ntrA*, *ntrC* and *glnA* genes from *A. vinelandii* suggests that the *ntr* system may be conserved among diverse bacterial genera. The results presented in this paper show that the Azotobacter genes can function in both *E. coli* and *K. pneumoniae*, share DNA sequence homology with the *K. pneumoniae* genes as detected by hybridization analysis, and have a genetic arrangement in *A. vinelandii* similar to that in enteric bacteria with *ntrC* adjacent to *glnA* and distant from *ntrA*. The finding that in *A. vinelandii ntrA* but not *ntrC* is essential for nitrogen fixation indicates a significant difference in the role of the *ntr* system in regulating *nif* in *A. vinelandii* as compared with *K. pneumoniae*, in which both *ntrA* and *ntrC* products are essential.

Table III. Expression of a p*nifL–lacZ* fusion in *A. vinelandii ntr* mutants

Strain	β-Galactosidase activity of cultures grown[a]	
	+Urea	$+NH_4^+$
UW136 (pMD23)[b]	100	100
MV700 (pMD23)	0	0
MV511 (pMD23)	3–7	2–10

[a]Activity reported as a percentage of UW136 (pMD23) grown under the same conditions. Range given for activity obtained from three separate determinations. Activity for UW136 (pMD23) was 842–5360 Miller units for urea-grown cultures and 1987–4422 Miller units for NH_4^+-grown cultures.
[b]pMD23 contains a p*nifL–lacZ* translation fusion and has been described elsewhere (Kennedy and Drummond, 1985).

The conservation of the *glnA–ntrC* linkage in *A. vinelandii* is of interest since, unlike the enteric bacteria, the amount of GS protein in *A. vinelandii* does not vary significantly with the nitrogen status of the cell (Kleinschmidt and Kleiner, 1981; Lepo *et al.*, 1982). This might be expected if GS is required for NH_4^+ assimilation under all growth conditions. Indeed, previous studies have shown that NH_4^+ is assimilated in *A. vinelandii* exclusively via GS and glutamate synthase (Kleiner and Kleinschmidt, 1976) and that glutamate dehydrogenase, which incorporates NH_4^+ into glutamate in enteric bacteria grown with high levels of NH_4^+, is not present (Kleiner, 1975). Therefore, in *A. vinelandii* there may be no need for regulation by fixed nitrogen at the *glnA* promoter.

The present work provides some evidence for the absence of *ntr* regulation at p*glnA* in *A. vinelandii*. Unlike most *ntrA* mutants of *E. coli* or *K. pneumoniae*, the *ntrA* mutant of *A. vinelandii* described in this paper did not require glutamine for growth nor did any of the *ntrC* mutants. *A. vinelandii ntrC* was also not necessary for expression of *A. vinelandii glnA* in *E. coli* or *K. pneumoniae* Δ(*glnAntrBC*) mutants since transconjugants carrying pAT512 with Tn*5* insertions in *ntrC* were still $GlnA^+$. However, it may be that there is both *ntr*-dependent and *ntr*-independent transcription from this promoter region, as is the case with *K. pneumoniae* and *E. coli* (Dixon, 1984b; Reitzer and Magasanik, 1985).

A separate promoter for the expression of *ntrC* independent of p*glnA* is proposed based on the results of subcloning. It is not yet evident that transcription can proceed from p*glnA* through *ntrC* in *A. vinelandii* as it does in enteric organisms. The physiology of *A. vinelandii* may make such a link unnecessary. Further studies will be necesary to determine if the linkage of *ntrC* to *glnA* has a physiological significance in *A. vinelandii*.

The existence of two promoters, p*glnA* and p*ntrC*, would explain the properties of cosmid pLV51, which was isolated by complementation of an *E. coli ntrC* mutant. This cosmid, which contained the 6.0-kb *Eco*RI fragment carrying *glnA* and *ntrC* but not the adjacent 10-kb *Eco*RI fragment carrying the *glnA* promoter, gave rare $GlnA^+$ transconjugants after transfer into the *E. coli* Δ(*glnAntrBC*) mutant. All transconjugants isolated had altered cosmids. It is likely that the rearrangements seen in these cosmids were necessary for and resulted in the expression of *glnA* from the cosmid, possibly by alignment with a vector promoter or by insertion of an element such as IS2 carrying a promoter from which *glnA* could be expressed.

Characterization of the nitrogen sources utilized by the *A. vinelandii ntrA* and *ntrC* mutants has shown that both genes

Table IV. Bacterial strains

Strain	Genotype	Source or reference
E. coli[a]		
ET8000	*ntr*$^+$ *rbs lacZ*::IS*1 gyrA hut*C_K[c]	MacNeil *et al.* (1982)
ET8045	*ntrA*208::Tn*10*	MacNeil *et al.* (1982)
ET8556	*ntrC*1488	Merrick (1983)
ET8894	Δ(*rha-ntrC*)1703::Mu*cts*62	MacNeil (1981)
K. pneumoniae[b]		
KP5022	*nif*$^+$ *ntr*$^+$ *his*D2	Streicher *et al.* (1974)
CK273	*ntrA*2273 hisD2	C.Kennedy
UNF1828	*ntrC*209 *his*D2 *recA*56	Espin *et al.* (1982)
UNF1848	Δ(*glnA-ntrC*)218 *his*D2 recA56 Δ(*lacZ*)2003	Alvarez-Morales *et al.* (1984)
CK2631	*nifA*2263	Dixon *et al.* (1977)
A. vinelandii		
UW	*nif*$^+$	Bishop and Brill (1977)
UW136	*nif*$^+$ *rif*-1	Bishop and Brill (1977)
MV511	*ntrC*51::Tn*5*	This work
MV512	*ntrC*52::Tn*5*	This work
MV513	*ntrC*54::Tn*5*	This work
MV516	*ntrC*58::Tn*5*	This work
MV600	*ntr*$^+$ *znc*26::Tn*5*	This work
MV700	*ntrA*1::Tn*5*	This work

[a]All ET strains are derivatives of ET8000 and have the same basic genotype.
[b]All *K. pneumoniae* strains carry *hsd*R1 and all *recA*56 strains carry a co-transducible *sbl*300::Tn*10*.

have roles in regulating nitrogen assimilation. The *A. vinelandii ntrA* product is required both for expression of genes which are dependent on the *ntrC* product for their expression, such as nitrate reductase genes, as well as others such as *nif*, which do not require *ntrC*. Mutants isolated by chemical mutagenesis unable to utilize N_2 or NO_3^- for growth were also characterized as *ntrA*$^-$ by Santero and co-workers (in preparation) using pLV72.

The *ntrC*-independent expression of nitrogen fixation in *A. vinelandii* indicates that positive control of *nif* regulation in Azotobacter is unlike that in *K. pneumoniae*, in which both *ntrC* and *ntrA* are essential. Nevertheless, the requirement of a *nifA*-like activator for *nif* expression in Azotobacter is suggested by the observation that *K. pneumoniae nifA* expressed constitutively on wide host range vectors can restore a Nif$^+$ phenotype to certain regulatory mutants of *A. vinelandii* and *A. chroococcum* (Kennedy and Robson, 1983). Furthermore, expression of the *nifHDK* genes of *A. chroococcum* in *K. pneumoniae* mutants is enhanced by multi-copy constitutive expression of the *K. pneumoniae nifA* gene (Jones *et al.*, 1984). Also, expression from a *A. chroococcum nifH*−*lacZ* fusion in an *E. coli* background requires *nifA* (Kennedy *et al.*, 1985).

This is consistent with DNA sequence analysis of the *nifH* promoter regions of *A. vinelandii* (Brigle *et al.*, 1985) and also *A. chroococcum* (Robson *et al.*, 1985) which show sequences very similar to the *ntrA*-dependent *nifA*-activated promoter consensus in *K. pneumoniae* and several Rhizobia (Ausubel, 1984; Dixon, 1984a). In *R. meliloti* a *nifA*-like gene which is necessary for nitrogen fixation in nodules has been identified (Szeto *et al.*, 1984) and it encodes a protein with high homology to the *K. pneumoniae nifA* product (Buikema *et al.*, 1985).

A similar *nifA*-like gene was found in *R. leguminosarum* (Rossen *et al.*, 1984). A probe carrying a portion of this gene was hybridized to *A. vinelandii* DNA and a number of restriction fragments showed homology (Kennedy *et al.*, 1985). Certain of the hybridizing fragments corresponded in size to the *A. vinelandii ntrC* gene and also to some fragments seen with the *K. pneumoniae ntrC* gene probe (Figure 3). It is therefore likely that one of these fragments carries the *A. vinelandii nifA*-like gene.

Although the *nif*-specific activator has not yet been characterized in Azotobacters, its expression apparently does not require the *ntrC* gene described in this paper. The existence of a second *ntrC* gene in *A. vinelandii* which fulfills this function is unlikely for two reasons. Firstly, expression of the *ntrC*- (and *ntrA*-) dependent *K. pneumoniae nifLA* promoter is greatly reduced or eliminated in an *ntrC*::Tn*5* (and *ntrA*::Tn*5*) mutant of *A. vinelandii* (Table III). Secondly, the cloned *A. vinelandii ntrC* gene (on pAT523) failed to hybridize to genomic *Eco*RI restriction fragments other than the 6-kb *Eco*RI *glnA*−*ntrC* fragment (Figure 4). These results preclude other genes with significant functional or structural similarity to the *glnA*-linked *ntrC* gene in *A. vinelandii* described here.

It will be of great interest to learn whether *ntrC* is involved in controlling nitrogen fixation in other diazotrophs. A requirement for *ntrC* in *Azospirillum lipoferum* was suggested by the isolation of Nif$^-$ mutants that could be complemented by the *glnAntrBC* region of *K. pneumoniae* or by constitutively expressed *K. pneumoniae nifA* (Pedrosa and Yates, 1984). Recently one of four regulatory genes necessary for *nif* gene expression in *Rhodopseudomonas capsulata* was found to hybridize to the *E. coli ntrC* gene (Kranz *et al.*, 1985). Additionally, *Rhizobium meliloti* contains an *ntrC*-like gene which is necessary for growth on poor nitrogen sources as well as for expression of a p*nifH*−*lacZ* fusion in free-living cells; interestingly, a mutant with Tn*5* inserted in this gene was only slightly impaired in symbiotic nitrogen fixation (Ausubel *et al.*, 1985). The way *nif* is regulated in different diazotrophs may be a reflection of the different physiological conditions under which the organisms fix nitrogen. For instance, the ability of *A. vinelandii* to fix nitrogen under conditions of Mo-deprivation may require activation of certain genes which are not needed for N_2 fixation in the presence of Mo. The expression of these alternative genes may impose certain constraints on the regulatory system(s) involved. Further studies both in *A. vinelandii* and in other nitrogen-fixing bacteria

will be necessary to determine the generality of the *ntrC–nif* relationship.

Materials and methods

Bacteria, media and growth conditions

Bacterial strains used in this study are listed in Table IV. For growth of *E. coli* and *K. pneumoniae* strains rich medium was LB (Kennedy, 1977); minimal medium consisted of Burk's nitrogen-free medium (Newton *et al.*, 1953) which was modified for growth of enterics by replacing the sucrose with 0.2% glucose and by adding 0.05% sodium dihydrogen citrate, 0.1 M potassium phosphate buffer (pH 7.4), trace elements from LNB5 medium (Kennedy *et al.*, 1981) and either 0.2% ammonium acetate or 0.2% arginine as the nitrogen source. Plates of this minimal medium without an added N source set with agar and incubated anaerobically were used to check for growth of *K. pneumoniae* strains on N_2. NFDM (Dixon *et al.*, 1977) was used to grow *K. pneumoniae* strains when acetylene reduction or glutamine synthetase activities were assayed. Cultures were incubated at 37°C except for *E. coli* strain ET8894 and *K. pneumoniae* strains grown on N_2 which were incubated at 30°C. Media were supplemented where appropriate with glutamine at 0.5 mg/ml (0.1 mg/ml when growth was on N_2), histidine at 25 μg/ml and antibiotics at previously described concentrations (Maniatis *et al.*, 1982).

A. vinelandii strains were grown at 30°C in Burk's nitrogen-free medium containing 2.0% sucrose (BS) with, as necessary, 0.2% ammonium acetate, 10 mM KNO_3 or 2 mM urea. Antibiotics, where appropriate, were used at the following concentrations: ampicillin 10 μg/ml, chloramphenicol 50 μg/ml, tetracycline 5 μg/ml and kanamycin 5 μg/ml.

Molecular methods

The *A. vinelandii* strain UW gene library was constructed by cloning genomic DNA, partially digested with *Eco*RI to give 15–35 kb fragments, into cosmid pLAFR1 (Friedman *et al.*, 1982). The bank represents ~2000 *E. coli* strain ED8767 transfectants (C.Kennedy and D.Dean, in preparation).

Unless otherwise noted, subclones were obtained by ligating under standard conditions agarose gel-purified insert DNA to phosphatase-treated vector. Plasmids were transferred by transformation or by conjugation using pRK2013 (Figurski and Helinski, 1979) or a Km::Tn*7* derivative, pRK2073, for mobilization.

Plate transformation of *A. vinelandii* was based on the methods described by Page and Sadoff (1976) and Page (1985). *A. vinelandii* cells were grown overnight with vigorous aeration on the Burk's medium described by Strandberg and Wilson (1968) which was modified by the omission of the Fe and Mo salts. Aliquots of cells were mixed with DNA on plates of Burk's medium (either formulation) with ammonium acetate as N source and incubated overnight at 30°C. The transformants were then plated on the appropriate selective medium.

K. pneumoniae DNA fragments used as probes in hybridization experiments were isolated from agarose gels after restriction enzyme digestion of the following plasmids: pMM17 digested with *Cla*I for *ntrA* (Merrick and Stewart, 1985); pAM51 digested with *Eco*RI for *glnA* (A.Alvarez-Morales, personal communication) and pMD114 digested with *Eco*RI for *ntrC* (Kennedy and Drummond, 1985). All other probes were whole plasmids and are as described in this paper. Probes were labelled with [^{32}P]dCTP by nick translation using a kit purchased from Besthesda Research Laboratories.

Tn*5* mutagenesis has been described elsewhere (Weaver *et al.*, 1981). Standard procedures were followed for all other methods used (Maniatis *et al.*, 1982).

Enzyme assays

Nitrogenase was measured using the acetylene reduction assay as previously described for *K. pneumoniae* (Dixon *et al.*, 1977) and *A. vinelandii* (Kennedy and Drummond, 1985).

Glutamine synthetase activity was measured using the Mn^{2+}-dependent γ-glutamyl transferase assay. *E. coli* cells were grown to a density of 4×10^8 cells per ml in the minimal medium described above except that glucose was at 0.4% and glutamine at 0.2% was the N source. *K. pneumoniae* cells were grown overnight on NFDM plus 50 μg/ml serine. Assays at pH 7.27 were performed for both organisms as described by Pahel *et al.* (1982); assays at pH 7.9 were as described by Espin *et al.* (1981).

Nitrate reductase was measured in *A. vinelandii* whole cells after overnight growth on BS (uninduced activity) or after an additional 5 h incubation in the presence of 10 mM KNO_3 (induced activity). Activity was assayed aerobically for 1 h at 30°C in a reaction mixture containing 0.1 M MOPS–KOH pH 7.0, 10 mM KNO_3, 0.15 mM methyl viologen, 1 mM KCNO and 4.6 mM sodium dithionite freshly prepared in 95 mM sodium bicarbonate. The reaction was terminated by vigorous aeration. Nitrite was measured by the method of Nicholas and Nason (1957) in an aliquot of the reaction mixture after removing cells by centrifugation. Urea was used as a non-repressing nitrogen source for growth of the *A. vinelandii ntrA* mutant when nitrogenase and nitrate reductase activities were measured.

β-Galactosidase activity was measured following the assay described by Miller (1972). Cultures to be assayed were taken from 1 day old BS plates containing 0.2% ammonium acetate, 50 μg/ml ampicillin and 50 μg/ml sulfathioazole (antibiotics selective for pMD23) and suspended in 2 ml BS liquid to a density of 2×10^8 cells/ml. 100 ml flasks containing 4 ml of BS plus ammonium acetate, ampicillin and sulfathioazole or 4 ml of BS plus 2 mM urea, ampicillin and sulfathioazole were each inoculated with 1.0 ml aliquots of this suspension. Cultures were incubated with aeration at 30°C for 16 h and then duplicate samples were removed for assaying activity.

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