Lethality of *glnD* null mutations in *Azotobacter vinelandii* is suppressible by prevention of glutamine synthetase adenylylation

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GlnD is a pivotal protein in sensing intracellular levels of fixed nitrogen and has been best studied in enteric bacteria, where it reversibly uridylylates two related proteins, PII and GlnK. The uridylylation state of these proteins determines the activities of glutamine synthetase (GS) and NtrC. Results presented here demonstrate that *glnD* is an essential gene in *Azotobacter vinelandii*. Null *glnD* mutations were introduced into the *A. vinelandii* genome, but none could be stably maintained unless a second mutation was present that resulted in unregulated activity of GS. One mutation, *gln-71*, occurred spontaneously to give strain MV71, which failed to uridylylate the GlnK protein. The second, created by design, was *glnAY407F* (MV75), altering the adenylylation site of GS. The *gln-71* mutation is probably located in *glnE*, encoding adenylyltransferase, because introducing the *Escherichia coli glnE* gene into MV72, a *glnD*⁺ derivative of MV71, restored the regulation of GS activity. GlnK-UMP is therefore apparently required for GS to be sufficiently deadenylylated in *A. vinelandii* for growth to occur. The Δ *glnD* GS⁻ isolates were Nif⁻, which could be corrected by introducing a *nifL* mutation, confirming a role for GlnD in mediating *nif* gene regulation via some aspect of the NifL/NifA interaction. MV71 was unexpectedly NtrC⁺, suggesting that *A. vinelandii* NtrC activity might be regulated differently than in enteric organisms.

Keywords: nitrogen fixation, nitrogen assimilation, GlnD, NtrC, *nif* gene regulation

INTRODUCTION

The *glnD* gene product is thought to be a component of the *Azotobacter vinelandii* fixed N signal transduction pathway that regulates *nif* gene expression. Certain Tn5 Nif⁻ regulatory mutations were located in a gene, originally named *nfrX*, that was shown by subsequent sequencing to encode a protein highly similar to the *glnD* gene product of *Escherichia coli* (Santero *et al.*, 1988; Contreras *et al.*, 1991). The only discernible phenotype of *nfrX* mutant MV17 was Nif⁻, which could be corrected by introducing either a *nifL*::kan mutation

or the *E. coli glnD* gene on a wide host range plasmid (Contreras *et al.*, 1991).

In enteric bacteria, GlnD and the small trimeric PII proteins, encoded by *glnB*, and GlnK, a PII paralogue, constitute a cellular N sensor. Biochemical experiments using wild-type cell extracts, and also *in vitro* systems, to examine the activities of purified proteins, have shown that the activity states of these proteins are dependent on small effector molecules and influence a cascade of events leading to the activation or inactivation of proteins involved in nitrogen metabolism (for reviews, see Merrick & Edwards, 1995; Reitzer, 1996). GlnD contains both uridylyltransferase and uridylyl-removing activities that are regulated largely by glutamine (Jiang *et al.*, 1998). In wild-type cells grown in N-limiting conditions, GlnD reversibly uridylylates PII (Son & Rhee, 1987) and GlnK (van Heeswijk *et al.*, 1995). PII-UMP or GlnK-UMP increase the rate of

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Abbreviations: ATase, adenylyltransferase; GS, glutamine synthetase.

Table 1. Strains and plasmids

Strain/plasmid	Characteristics	Source/reference
<i>A. vinelandii</i>		
MV17	<i>nfrX16(glnD)::Tn5</i> (Nif ⁻)	Santero <i>et al.</i> (1988)
MV71	<i>glnD1::Ω gln-71</i> (Nif ⁻)	This work
MV72	<i>gln-71</i> (Nif ⁺)	This work
MV73	<i>glnD1::Ω gln-71 nifL1::KIXX</i> (Nif ⁺)	This work
MV74	<i>glnA8</i> (GSY407F) (Nif ⁺)	This work
MV75	<i>glnD1::Ω glnA8</i> (GSY407F) (Nif ⁻)	This work
UW136	<i>rif1</i> (Nif ⁺)	Bishop & Brill (1977)
<i>E. coli</i>		
DH5α	<i>supE44 ΔlacU69 (φ80 lacZ ΔM15) hsdR17 recA1 gyrA96 thi1 relA1</i>	Sambrook <i>et al.</i> (1989)
71//18	<i>supE thi Δ(lac-proAB) F' [proAB⁺ lacI^a lacZ ΔM15]</i>	Sambrook <i>et al.</i> (1989)
Plasmids		
pAG401	<i>PstI-EcoRI</i> fragment from pAT512 containing the <i>glnA</i> gene cloned in pTZ19R	This work
pAG444	<i>PstI-EcoRI</i> fragment cloned into pTZ19R containing the mutated <i>glnA</i> gene	This work
pAT512	12.5 kb <i>HindIII-ClaI</i> fragment from cosmid pVL51 containing <i>A. vinelandii glnA</i> gene	Toukdarian & Kennedy (1986)
pBluescript	Cloning and sequencing vector (Amp ^r)	Stratagene
pDK805	<i>E. coli glnE</i> cloned in pUC18	van Heeswijk <i>et al.</i> (1993)
pLAFR3	22 kb Cosmid (Tet ^r)	Peet <i>et al.</i> (1986)
pPR803	<i>E. coli glnE</i> cloned in pLAFR3	This work
pPH45	Plasmid containing Ω (Str ^r Spc ^r) cassette	Prentki and Krisch (1984)
pRCS1	3.3 kb <i>BamHI-KpnI</i> fragment from pSS166 cloned in pBluescript	This work
pRCS1::Ω	Ω cassette inserted between the two <i>PstI</i> sites in <i>glnD</i> in pRCS1	This work
pSS166	4.7 kb <i>KpnI</i> fragment containing <i>glnD</i> in pTZ18	Santero <i>et al.</i> (1988)
pSS1663	KIXX cassette inserted between <i>BamHI</i> and <i>BglII</i> sites in pSS166 (<i>glnD2::KIXX</i>)	This work
pSSK	KIXX cassette inserted between the two <i>SalI</i> sites in pSS166 (<i>glnD3::KIXX</i>)	This work
pTU13	KIXX cassette inserted in the <i>BglII</i> site downstream of <i>glnD</i>	This work
pTZ18R/pTZ19R	Cloning and sequencing vectors (Amp ^r)	Pharmacia
pUC4::KIXX	Plasmid containing KIXX (Kan ^r) cassette	Pharmacia

deadenylation (activation) of glutamine synthetase (GS) by stimulation of the adenylyl-removing activity of adenylyltransferase (ATase), the product of the *glnE* gene (van Heeswijk *et al.*, 1995; Edwards & Merrick, 1995; Jaggi *et al.*, 1997). In cells transferred to N-rich medium, GlnD deuridylylates PII and GlnK (van Heeswijk *et al.*, 1996). PII stimulates the ATase activity of GlnE (Jaggi *et al.*, 1997) and also the phosphatase activity of NtrB (Kamberov *et al.*, 1995; Jiang & Ninfa, 1999), leading to the dephosphorylation and inactivation of the transcriptional activator, NtrC. Because the *glnK* gene in enteric bacteria requires phosphorylated NtrC (NtrC-P) for expression, GlnK synthesis does not occur

in *E. coli* cultures grown with ammonium (He *et al.*, 1998; van Heeswijk *et al.*, 1996).

This work further examines roles of GlnD in *A. vinelandii*. Attempts were made to isolate true *glnD* null mutants, with internal deletion/insertion mutations, because the *glnD* mutant, MV17, that was initially studied contained Tn5 inserted about 80 bp upstream of the stop codon, very near to the 3' end of this large gene (2700 bp) (Contreras *et al.*, 1991). While MV17 was Nif⁻, both GS and NtrC activities appeared to be normally regulated. The truncated GlnD protein in MV17 may therefore have retained GlnD function(s)

associated with GS and NtrC activities but not with its influence on the NifL/NifA regulatory interaction. It is likely that GS and NifL/NifA activities are determined by the uridylylation state of GlnK, the only PII-like protein apparently present in *A. vinelandii* (P. Rudnick, unpublished). The designation of *glnK* in *A. vinelandii* was based on the greater similarity of its gene product to GlnK proteins in other organisms and on the location of *glnK* upstream of *amtB*, encoding a methylammonium membrane transport protein, an organization that occurs in several other proteobacterial species (Meletzus *et al.*, 1998; Jack *et al.*, 1999; Thomas *et al.*, 2000). The use of MV17 to define a role for GlnD in *nif* gene regulation in *A. vinelandii* was further compromised by the occurrence of an uncharacterized *glnD*-linked suppressor mutation that restored good growth on N-sufficient medium in this strain as compared to the original poor growth phenotype in MV16 from which MV17 arose (Santero *et al.*, 1988). The *glnD* null mutations reported here, with interposon mutations in the 5' and central regions, could replace the wild-type *glnD* gene after transformation into *A. vinelandii*, but strains carrying only the mutated *glnD* gene could not be isolated unless a second mutation occurred, either spontaneously or by design, resulting in the inability of GS to be adenylated.

METHODS

Bacterial strains and growth conditions. Strains and plasmids are shown in Table 1. *Azotobacter vinelandii* strains were grown aerobically at 30 °C in Burk's medium (Newton *et al.*, 1953) with 1% sucrose (BS); BSN contained 15 mM ammonium acetate, BSU contained 10 mM urea; BSNO₃ contained 10 mM KNO₃. Glutamine was included in competence and selective media at 1 mg ml⁻¹ where indicated. Antibiotics for selection of recombinant *A. vinelandii* strains were added at the following concentrations: ampicillin 50 µg ml⁻¹; kanamycin 2 µg ml⁻¹; streptomycin 0.5 µg ml⁻¹; tetracycline 2.5 µg ml⁻¹.

Construction and transformation of mutations in the *glnD* gene of *A. vinelandii*. Plasmids pSS166 or pRCS1 were mutagenized with either the KIXX (Kan^r) or the Ω (Str^r) cassettes at or between different restriction sites, thereby creating insertion or deletion/insertion mutations (Fig. 1). Plasmids were transformed into *A. vinelandii* competent cells on solid competence medium (Bali *et al.*, 1992) with and without added glutamine. Amp^s transformants were those in which a double crossover event leading to gene replacement had occurred (*bla* was located on the suicide vectors). These were serially subcultured several times on BSN+Kan or BSN+Str. DNA was prepared from transformant or wild-type strains as described by Hopwood *et al.* (1985).

For complementation experiments plasmids pLAFR3 and pPR803 were conjugated into *A. vinelandii* MV72 and MV71 strains by triparental mating with *E. coli* and the transformants were selected on the basis of resistance to tetracycline (Kennedy & Drummond, 1985).

Construction of *glnA* (GSY407F) mutants. Site-directed substitution of codon TAC (Tyr) with codon TTC (Phe) was achieved by elongation of a mutagenic primer. Plasmid pAG401, in which the *glnA* gene of *A. vinelandii* was cloned by ligation of a 0.6 kb *Pst*I-*Eco*RI fragment from pAT512 into pTZ19, was used as template. It was confirmed by sequencing

Fig. 1. Map of mutations in the *A. vinelandii* *glnD* region. The 4.7 kb *Kpn*I fragment containing the *glnD* gene was cloned in pTZ18 to give pSS166; the 3.3 kb *Kpn*I-*Bam*HI fragment was cloned into pBluescript to give pRCS1. Sites of deletion and insertion of Ω and KIXX cassettes and the position of insertion of Tn5 in the original *glnD* mutant MV17 are indicated. The horizontal arrow indicates direction of transcription of the *glnD* gene. Restriction sites are abbreviated as follows: B, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I; P, *Pst*I; S, *Sal*I.

that one plasmid, pAG444, contained the mutated *glnA* gene. Plasmid pAG444 was transformed into *A. vinelandii* in order to insert the mutated *glnA* gene into the bacterial chromosome by a single cross-over event, leading to co-integration of the *glnAY407F* plasmid. One such strain was named MV74.

GS assays. *A. vinelandii* cultures for GS activity measurement were typically grown for 20–30 h in 20 ml BSU; 15 mM ammonium acetate was added when they reached an OD₆₀₀ of 0.6–0.8 and then removed 30 min later by centrifugation of cells and resuspension in N-free medium. Six-millilitre samples of culture were taken before addition of ammonium, 30 min after its addition and 30 min after its removal. Cells were harvested and resuspended in 200 µl 1% KCl. GS Mn²⁺-dependent transferase and Mg²⁺-dependent synthetic activities were measured at pH 7.5 and 7.6, respectively, using the reaction mixture (containing 0.1 mg cetyltrimethylammonium bromide ml⁻¹ for cell lysis) described by Bender *et al.* (1977). At these pHs, the transferase and synthetase activities showed the strongest changes in response to the degree of GS adenylation (Kleinschmidt & Kleiner, 1978, 1981). One unit of GS activity is the amount of enzyme producing 1 µmol γ-glutamyl hydroxamate min⁻¹. Activities for all strains were determined several times; the data in Fig. 3 are from a single representative experiment.

In vitro uridylylation and Western blot analysis. Crude protein extracts were prepared from 25 ml of saturated BSN cultures (OD₆₀₀ 0.8–1.0). Cells were harvested and resuspended in 500 µl 50 mM Tris/HCl, pH 7.5, containing 100 mM KCl and 10 mM MgCl₂, then disrupted by sonication. Uridylylation of PII in extracts was determined as described previously (Atkinson *et al.*, 1994; Kamberov *et al.*, 1995). To 92 µl crude extract were added 0.1 mM ATP, 1 mM DTT, 0.1 mM 2-oxoglutaric acid, pH 7.5, and 5 µl [α-³²P]UTP (1.1 × 10⁴ Bq) (Amersham). Mixtures were incubated for 2 h at 30 °C and the reaction stopped by the addition of EDTA (10 mM). Proteins were separated by SDS-PAGE and transferred to a Duralose membrane for subsequent exposure to X-ray film. Western

analysis was performed using the Immun-Blot Assay Kit (Bio-Rad). Treated membranes were incubated with *E. coli* anti-Pil (gift from W. van Heeswijk), diluted 1:2500, for 2.5 h at room temperature. Anti-rabbit IgG alkaline phosphatase conjugate was added and after 2 h, cross-reacting proteins were identified by adding substrate for alkaline phosphatase activity. Signal intensities were quantified using the Molecular Dynamics ImageQuant software package.

RESULTS

Attempts to construct stable *glnD* null mutants

A. vinelandii wild-type strain UW136 was transformed with *glnD* insertion/deletion mutations, removing the central, entire or 5' regions of *glnD*, carried on the Amp^r suicide vectors pTZ18R or pBluscriptII unable to replicate in this host: pRCS1::Ω (*glnD*1::Ω; Str^r), pSS1663 (*glnD*2::KIXX; Kan^r) and pSSK (*glnD*3::KIXX; Kan^r) (Fig. 1). Kan^r or Str^r transformants (grown with or without 10 mM glutamine) were screened for ampicillin sensitivity; Amp^s (vector-free) isolates were subsequently subcultured at least three times on the antibiotic used for initial selection. Several Str^r Amp^s pRCS1::Ω transformants in which the mutated *glnD* gene had presumably replaced the wild-type allele by a double recombination event were analysed by Southern hybridization. In all colonies, two *Kpn*I fragments hybridized to a *glnD* probe: a 4.7 kb band corresponding to the wild-type *glnD* region and a 5.2 kb band, representing the *glnD*1::Ω mutated region (Fig. 2a). These results indicate that the transformants contained both wild-type and mutated copies of *glnD*. It is possible for both to be present in the same cell on different chromosomes because *A. vinelandii* often carries multiple chromosomal copies (Maldonado *et al.*, 1994). In addition, 10 to 50 individual colonies of pRCS1::Ω, pSS1663 and pSSK transformants were tested for retention of Kan^r or Str^r after three subcultures on antibiotic-free medium. All *A. vinelandii* transformants obtained lost antibiotic resistance after only one subculture on BSN agar without antibiotics. These two criteria, instability of antibiotic resistance and retention

of wild-type and mutated copies of chromosomes after many generations of selective growth, were previously used to establish the lethality of other cassette-insertion null mutations in *A. vinelandii* (Toukdarian *et al.*, 1990; Meletzus *et al.*, 1998; Zheng *et al.*, 1998).

The pTU13 transformants (KIXX inserted downstream of the *glnD* coding region, Fig. 1) were stably Nif⁺ and retained Kan^r after many generations in non-selective medium. Southern blot analysis of the DNA of one isolate tested showed that only the mutated region, and not the wild-type, was present in its genome (data not shown). This indicates that the instability of antibiotic resistance observed in transformants derived using pSS1663, pSSK or pRCS1::Ω is due to interruption of *glnD* itself and not due to interference with expression of any gene downstream from *glnD*.

Isolation and analysis of a stable *A. vinelandii glnD* internal deletion mutant

In one experiment in which *A. vinelandii* was transformed with pRCS1::Ω, a single Str^r Amp^s transformant appeared as a much larger colony than usual. In this isolate, MV71, Str resistance was not lost as had usually occurred with pRCS1::Ω Str^r Amp^s transformants after cycles of growth on a medium without Str. In addition, while unstable *glnD*1::Ω mutant transformants were small and irregularly shaped, MV71 colonies looked nearly the same as wild-type, though they were smaller on ammonium-containing medium. In Southern hybridization analysis of MV71 genomic DNA, the *glnD* probe hybridized only to a 5.2 kb *Kpn*I fragment, the size of the *glnD*1::Ω fragment (Fig. 2b). These results show that replacement of the wild-type gene with the *glnD*1::Ω allele had occurred in MV71, and that chromosomes carrying wild-type *glnD* genes were undetectable. In addition, MV71 colonies were Nif⁻.

The appearance of this rare, stable *glnD*1::Ω null mutant, MV71, among a background of several hundred unstable mutants suggested that a second, compensating mutation in an unknown gene had occurred in this strain; this mutant allele was designated *gln-71*. To separate the *glnD*1::Ω and *gln-71* alleles, MV71 was first transformed with plasmid pSS166 carrying the wild-type *glnD* gene, followed by selection on BS medium without antibiotics. All Nif⁺ transformants were Amp^s Str^s and represented strains in which the *glnD*1::Ω allele was replaced with wild-type *glnD* by homologous recombination. One such transformant carrying only the *gln-71* mutation and not the *glnD*1::Ω allele was named MV72. The only phenotype noted for MV72 was a longer lag phase and slower growth rate in liquid BSN and smaller colony formation on solid BSN than occurred with the wild-type strain. The growth rate of MV72 on N-free medium was similar to that of the wild-type strain. MV72 transformed with pRCS1::Ω (*glnD*1::Ω) resulted in Str^r Amp^s transformant colonies (30 tested) that were unable to fix nitrogen and had the same colony morphology as the original MV71 mutant, confirming that MV72 contains a mutation capable of

Fig. 2. Southern blot analysis of *glnD*::Ω transformants. DNA was prepared from strains, digested with *Kpn*I and run on 0.8% agarose gels for fragment separation. The probe was a 700 bp *Bam*HI–*Eco*RI fragment from pSS166 labelled with [³²P]dCTP in a random-primed polymerase reaction (Stratagene). (a) Lane A, 1 kb ladder (Gibco); lane B, wild-type UW136; lanes C and D, two 'unstable' *glnD*::Ω transformants. (b) lane A, UW136; lane B, MV71; lane C, 1 kb ladder (Gibco).

Fig. 3. GS activities in wild-type, mutant and transformant strains. Strains were grown and assayed as described in Methods. (a) Mg²⁺-dependent transferase activity; (b) Mn²⁺-dependent synthetic activity; (c) activities ratio (Mn²⁺-dependent/Mg²⁺-dependent). □, Activity before addition of NH₄⁺; ▨, activity after addition of NH₄⁺; ■, activity after removal of NH₄⁺.

stabilizing the *glnD* null mutation by suppressing its very deleterious or lethal effect in *A. vinelandii*.

GS activity of MV71 and MV72

Two other deleterious/lethal insertion mutations were those in *glnA* encoding GS or in *glnK* (Toukdarian *et al.*, 1990; Meletzus *et al.*, 1998). Because GS is an essential enzyme in *A. vinelandii*, and since GlnD in enteric bacteria influences the activity state of GS through modulation of the activity of the GlnE protein, which adenylylates/deadenylylates GS, it seemed possible that the *gln-71* mutation in MV71 and MV72 might affect GS catalytic behaviour. The degree of GS adenylylation was qualitatively estimated from the ratio of the Mn²⁺-dependent transferase activity at pH 7.15 to the Mg²⁺-

dependent synthetic activity at pH 7.6 (Kleinschmidt & Kleiner, 1978, 1981). At these pH values both activities showed the strongest and inverse changes in response to the degree of GS adenylylation (Kleinschmidt & Kleiner, 1978). When ammonium was added to cultures of the wild-type strain UW136, the activities ratio increased about fivefold, indicating GS adenylylation (Fig. 3c). After removal of ammonium, the activities ratio decreased to approximately the original value, indicating GS deadenylylation. In contrast, the activities ratio of both MV71 and MV72 strains remained very low and little change was observed upon addition and subsequent removal of ammonium from cultures, indicating that in the *gln-71* strains, the regulation of GS activity via adenylylation was altered. The Mg²⁺-dependent synthetic activity and the Mn²⁺-dependent transferase activity of either MV72 or MV71 were respectively higher and lower than wild-type (Fig. 3a, b). This can be explained by considering the fact that in both mutant strains GS is hyperactive.

The *gln-71* mutation is probably located in the *glnE* gene

Candidate genes in which the *gln-71* mutation might have occurred include *glnA* (e.g. a mutation preventing adenylylation of Tyr407), *glnK* (e.g. a mutation resulting in a GlnK-UMP-like conformation), or *glnE* (e.g. a null mutation inactivating the GS-adenylylation function of GlnE). Another possibility was that the *gln-71* mutation was in an unknown gene or regulatory region linked to *glnD*.

Linkage between *gln-71* and *glnD* was ruled out by transformation experiments in which MV71 DNA was used to transform the wild-type strain UW136. When the amount of donor DNA added to competent cells was just sufficient to yield 50–100 Str^r transformants per plate, all of the approximately 1000 colonies obtained were small in size and unstable with respect to streptomycin resistance, indicating that cotransformation of *gln-71* with *glnD* did not occur with significant frequency.

To determine whether the *gln-71* mutation occurred within *glnA* or *glnK*, DNA from these regions of MV72 was sequenced. Each sequence in both strains was identical to the wild-type gene. Attempts to identify and clone the *glnE* gene of *A. vinelandii* by Southern hybridization using the *E. coli glnE* gene as probe or by PCR strategies based on GlnE amino acid similarities in other prokaryotes were unsuccessful. An alternative strategy was to try to complement the unregulated GS activity phenotype of MV72 by introducing the *E. coli glnE* gene on a wide-host-range plasmid. To achieve this, the 5 kb *Bam*HI–*Pst*I fragment from plasmid pDK805 (van Heeswijk *et al.*, 1993) carrying the *E. coli glnE* gene was subcloned into vector pLAFR3 (Peet *et al.*, 1986), resulting in pPR803. Plasmid pPR803 restored adenylylation/deadenylylation of GS in MV72 (Fig. 3c), indicating that the mutation stabilizing the *glnD* phenotype is most probably located in *glnE*.

An experiment to further test the idea that the *gln-71* mutation is located in *glnE* was based on the idea that if the lethality of the *glnD1::Ω* allele was suppressed by a mutation in *glnE*, then the stable mutant MV71 carrying both the *glnD1::Ω* and *gln-71* alleles should be unable to yield viable transformants carrying pPR803. Plasmids pPR803 and pLAFR3 were independently conjugated into both MV71 (*glnD1::Ω gln-71*) and MV72 (*gln-71*) strains by triparental mating (see Methods). MV72 yielded several hundred Tet^r colonies after conjugation with either pPR803 or pLAFR3. MV71(pLAFR3) Tet^r transformants also numbered in the hundreds but no Tet^r colonies arose from conjugation of MV71 cells with pPR803, indicating that introduction of the *E. coli glnE* gene which restored the ability of GS to be adenylylated was 'lethal' and could not be harboured in the *glnD1::Ω gln-71* background.

Consistent with the conclusion that the *gln-71* mutation is in the *glnE* gene is the fact that the poor growth phenotype of MV71 and MV72 on BSN medium is similar to that shown by *glnE* mutants of *Salmonella typhimurium* (Kustu *et al.*, 1984). Poor growth by these mutants was attributed to a deficiency of glutamate due to its hyperconversion to glutamine. In addition, mutations in *glnE* suppressed the glutamine auxotrophy of *glnD* mutants in *S. typhimurium* (Bancroft *et al.*, 1978).

Construction of *glnA* (GSY407F) *glnD::Ω* double mutants

In parallel experiments to test the hypothesis that *glnD* null mutations were lethal because GS cannot be deadenylylated in the absence of GlnD, the site of GS adenylylation was altered by mutagenesis of *glnA* (see Methods). A *glnAY407F* strain, MV74, grew well in BS medium but not in liquid BSN medium and only very small colonies formed on BSN agar; again this is probably related to a glutamate deficiency as for the *gln-71* (*glnE*) mutant. As in MV71 and MV72, little change of GS catalytic behaviour occurred in MV74 upon addition or removal of NH₄⁺ from the medium (data not shown). MV74 was then transformed with the *glnD1::Ω* plasmid pRCS1::Ω. Several hundred Str^r transformants, about half of which were Amp^s indicating *glnD* gene replacement by a double crossover event, appeared as large colonies on BSU Str medium and did not lose their Str resistance after several cycles of growth on non-selective medium. Southern blot analysis confirmed the complete substitution of wild-type *glnD* with the *glnD1::Ω* allele in a representative Str^r Amp^s isolate, MV75 (data not shown), indicating that like *gln-71*, *glnAY407F* can stabilize a *glnD* null mutation.

Influence of *glnD* mutations on *nif* gene regulation and NtrC phenotype

As with the original *glnD::Tn5* mutant MV17 (Santero *et al.*, 1988), neither MV71 nor MV75, the two stable *glnD* null mutants characterized here, was able to grow

Fig. 4. *In vitro* uridylylation and immunoblotting analysis of *A. vinelandii* GlnK. Following an *in vitro* uridylylation reaction, proteins of the cell-free extracts were separated by SDS-PAGE and transferred to Duralose membranes for exposure to X-ray film (lanes D to F). The same blot was then incubated with *E. coli* anti-P_{II} serum for Western analysis (lanes A to C). Lanes A and D, UW136 (wild-type); lanes B and E, MV17 (*glnD16::Tn5*); lanes C and F, MV71 (*glnD1::Ω gln-71*).

on N-free media (solid or liquid) and they had little or no detectable nitrogenase activity, as measured by acetylene reduction (data not shown). The Nif⁻ phenotypes of MV71 and MV75 were suppressed (i.e. became Nif⁺) by introduction of the *nifL1::K1XX* deletion/insertion mutation (Bali *et al.*, 1992; Blanco *et al.*, 1993), or, in the case of MV71, by introduction of pCK1 expressing the *Klebsiella pneumoniae nifA* gene constitutively (Kennedy & Robson, 1983). This result confirms that the *A. vinelandii glnD* gene product is required for the relief of the NifL inhibition of NifA that normally occurs in cells grown under N-limiting conditions.

Because *glnD* null mutants of enteric bacteria are generally Ntr⁻, the stable *glnD* null mutants MV71 and MV75, and also MV17 [*glnD16::Tn5*], were examined for growth on nitrate as an indicator of NtrC activity (Toukdarian & Kennedy, 1986). Tests included growth on BSNO₃ agar medium and also liquid medium derepression experiments in which the lag time and subsequent growth rate after shift from BSN to BSNO₃ was determined. All three *glnD* mutants grew on BSNO₃ at a rate indistinguishable from wild-type UW136 and had no longer lag time after the shift from BSN to BSNO₃ (data not shown), indicating an NtrC⁺ phenotype.

Uridylylation of GlnK and Western blot analysis

The GlnK protein of *A. vinelandii* was labelled in cell-free extracts to which [α -³²P]UTP and 2-oxoglutarate were added. As shown in Fig. 4, labelling of a 12 kDa protein was observed in cell-free extracts of wild-type and MV17 strains; the extent of label in the 12 kDa

protein in MV17 was about 60% of that appearing in wild-type extracts (determined by Quant Image Analysis). No [32 P]UTP incorporation was detected in the stable Δ *glnD gln-71* strain MV71. Similar amounts of GlnK protein were present in wild-type and in MV17 extracts, as shown by Western blots treated with polyclonal antibody prepared against the PII protein from *E. coli*. Slightly less 12 kDa protein was detected in MV71 extracts, approximately 75% of that found in the other two strains. That uridylyltransferase activity of GlnD was present in MV17 confirms the hypothesis that GlnD in this mutant was not completely inactivated by the Tn5 insertion or that the activity was at least partially restored by the uncharacterized suppressor mutation that occurred in MV16, leading to MV17. Unfortunately, stored cultures of MV16 were not viable and it was not possible to distinguish between these two possibilities.

DISCUSSION

A. vinelandii represents a model system among Eubacteria for analysing mutations in essential genes. Retention of both mutant and wild-type copies of chromosomes can occur and be detected in *A. vinelandii* because it can harbour a large number of chromosomes, up to 80 or more, depending on growth conditions (Maldonado *et al.*, 1994). The great majority of null mutations in *A. vinelandii* genes have been easily introduced and have fully replaced the wild-type alleles; these are too numerous to detail here but include many *nif*, *ntr*, *cyd*, *vnf*, *anf* and *hup* genes. The first case of an apparently lethal mutation in *A. vinelandii* was the finding that mutations in the *glnA* gene encoding GS, created by insertion of Tn5 or other elements, could replace the wild-type gene but were maintained in only a fraction of the chromosomes and only under antibiotic selection for the insertion element (Toukdarian *et al.*, 1990). The lethality of *glnA* null mutations in *A. vinelandii* was explained by the inability of this organism to transport glutamine and the absence of other pathways for ammonium assimilation (Kennedy & Toukdarian, 1987). Other examples of essential genes in *A. vinelandii* include *glnK* (Meletzus *et al.*, 1998) and also *isc* genes encoding proteins involved in iron-sulphur cluster formation (Zheng *et al.*, 1998). *glnD* can now be added to the list.

The most probable explanation for the failure to establish *glnD* null mutations in *A. vinelandii* is the requirement of GlnD protein for uridylylation of GlnK, which in turn is required for deadenylylation of GS. Alteration of the adenylylation site Tyr407 to Phe in the GS protein allows the *glnD* null mutations to stably replace the wild-type allele. Also, the spontaneously arising stable *glnD* mutant strain, MV71, carries a second-site mutation, *gln-71*, which is probably located within *glnE* and results either in a lack of both ATase and adenylyl-removing activities of GlnE or in a GlnE which is locked in the latter conformation and fails to respond to ammonium. It was previously shown that

glnA null mutations could not be established in *A. vinelandii*, even in the presence of added glutamine (Toukdarian *et al.*, 1990) and that glutamine was not significantly transported into *A. vinelandii* cells in [14 C]glutamine uptake experiments. In the current work, the addition of glutamine also did not allow establishment of the *glnD* null mutations (Toukdarian *et al.*, 1990). Taken together, these results confirm that GS in *A. vinelandii* is an essential enzyme for the biosynthesis of glutamine and ammonium assimilation.

Amongst enteric bacteria, *glnD* mutants, which are generally NtrC⁻, adenylylate GS faster and deadenylylate it more slowly than wild-type organisms, and the extent to which glutamine is required for growth varies considerably (Bancroft *et al.*, 1978; Foor *et al.*, 1978; Bueno *et al.*, 1985; Edwards & Merrick, 1995; Atkinson & Ninfa, 1998). Based on these general phenotypes, it was expected that *A. vinelandii glnD* null mutants might be less able to grow on nitrate (the only NtrC⁻ phenotype identified in this organism: Toukdarian & Kennedy, 1986), and that GS might be deadenylylated at a rate slower than in the wild-type. However, no effect on growth on nitrate was observed in the viable *glnD* mutants MV71 (*glnD1::Ω gln-71*) or MV75 (*glnD1::Ω glnAY407F*). In contrast, the physiological effect of *glnD* mutations in *A. vinelandii* on the inability of GS to be deadenylylated appears to be more drastic than in the enteric *glnD* mutants. The inability to isolate stable *glnD* mutants of *A. vinelandii* at all unless a second mutation occurred resulting in inability of GS to be adenylylated is evidence for this. The target proteins, NtrB and GlnE, of *A. vinelandii* may be less and more, respectively, sensitive to the influence of GlnK or GlnK-UMP in *A. vinelandii* than to GlnB or GlnK (or their uridylylated forms) in enteric organisms. Alternatively, the apparent NtrC⁺ phenotype of *A. vinelandii glnD* mutants might be explained by there being sufficient 2-oxoglutaric acid in cells grown in nitrate to inhibit the phosphatase activity of NtrB, resulting in the inability of NtrC to be dephosphorylated (Jiang & Ninfa, 1999).

That the *glnD::Tn5* mutation located at the 3' end of the *glnD* gene in MV17 did not severely influence the ability of the mutant strain to uridylylate GlnK indicates that the transferase activity does not involve the C-terminal region of the protein. GlnD is very large, > 100 kDa and was shown by Jiang *et al.* (1998) to have both uridylyltransferase and uridylyl-removing activities located within the same active site. It is relevant in this context that, as for *A. vinelandii*, *glnD* null mutants also could not be isolated in *Sinorhizobium* (*Rhizobium*) *meliloti* (P. Rudnick and others, unpublished) or in the Gram-positive *Corynebacterium glutamicum* (Jakoby *et al.*, 1999). In the case of *R. meliloti*, the apparent lethality is not related to the adenylylation state of GS, as is shown here for *A. vinelandii*, because a *glnA1* mutant with a Y407F substitution that prevents GS adenylylation (Arcondeguy *et al.*, 1996) did not provide a background in which stable and viable *glnD::spc* mutants could be isolated (P. Rudnick & D. Kahn, unpublished results). Also, in *Acetobacter diazo-*

trophicus, *glnD* insertion mutations resulted in formation of very tiny colonies after several days of growth (Perlova *et al.*, 2000). Whether this suggests an essential or near-essential role for GlnD other than that for PII- or GlnK-uridylation, or one for PII-UMP or GlnK-UMP, remains to be determined. Recent connections between GlnD and cell division, in which *glnD99::Tn10* suppressed the lethality of a temperature sensitive *ftsZ* mutation (Powell & Court, 1998), and between GlnD and polyphosphate accumulation, which is believed to prevent stress-induced damage (Ault-Riche *et al.*, 1998), may provide clues. Also, a transposon insertion in the *Vibrio fischeri glnD* gene resulted in slower but variable growth rates depending on N source and, very interestingly, on an inability to synthesize siderophores for iron uptake (Graf & Ruby, 2000). In contrast, *glnD* null mutants of *Rhizobium tropici* were apparently unaffected in general growth properties; however, only those with insertion mutations in the 5' or central region of *glnD* were unable to utilize nitrate as a N source and did not induce chlorosis in plants, a process that is regulated by fixed N (O'Connell *et al.*, 1998). This result again reflects the complexity of GlnD and the apparent dispensability, at least in some cases, of the C-terminal region of the protein.

The Nif⁻ phenotype of the *glnD* null mutants was, as for the original *glnD::Tn5* strain MV17, corrected to Nif⁺ by the introduction of the *nifL1::KIXX* mutation (Bali *et al.*, 1992) or the plasmid pCK1, which constitutively expresses the *nifA* gene of *K. pneumoniae* (Kennedy & Robson, 1983) (MV75 was not tested). This confirms the involvement of GlnD in relieving the inhibition of NifA by NifL in *A. vinelandii*. He *et al.* (1998) and Arcondeguy *et al.* (1999) recently showed that GlnD is required for relief of *K. pneumoniae* NifL inhibition of NifA activity in an *E. coli* background. The GlnD requirement is for uridylation of PII, and PII-UMP is required to eliminate the PII-stimulated dephosphorylation of NtrC (Jiang & Ninfa, 1999). NtrC-P is in turn needed for the expression of *glnK*. GlnK appears to be the only NtrC-P-activated gene required to modulate the NifL/NifA interaction and is required for relief of NifA inhibition by NifL in enteric bacteria (He *et al.*, 1998; Jack *et al.*, 1999). However, the uridylation state of GlnK appears to be immaterial with respect to the NifL/NifA interaction (He *et al.*, 1998; Arcondeguy *et al.*, 1999). Therefore the only role for GlnD is apparently in ensuring that NtrC is phosphorylated and available for *glnK* transcription. Edwards & Merrick (1995) had shown that GlnD was not required for relief of inhibition of NifA by NifL in an *ntrB6* strain that should express *glnK* constitutively. In contrast, the requirement in *A. vinelandii* for GlnD to relieve inhibition of NifA activity by NifL does not involve NtrC, because NtrC is required neither for expression of the *nifLA* operon (Toukdarian & Kennedy, 1986; Blanco *et al.*, 1993), as it is in *K. pneumoniae*, nor for *glnK* expression, implied from the fact that the *glnKamtB* operon in *A. vinelandii* is expressed at very low levels under all conditions, regardless of fixed N supply (Meletzus *et al.*, 1998).

Also, the *glnK* promoter region shows no sequences similar to those recognized by NtrC and σ^N , as are found upstream of the *glnK* gene in *E. coli* and in *K. pneumoniae*. Therefore, the requirement of GlnD to relieve inhibition of NifA activity by NifL apparently occurs by a different mechanism in *A. vinelandii* than in the enteric organisms.

Is uridylylated GlnK protein in *A. vinelandii* required for relief of inhibition of NifA activity by NifL? Both *glnD* mutant strains MV17, which can at least partially uridylylate GlnK, and MV71, which cannot detectably uridylylate GlnK, are Nif⁻ and are restored to Nif⁺ by introduction of a *nifL::KIXX* mutation. These results might suggest that GlnK is not involved in the relief of inhibition of NifA activity by NifL or that its uridylation state is irrelevant, as reported to be the case for enteric bacteria (He *et al.*, 1998; Arcondeguy *et al.*, 1999). It is conceivable that levels of GlnK-UMP in MV17 are sufficient to promote deadenylation of GS, but not sufficient to relieve NifL inhibition of NifA under N-fixing conditions. Recent results with a *glnKY51F* mutant indicate that uridylylated GlnK is required for relief of inhibition of NifA activity by NifL in wild-type *A. vinelandii* but not in the *nifL1::KIXX* mutant strain (P. Rudnick and others, unpublished). In addition, Little *et al.* (2000) recently demonstrated that unuridylylated PII-like proteins cause NifL to be inhibitory to NifA in an *in vitro* system.

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