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Cloning of a positive regulatory element involved in nitrogen fixation in *Azotobacter vinelandii*

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Abstract. A positive regulatory gene has been cloned from *Azotobacter vinelandii*, using its ability to activate the expression of *nifH* :: *lacZ* fusion in *E. coli*. The gene has also been shown to activate the transcription from the P_1 promoter of *Rhizobium meliloti*. It did not, however, hybridize with the *nifA* gene of *R. meliloti*.

Keywords. *Azotobacter vinelandii*; *nifH*::*lacZ* fusion; positive regulatory element; nitrogen fixation.

1. Introduction

In *Klebsiella pneumoniae*, the most extensively studied nitrogen fixing bacteria, the product of the gene *nifA* acts as a positive regulatory element for the expression of the other *nif* genes (Dixon 1984). The possibility of the presence of a *nifA*-like gene in *Azotobacter vinelandii* was suggested by the ability of cloned *K. pneumoniae nifA* gene to complement the lesion in *A. vinelandii* UW1, a regulatory mutant (Kennedy and Robson 1983). In addition, the *K. pneumoniae nifA* gene product has been shown to activate in *Escherichia coli*, the *nifH*::*lacZ* fusion from the closely related bacterium, *Azotobacter chroococcum* (Kennedy *et al* 1985). The sequences upstream of *nifH* and *nifE* of *A. vinelandii* have also been found to be similar to the sequences upstream of the *nif* operons of *K. pneumoniae*, which are thought to be involved in the binding of the *nifA* gene product (Brigle *et al* 1985; Dean and Brigle 1985). The *nifA* gene of *A. vinelandii* has, however, eluded cloning. The *nifH*::*lacZ* fusion of *K. pneumoniae* does not get activated in *A. vinelandii* (Kennedy and Drummond 1985). There is no DNA sequence in *A. vinelandii* which is homologous to *K. pneumoniae nifA* (Kennedy *et al* 1984). These properties of *A. vinelandii nifA*, thus, do not appear to be very similar to that of *K. pneumoniae*. Attempts to select the *A. vinelandii nifA* clone from a genomic library in the broad host range cosmid pLAFR1 by complementation of *A. vinelandii* UW1 were unsuccessful (unpublished results). We have made use of the expected ability of *A. vinelandii nifA* to activate the *nifH*::*lacZ* fusion from *A. vinelandii* in *E. coli*, to successfully clone this gene.

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Table 1. List of bacterial strains and plasmids.

Strain/ plasmid	Relevant characteristics	Reference
E. coli		
TB1	*ara*, *lac*, *proAB*, *rspL*, *lacZ*, M15, hsdR	Ditta *et al* (1985)
DH1	F^-, *recA*1, *endA*1, *gyrA*96, *thi*-1 hsdR-17 ($r_k^- m_k^-$), SupE44	Hanahan (1983)
Azotobacter vinelandii		
UW	nif^+ (wild type)	Bishop and Brill (1977)
MV551	*ntrC*::Tn5	Toukdarian *et al* (1986)
Plasmids:		
pGD499	Tc^r, Ap^r, *lacZ*, *lacY*	Ditta *et al* (1985)
pHC79	Tc^r, Ap^r, *cos*	Hohn and Collins (1980)
pMB210	Tc^r, *nifH*::*lacZ* (*R. meliloti*)	Better *et al* (1985)
pMB1107	Tc^r, negative control for pMB210	Better *et al* (1985)
pDC2	Tc^r, *R. meliloti nifA*	(Kim *et al* 1986)
pMC71A	Cm^r, *K. pneumoniae nif A* cloned under *tet* promoter	Buchanan-Wollaston *et al* (1981)
pAM60	Tc^r, *A. vinelandii nifH*::*lacZ*	This study
pRJ24	Ap^r, pHC79 carrying *A. vinelandii* DNA	This study
pRJ13	Ap^r, pHC79 carrying *A. vinelandii* DNA	This study

2. Materials and methods

2.1 *Bacterial strains and plasmids*

A list of bacterial strains and plasmids used are given in table 1.

2.2 *Growth of bacteria*

E. coli strains were grown on LB or minimal salts medium (Miller 1972), at appropriate temperature. Concentration of antibiotics used was, ampicillin (100 μg/ml), tetracycline (15 μg/ml) and chloramphenicol (30 μg/ml). *Azotobacter vinelandii* cells were grown at 30°C on Burk's nitrogen-free (BNF) medium (Strandberg and Wilson 1968), supplemented with ammonium acetate (0·11%). Urea (2 mM) was used as derepressing nitrogen source. Tetracycline was used at 10 μg/ml.

2.3 *Bacterial matings*

Conjugal transfer of plasmids from *E. coli* to *A. vinelandii* was done using pRK2013 as the helper plasmid in a triparental mating (Ditta *et al* 1980) on BNF agar plates supplemented with ammonium acetate (0·11%), casamino acids (0·2%) and thiamine (17 μg/ml) at 30°C for 18–24 hours. Exconjugants were selected on BNF agar plates supplemented with ammonium acetate (0·11%) and tetracycline (10 μg/ml) at 30°C.

2.4 *DNA manipulation*

Plasmids were isolated according to Birnboim (1979) and were further purified on an ethidium bromide–cesium chloride gradient. *A. vinelandii* and *E. coli*

chromosomal DNA was isolated according to Ditta *et al* (1985). DNA samples were digested with restriction endo-nucleases according to manufacturer's specifications.

Ligation of DNA fragments and *in vitro* packaging were done according to Maniatis *et al* (1982). *E. coli* was transformed with plasmid DNA according to Cohen *et al* (1972).

2.5 *DNA hybridisation on membranes*

DNA fragments were transferred from agarose gels to nitrocellulose membranes by blotting (Southern 1975). DNA probe was labelled with ^{32}P-α-dCTP by nick translation (Rigby *et al* 1977). DNA fragments on nitrocellulose membranes were hybridized to labelled probe according to Maniatis *et al* (1982). Hybridization conditions used were: $6 \times$ SSC, $5 \times$ Denhardt's, 0·5% SDS, calf thymus DNA (200 μg/ml) and labelled DNA probe (10^6 cpm/ml of specific activity, 10^8 cpm/μg). After washing the membrane, it was exposed to Indu X-ray film.

2.6 *Assay of β-galactosidase*

β-Galactosidase activity in *E. coli* and *A. vinelandii* cultures was assayed according to Miller (1972).

3. Results and discussion

The *nifHDK* cluster of *A. vinelandii* is present in a 6·2 kb *Sma*I fragment (Brigle *et al* 1985). From this fragment, a 1·2 kb *Sma*I-*Sal*I segment that contains the *nifH* promoter and the region coding for 250 amino acids of the Fe-protein (Brigle *et al* 1985; Reddy *et al* 1985) was cloned into the broad host range *lacZ* fusion vector

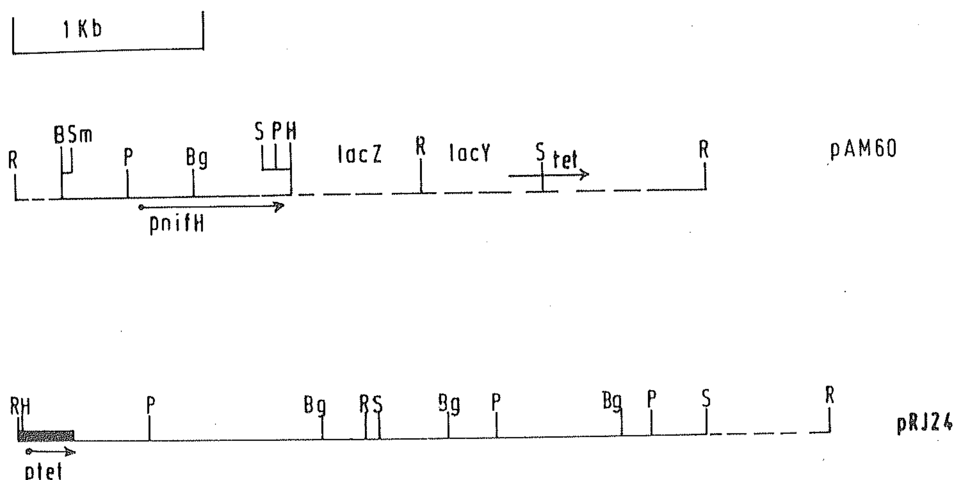

Figure 1. Restriction maps of plasmids pAM60 and pRJ24. Vector portions (pGD499 and pHC79, broken lines) are not drawn to scale. The thick region in pRJ24 represents the *Eco*RI–*Bam*HI segment of pHC79, containing the *tet* promoter. The *Bam*HI site is destroyed due to cloning of *Sau*3A fragments. Arrows indicate the direction of transcription. Restriction enzymes are indicated by: B–*Bam*HI; Bg–*Bgl*II; H–*Hind*III; P–*Pst*I; S–*Sal*I; Sm–*Sma*I.

Table 2. Expression of *nifH*::*lacZ* fusion (pAM60) in different backgrounds.

Strain (plasmid)	Growth conditions	β-galactosidase units
E. coli TB1 (pAM60)	M9	40
E. coli TB1 (pAM60 + pMC71A)	M9	3550
A. vinelandii UW (pGD499)	BN^+F	170
A. vinelandii UW (pGD499)	BNF	160
A. vinelandii UW (pAM60)	BN^+F	380
A. vinelandii UW (pAM60)	BNF	5610
A. vinelandii MV551 (pAM60)	BN^+F	190
A. vinelandii MV551 (pAM60)	BNF	2315

BNF: Burk's nitrogen-free medium (Strandberg and Wilson 1968)
BN^+F: BNF supplemented with 0·11% ammonium acetate.
M9: minimal medium (Miller 1972).

pGD499, upstream of the *lac* operon in proper orientation. The resultant derivative pAM60 (figure 1 for restriction map) was used for checking the activity of *nifH* promoter. A lac^- *E. coli* strain, TB1 containing pAM60 was transformed with the plasmid pMC71A, which contains *nifA* of *K. pneumoniae* under the control of *tet* promoter (Buchanan-Wollaston *et al* 1981). The transformants showed a 100-fold increase in the levels of β-galactosidase (table 2). Plasmids pAM60 and pGD499 were then conjugated into *A. vinelandii* UW. The Tc^r exconjugants were assayed for β-galactosidase activity in the presence and the absence of a fixed nitrogen source. In the absence of ammonium acetate, β-galactosidase activity with pAM60 was found to be enhanced 12-fold, whereas in UW containing pGD499 there was no enhancement (table 2). These data show that pAM60 contains the β-galactosidase gene under the control of *A. vinelandii nifH* promoter. The *nifH*::*lacZ* fusion also expressed in the *ntrC*$^-$ mutant MV551, thus precluding its possible activation by the *ntrC* gene. It also supports the earlier report of Nif^+ phenotype of this mutant (Toukdarian *et al* 1986).

In order to isolate the *nifA* gene, *A. vinelandii* DNA was partially digested with *Sau*3A and the 30–35 kb fragments isolated by sodium chloride density gradient centrifugation (1·25–5M) were ligated to the cosmid pHC79 linearized with *Bam*HI. The ligated DNA was packaged *in vitro*. *E. coli* TB1 containing pAM60 was infected with the phages. Transductants were selected on minimal agar plates supplemented with ampicillin (100 μg/ml), tetracycline (10 μg/ml), urea (5 mM) and X-gal (30 μg/ml) at 30°C. Urea was used as a nitrogen source to avoid any possible repression by a gene analogous to *nifL*. Screening of 30,000 single colonies yielded 3, which were dark blue in colour. β-Galactosidase activity in these colonies was found to be 10-fold higher than that of *E. coli* TB1 containing pAM60 alone (table 3). Plasmid DNA from these three colonies was isolated and *E. coli* DH1 was transformed. Ap^r Tc^s transformants were selected. The plasmids isolated from these colonies were found to have similar restriction patterns after digestion with *Sal*I, *Bgl*II and *Eco*RI. One of these plasmids, pRJ13 was used for further investigations. When *E. coli* TB1 containing pAM60 was transformed with pRJ13, β-galactosidase activity of the fusion plasmid was stimulated 10-fold. This

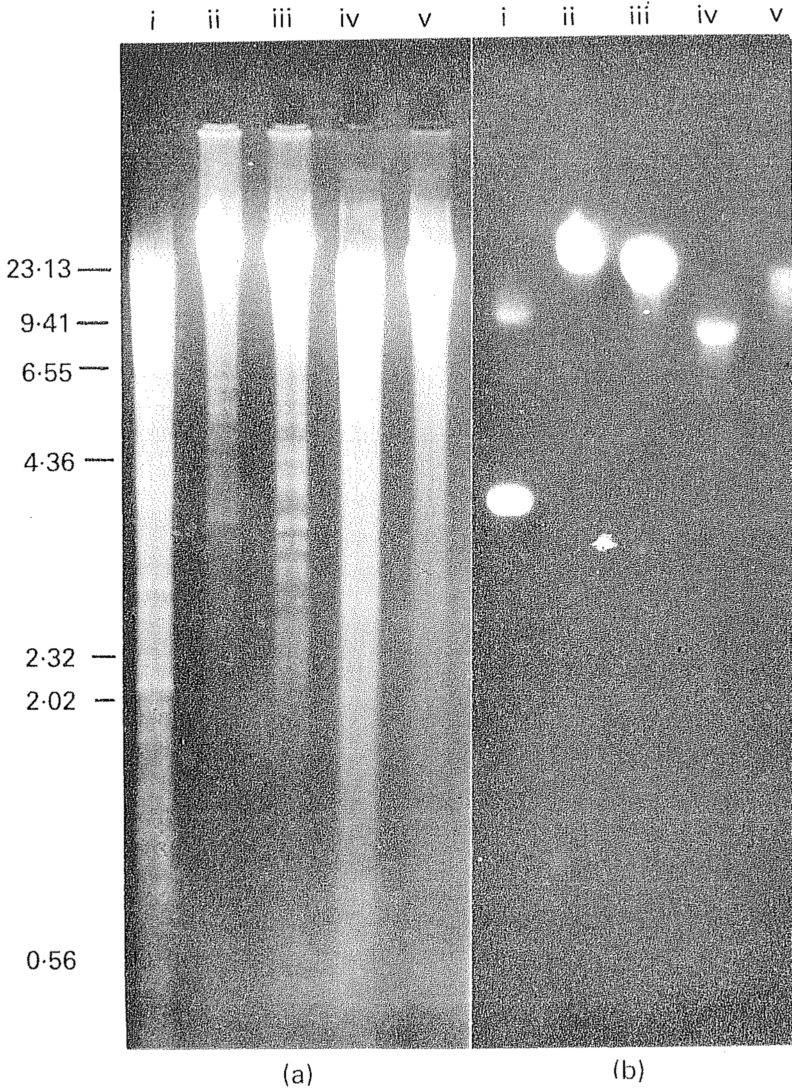

Figure 2. Hybridization of *A. vinelandii* chromosomal DNA with the 3·6 kb insert in pRJ24. **(a).** Gel photograph showing the *A. vinelandii* chromosomal DNA digested with: i–*Eco*RI; ii–*Bam*HI; iii–*Hind*III; iv–*Sac*I and v–*Kpn*I. **(b).** Autoradiogram of the Southern blot of the gel in (a) after hybridization.

Sizes of DNA markers (lambda DNA digested with *Hind*III) are indicated on the left side

eliminated the possibility of any mutations in *nifH* promoter present in pAM60 that could lead to the constitutive expression of β-galactosidase. Partial digestion of pRJ13 with *Sal*I allowed us to localize the gene responsible for stimulation on a 3·6 kb terminal fragment proximal to the *Hind*III site of the vector pHC79, thereby indicating the possibility of this gene expressing from the *tet* promoter. Figure 1 shows the restriction map of this smaller subclone pRJ24. The plasmid pAM60 produces a 100-fold excess of β-galactosidase in the presence of pRJ24, which is

Table 3. Activation of *nifH* promoter in *E. coli* by the cloned *nifA* gene of *A. vinelandii*.

Plasmid (s)	β-galactosidase activity
pAM60	40
pAM60 + pRJ13	410
pAM60 + pRJ24	3420
pMB210	50
pMB210 + pRJ24	3460
pMB1107 + pRJ24	50

Plasmid pMB210 contains the *lacZ* fusion of P_1 promoter (*nifH*) of *R. meliloti*. Plasmid pMB1107 is negative control for pMB210 where the promoter has been deleted (Better *et al* 1985).

10-fold higher than that produced in the presence of the parent plasmid pRJ13. This may be due to the larger size of pRJ13, and hence, the lesser number of copies. To further test whether the activator gene in pRJ24 can stimulate the promoter of a *nif* operon from another bacterium, a plasmid pMB210 containing *lac*Z fusion of *Rhizobium meliloti* promoter P_1 (Better *et al* 1985) was tested. As can be seen from table 3, pRJ24 did stimulate the promoter P_1 which is upstream of *R. meliloti nifHDK* operon. It also confirmed the *nifA*-like activity of this cloned gene.

A Southern blot containing *A. vinelandii* chromosomal DNA digested with different restriction enzymes was hybridized with ^{32}P-labelled 3·6 kb fragment from pRJ24. The autoradiogram (figure 2) shows that two *Eco*RI fragments of 12 kb and 4·0 kb, an 8 kb *Sac*I fragment and a 20 kb *Kpn*I fragment hybridized. In *Bam*HI and *Hind*III digests, sizes of hybridizing fragments were above 23·0 kb.

Recently it was reported that multiple fragments of *A. vinelandii* chromosomal DNA hybridize with *R. leguminosarum nifA* gene (Toukdarian and Kennedy 1986) We, however, failed to observe any hybridization with *R. meliloti nifA* gene cloned in pDC2 (Kim *et al* 1986), under the stringency conditions we used for the hybridization. Possibly, they may not be very homologous at the DNA level.

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