The *Rhizobium leguminosarum* bv. *viciae* *glnD* gene, encoding a uridylyltransferase/uridylyl-removing enzyme, is expressed in the root nodule but is not essential for nitrogen fixation

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A *Rhizobium leguminosarum* bv. *viciae* VF39 gene (*glnD*) encoding the uridylyltransferase/uridylyl-removing enzyme, which constitutes the sensory component of the nitrogen regulation (*ntr*) system, was identified, cloned and characterized. The deduced amino acid sequence contains the conserved active site motif of the nucleotidyltransferase superfamily and is highly homologous to the *glnD* gene products of other bacterial species. Downstream of the VF39 *glnD* resides an open reading frame with similarity to the *Salmonella typhimurium* virulence factor gene *mviN*. Mutation of the *glnD* gene abolished the ability to use nitrate as a sole nitrogen source but not glutamine. In addition, neither uridylylation of P_{II} nor induction of the *ntr*-regulated *glnII* gene (encoding glutamine synthetase II) under ammonium deficiency could be observed in mutant strains. This strongly suggests that *glnD* mutants harbour a permanently deuridylylated P_{II} protein and as a consequence are unable to activate transcription from NtrC-dependent promoters. The *glnD* gene itself is expressed constitutively, irrespective of the nitrogen content of the medium. A functional GlnD protein is not essential for nitrogen fixation in *R. leguminosarum* bv. *viciae*, but *in situ* detection of *glnD* expression in the symbiotic and infection zone of the root nodule and quantitative measurements suggest that at least part of the *ntr* system functions in symbiosis. The results also indicate that the N-terminal part of GlnD is essential for the cell, as deletions in the 5'-region of the gene appear to be lethal and mutations possibly affecting the expression of the first half of the protein have a significant effect on the vitality of the mutant strain.

Keywords: nitrogen regulation, uridylyltransferase, P_{II} uridylylation, glutamine synthetase, *mviN*

INTRODUCTION

The soil bacterium *Rhizobium leguminosarum* bv. *viciae* fixes dinitrogen in symbiosis with host plants belonging to the genera *Pisum*, *Vicia*, *Lathyrus* and *Lens*

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Abbreviation: GS, glutamine synthetase.

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(van Rhijn & Vanderleyden, 1995). The symbiotic interaction is initiated by an exchange of specific signal molecules between the plant and the microsymbiont (the bacterium) and culminates in the formation of specialized plant organs called root nodules which are colonized by the rhizobia (Bladergroen & Spaink, 1998; Lorkiewicz, 1997; Dénarié & Debelle, 1996; Downie, 1994). Upon release into the plant cell, rhizobia differentiate into bacteroids in which the nitrogen fixation (*nif/fix*) genes are induced under the control of the central nitrogen fixation regulatory gene

nifA. It is generally accepted that during the onset of nitrogen fixation, the bacterial nitrogen metabolism changes from ammonium assimilation to ammonium export, which is a prerequisite for an effective symbiosis in which the plant benefits from the fixed nitrogen (Brown & Dilworth, 1975; Glenn & Dilworth, 1985; Udvardi & Day, 1997).

Under free-living conditions, rhizobia assimilate ammonium by the glutamine synthetase (GS)/glutamate synthase (GOGAT: glutamine-oxoglutarate aminotransferase) pathway leading to the production of glutamate from ammonium and 2-oxoglutarate (Bravo & Mora, 1988). *R. leguminosarum* possesses three GSs encoded by *glnA* (GSI), *glnII* (GSII) and *glnT* (GSIII) (Espin *et al.*, 1994). GSI is homologous to GS from enteric bacteria and its enzymic activity is regulated by reversible adenylation/deadenylation (Filser *et al.*, 1986; Colonna-Romano *et al.*, 1987; Bravo & Mora, 1988). GSII is similar to eukaryotic GSs and its expression is most probably turned off in symbiosis (Shatters *et al.*, 1989). The function of GSIII is not quite clear in *R. leguminosarum* but for *Sinorhizobium meliloti* it is known that GSIII is not expressed when GSI and GSII are present (de Bruijn *et al.*, 1989; Chiurazzi *et al.*, 1992; Shatters *et al.*, 1993). None of the GS isoenzymes plays an essential role in symbiosis.

Activity of GSI and synthesis of GSI and GSII are controlled by the global nitrogen regulation (*ntr*) system, which modulates nitrogen metabolism in response to the prevailing nitrogen source and the needs of the cell (Merrick & Edwards, 1995). In enteric bacteria, this system is composed of four components: the GlnD protein (uridylyltransferase/uridylyl-removing enzyme, UTase/UR), the signal transduction protein P_{II} (encoded by *glnB*) and the two-component regulatory system NtrB/NtrC (Merrick & Edwards, 1995; Magasanik, 1982; Adler *et al.*, 1975; Mangum *et al.*, 1973). The activity of the P_{II} protein is stimulated by glutamine and inhibited by 2-oxoglutarate (Engleman & Francis, 1978). Under high concentrations of fixed nitrogen, P_{II} activates an adenylyltransferase (ATase, encoded by the *glnE* gene), which in turn inactivates GSI by adenylation. At the same time, P_{II} causes NtrB, a sensory histidine protein kinase, to dephosphorylate and thereby inactivate the corresponding response regulator NtrC. As a consequence, NtrC-dependent (σ^{54}) promoters are switched off. Under glutamine-limiting conditions, P_{II} is uridylylated by the *glnD* gene product, which leads to deadenylation (activation) of GSI by the ATase and to phosphorylation of NtrC by NtrB. NtrC-P then stimulates transcription of NtrC-dependent promoters (Jiang *et al.*, 1998a, b, c).

The NtrB/NtrC regulatory system has also been identified in *R. leguminosarum* (Moreno *et al.*, 1992), where it is cotranscribed with an open reading frame of unknown function from two $-35/-10$ -like promoters (Patriarca *et al.*, 1993). In bacteroids, transcription was found to be down-regulated (Patriarca *et al.*, 1996). Expression of the *glnII* gene, which is preceded by a characteristic σ^{54} -

dependent promoter, is strictly regulated by NtrB/NtrC (Patriarca *et al.*, 1992) and most probably turned off in symbiosis (Shatters *et al.*, 1989). It was shown that the P_{II} protein, which is encoded upstream of *glnA* (Chiurazzi & Iaccarino, 1990), negatively controls the transcriptional activity of NtrC and that it is subject to uridylation, suggesting the presence of a UTase/UR-activity also in *R. leguminosarum* (Amar *et al.*, 1994; Colonna-Romano *et al.*, 1993).

Here we describe the identification and characterization of a *R. leguminosarum* bv. *viciae* gene (*glnD*) encoding such a uridylyltransferase/uridylyl-removing enzyme. We constructed mutants and tested the function and expression of this gene under free-living and symbiotic conditions.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* was grown at 37 °C in Assay Broth No. 3 (Oxoid) supplemented as needed with antibiotics at the following final concentrations: tetracycline, 10 µg ml⁻¹; gentamicin, 10 µg ml⁻¹; ampicillin, 100 µg ml⁻¹; kanamycin, 25 µg ml⁻¹. *Rhizobium* strains were grown in TY medium (Beringer, 1974) or *Rhizobium* minimal medium (RMM) (Hooykaas *et al.*, 1977) at 30 °C. When needed, antibiotics were added at the following concentrations: streptomycin, 400 µg ml⁻¹; tetracycline, 10 µg ml⁻¹; gentamicin, 30 µg ml⁻¹; neomycin, 100 µg ml⁻¹. KNO₃, NH₄Cl or glutamine was used as nitrogen source at a concentration of 1 g l⁻¹. Indicator medium for strains expressing an active β -galactosidase was supplemented with 40 µg X-Gal ml⁻¹ (final concentration). β -Glucuronidase activity was detected on agar medium containing 5-bromo-4-chloro-3-indolyl β -D-glucuronide at a final concentration of 40 µg ml⁻¹.

Genetic methods. The protocols for mating experiments, mobilization of plasmids between *E. coli* and *Rhizobium* strains as well as for Tn5 mutagenesis were as previously described (Simon, 1984; Priefer, 1989).

Standard DNA techniques. Total DNA was isolated by the CTAB (cetyltrimethylammonium bromide) method described by K. Wilson in Ausubel *et al.* (1987). Plasmid DNA and DNA fragments were purified using the QIAprep Spin Miniprep kit (Qiagen) and the USBioclean kit (USB) containing a glass powder suspension according to the manufacturers' instructions. Restriction enzyme digestion, agarose gel electrophoresis, DNA cloning and transformations were carried out according to Maniatis *et al.* (1982). For hybridization, separated restriction fragments were transferred onto QIABRANE nylon membranes (Qiagen) by vacuum blotting (Pharmacia) and probes were labelled with digoxigenin-11-UTP (DIG DNA Labelling kit; Boehringer). Detection of hybridizing bands was achieved using the DIG Nucleic Acid Detection kit (Boehringer) based on an enzyme-catalysed colour reaction.

Sequence analysis. Appropriate restriction sites within the *glnD*-containing region were used to clone 200–700 bp fragments into sequencing vectors pUC18/19, pUC57, pBluescript II KS and pK18/19mob. Sequencing of the resulting recombinant plasmids was done for both strands by the chain-termination method using IRD-700 or IRD-800 labelled standard primers (universe and reverse) according to the

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics	Reference
Strains		
<i>Escherichia coli</i>		
S17-1	Modified RP4 plasmid integrated into the genome of <i>E. coli</i> 294 <i>recA</i>	Simon <i>et al.</i> (1983)
JM83	$\Delta(lac\ pro)\ thi\ \phi 80dlacZ\ \Delta M15$	Vieira & Messing (1982)
XL-1 Blue	<i>recA1 endA1 gyrA96 hsdR17 supE44 lac</i> [F' <i>proAB lacI^a</i> $\Delta M15$ Tn10 (<i>tet^r</i>)] <i>thi</i>	Bullock <i>et al.</i> (1987)
DH5 α	<i>supE44Δ lacU169 ($\phi 80lacZ\Delta M15$) hsdR17 recA endA1 gyrA96 thi-1 relA1</i>	Hanahan (1983)
<i>Rhizobium leguminosarum</i>		
bv. <i>viciae</i>		
VF39	Wild-type, Sm ^r	Priefer (1989)
VF39-B1	<i>glnD::Tn5</i> mutant, Nm ^r	This work
VF39-BB1	<i>glnD::Nm^r ($\leftarrow$)*</i> mutant, Nm ^r	This work
VF39-BB3	<i>glnD::Nm^r ($\rightarrow$)*</i> mutant, Nm ^r	This work
VF39-C	<i>fixC::Nm^r ($\rightarrow$)*</i> mutant, Nm ^r	This work
LPR1105	Wild-type	Hooykaas <i>et al.</i> (1977)
BS11	<i>glnB</i> mutant, Nm ^r	Amar <i>et al.</i> (1994)
Plasmids		
pBluescript II KS	Cloning and sequencing vector, Ap ^r	Stratagene
pUC18/19	Cloning and sequencing vector, Ap ^r	Yanisch-Perron <i>et al.</i> (1985)
pUC57	Cloning and sequencing vector, Ap ^r	Markausakas & Dreguniene, Fermentas
pK18/19mob	pK18/19 derivative, <i>mob</i> , Km ^r	Schäfer <i>et al.</i> (1994)
pSUP202	pBR325 derivative, <i>mob</i> , Tc ^r Ap ^r Cm ^r	Simon <i>et al.</i> (1983)
pSUP202-Gm	pSUP202 derivative, Tc ^r Gm ^r Cm ^r	Colonna-Romano <i>et al.</i> (1990)
pAS269	pSUP301 derivative, <i>mob</i> , Ap ^r Cm ^r Tc ^r	Patschkowski <i>et al.</i> (1996)
pSUP1021	Tn5-carrier suicide vector, <i>mob</i> , Cm ^r Tc ^r Km ^r	Simon <i>et al.</i> (1986)
pBI221.1	pUC19 derivative, <i>gusA</i> cassette	Jefferson (1987)
pJP2	Broad-host-range promoter probe vector, Tc ^r	J. Prell, RWTH Aachen
pMK97.2B	pUC19 derivative, 7.7 kb <i>EcoRI</i> fragment carrying ' <i>glnD::Tn5</i> , Ap ^r	This work
pUC57-5/6	pUC57 derivative, 16 kb <i>KpnI</i> fragment carrying the <i>glnD-mviN'</i> region (both orientations), Ap ^r	This work
pAS1298	pK19mob derivative, 1.2 kb <i>HindIII-EcoRI</i> fragment carrying <i>pglnD'</i> , Km ^r	This work
pAS1098	pSUP202-Gm derivative, 2346 bp <i>SphI-BglII</i> fragment carrying ' <i>glnD</i> ', Gm ^r	This work
pNE1	pK19mob derivative, 1041 bp <i>HindIII</i> fragment carrying ' <i>glnD</i> ', Km ^r	This work
pNE3	pK19mob derivative, 926 bp <i>BamHI-BglII</i> fragment carrying ' <i>glnD</i> ', Km ^r	This work
pAS1198-BB3	pSUP202-Gm derivative, <i>glnD::Nm^r ($\rightarrow$)*</i> , Gm ^r Cm ^r	This work
pAS1198-BB1	pSUP202-Gm derivative, <i>glnD::Nm^r ($\leftarrow$)*</i> , Gm ^r Cm ^r	This work
pAS1198-2	pSUP202-Gm derivative, <i>glnD</i> deletion/insertion, Gm ^r Cm ^r	This work
pAS1092	pSUP202 derivative, <i>fixC::Nm^r</i> , Tc ^r Nm ^r	This work
pAR36A	<i>glnII-lacZ</i> transcriptional fusion, Tc ^r	Patriarca <i>et al.</i> (1992)
pAS1299	pJP2 derivative, <i>pglnD-gusA</i> transcriptional reporter gene fusion, Tc ^r	This work

* Arrows denote the orientation of the neomycin-resistance gene cassette (Nm^r) in relation to the corresponding gene.

instructions of a Thermo-Sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech). Extension products were separated and detected on a LI-COR 4200L DNA Sequencer. An IRD-700 labelled IS50 specific primer (5'-CGGGAAAGTTCCGTT-CAGGACG-3') was used to generate sequences adjacent to the Tn5 insertion. Sequence analysis and determination of the coding probability was done using the computer programs of

Staden (1986). The deduced amino acid sequences were compared to the protein database NCBI BLAST E-Mail Server (Altschul *et al.*, 1990).

Construction of mutant strains and recombinant plasmids

Construction of *R. leguminosarum* bv. *viciae* VF39 *glnD* mutants. A 2346 bp *SphI-BglII* fragment carrying the 5'-part of the *R. leguminosarum* bv. *viciae* VF39 *glnD* region was

Fig. 1. Physical and genetic map of the *R. leguminosarum* bv. *viciae* VF39 *glnD*–*mviN* gene region. The *glnD* and *mviN* coding regions are indicated by arrows; only the 5'-end of the putative *mviN* gene is present on the cloned fragment. The location of the Tn5 insertion in the originally isolated *glnD* mutant is marked by a triangle. The *Sph*I–*Bgl*II fragment cloned in plasmid pAS1198 was mutagenized by insertion of the neomycin-resistance gene cassette (*aph* – aminoglycoside-phosphotransferase) in both orientations. The arrows indicate direction of transcription of the *aph* gene. The resulting plasmids pAS1198-BB3 and pAS1198-BB1 were used for the construction of *R. leguminosarum* bv. *viciae* *glnD* insertion mutants. The fragment carried by pAS1198-2, in which the internal 1041 bp *Hind*III fragment was replaced by a 3424 bp *Hind*III neomycin-resistance gene cassette and which did not yield any viable mutants, is also shown. pAS1299 is a mobilizable broad-host-range plasmid that carries a transcriptional *glnD*–*gusA* reporter gene fusion generated at the *Xho*I restriction site within *glnD*. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; EV, *Eco*RV; H, *Hind*III; Kp, *Kpn*I; Sa, *Sac*II; Sp, *Sph*I; St, *Stu*I; X, *Xho*I. Only one *Sac*II site (upstream of *glnD*) is shown.

subcloned into the mobilizable plasmid pSUP202-Gm, resulting in plasmid pAS1098. A 1861 bp *Hind*III–*Bam*HI fragment originating from Tn5 and carrying the neomycin-resistance gene was converted into a *Bam*HI fragment and subsequently cloned in both orientations into the single *Bam*HI site in the centre of the *glnD* gene (Fig. 1). The resulting constructs pAS1198-BB3 and pAS1198-BB1 were transferred from *E. coli* S17-1 to *R. leguminosarum* bv. *viciae* VF39 and recombined into the genomic *glnD* region via double crossover. The corresponding mutants were designated VF39-BB3 and VF39-BB1 (Fig. 1). Correct homogenotization of the *glnD*::Nm^r mutation was verified by Southern hybridization analysis. For this purpose, total DNA restricted with *Bgl*II and *Xho*I was hybridized using labelled pAS1099, which carries the almost complete *glnD* gene on a *Sph*I fragment (Fig. 1). The wild-type DNA restricted with *Bgl*II showed two hybridizing bands of 4600 and 12000 bp; insertion of the 1881 bp neomycin-resistance cassette, which also carries a *Bgl*II site, into the 12000 bp *Bgl*II fragment resulted in two hybridizing bands (1266 and 12600 bp in VF39-BB1; 2467 and 11400 bp in VF39-BB3). Wild-type DNA restricted with *Xho*I showed two hybridizing bands of 4400 and 9000 bp. Insertion of the resistance cassette introduced another *Xho*I site into the 9000 bp fragment, yielding two bands in the mutant strains (1012 and 9900 bp in VF39-BB1 and 2445 and 8400 bp in VF39-BB3).

To construct a *glnD* deletion/insertion mutant, an internal 1041 bp *Hind*III fragment was replaced by a 3423 bp *Hind*III neomycin-resistance cassette in plasmid pAS1098. The resulting plasmid pAS1198-2 (Fig. 1) was transferred to *R. leguminosarum* bv. *viciae* VF39 and exconjugants were selected on medium containing neomycin. In order to identify homogenotization events mediated by double crossover, about

2000 exconjugants were tested for loss of the plasmid-encoded gentamicin resistance. No such mutants could be obtained.

Construction of a *R. leguminosarum* bv. *viciae* VF39 *fixC* mutant. A 3400 bp *Pst*I–*Eco*RI fragment carrying the *R. leguminosarum* bv. *viciae* 'fixB-fixC-fixX-nifA' region was cloned into plasmid pSUP202. A 1900 bp *Bam*HI neomycin-resistance gene cassette was then inserted into the single *Bgl*II site located in *fixC*. The resulting plasmid pAS1092 was mobilized into VF39 and the *fixC*::Nm^r mutation was homogenotized. The *fixC* mutant (VF39-C) was verified by Southern hybridization and by its complete inability to fix nitrogen.

Construction of a *R. leguminosarum* bv. *viciae* VF39 *glnD*–*gusA* reporter gene fusion. A 3500 bp *Bam*HI–*Xho*I fragment carrying the 5'-end of the *glnD* gene and the putative *glnD* promoter region was cloned into the broad-host-range promoter probe vector pJP2, thus creating a transcriptional p*glnD*–*gusA* reporter gene fusion at the *Xho*I site located 630 bp downstream of the *glnD* ATG start codon. The resulting plasmid was designated pAS1299 (Fig. 1).

Uridylation of P_{II}. The assay for P_{II} uridylation was performed according to Colonna-Romano *et al.* (1993). Strains were grown in minimal media (RMM) containing glutamate as a nitrogen source. For every strain, three aliquots were taken in exponential growth phase, incubated for 2 h in 2-methyl-imidazole buffer containing [α -³²P]UTP (800 Ci mM⁻¹; 29.6 TBq mM⁻¹), and subsequently either 30 μ l 2-oxoglutarate (180 mM), glutamine (200 mM) or water, respectively, was added to individual samples. All samples were incubated further for 2 h more and then analysed on a 15% SDS-PAGE gel.

Enzyme tests. Quantitative determination of β -galactosidase activity was performed according to Miller (1972). The *glnI* promoter activity was tested by growing the strains first in rich medium (TY) and then transferring them into minimal medium with different nitrogen sources. Although the *glnD* mutant strains are unable to use nitrate as sole nitrogen source, they still could reach exponential phase due to nitrogen carry-over from the rich medium. Exponential-phase cultures were taken for the assays. β -Glucuronidase activity was measured using the substrate *p*-nitrophenyl glucuronide (PNPG) as described by Jefferson (1987). β -Glucuronidase units were calculated according to Miller (1972). Determination of the β -glucuronidase activity of bacteroids was done by crushing nodules of about 10 plants in 1 ml 50 mM phosphate buffer with 10 μ l 10% Triton. Plant material was removed by centrifugation (5 min, 1000 r.p.m.). The supernatant was then centrifuged (5 min, 4500 r.p.m.) to sediment the bacteroid fraction, which was suspended in 650 μ l 50 mM phosphate buffer and used for PNPG tests.

Nodulation tests and determination of nitrogen fixation activity. Nodulation and nitrogen fixation tests using *Vicia hirsuta* as a host plant and acetylene reduction assays for the determination of nitrogenase activity were as previously described (Priefer, 1989).

GUS-specific staining of nodule sections. Nodules were excised from the roots of *V. hirsuta* plants 21 d after inoculation and embedded in 6% (w/v) agarose in 50 mM phosphate buffer. Sections of 90 μ m thickness were cut using a vibratome (VT 1000 S; Leica). Nodule sections were stained as described previously (Schlüter *et al.*, 1997).

RESULTS AND DISCUSSION

Identification and cloning of the *R. leguminosarum* bv. *viciae* *glnD* gene

For several *Rhizobium* species it has been shown that mutations in genes of the *ntr* system cause defects in nitrate assimilation (Merrick & Edwards, 1995). *R. leguminosarum* bv. *viciae* strain VF39 was mutagenized with transposon Tn5 using pSUP1021 as a suicide transposon carrier and Tn5-containing exconjugants were screened for growth on minimal medium (RMM) with different nitrogen sources. Two mutants out of approximately 10000 were identified to be unable to utilize nitrate but grew normally on RMM containing either ammonium or glutamine.

One of these mutants, designated VF39-B1, was characterized in more detail. A 7700 bp *Eco*RI fragment containing the Tn5 insertion was cloned from the mutant genome taking advantage of the Tn5-encoded neomycin resistance. Partial sequencing of the cloned fragment using ISS0-specific primers revealed that the transposon was inserted in a gene that showed considerable similarity to the *glnD* genes of *Rhizobium tropici*, *Azotobacter vinelandii*, *Klebsiella pneumoniae* and *E. coli* (O'Connell *et al.*, 1998; Contreras *et al.*, 1991; Edwards & Merrick, 1995; van Heeswijk *et al.*, 1993). Therefore, the corresponding *R. leguminosarum* bv. *viciae* gene was designated *glnD*.

The observed phenotype of VF39-B1 is consistent with *Rhizobium* mutants described in the literature: a *R.*

tropici *glnD* mutant similarly fails to grow on nitrate as a sole nitrogen source (O'Connell *et al.*, 1998), and a mutation in the *R. leguminosarum* bv. *viciae* *glnB* gene (encoding P_{II}, the target protein of GlnD activity) also abolishes the ability to utilize nitrate (Amar *et al.*, 1994). Glutamine prototrophy of the *glnD* mutant can be explained by the fact that the *glnA* gene (encoding GSI) is not strictly *ntr*-regulated since it contains in addition to the NtrC-dependent promoter a -35/-10-like promoter sequence in its upstream region (Chiurazzi & Iaccarino, 1990).

Sequence analysis of the *R. leguminosarum* bv. *viciae* *glnD* gene

The Tn5 insertion was mapped precisely by sequencing and found to be located in the outermost 3'-end of the gene, only 197 bp upstream of the stop codon (Fig. 1). Hybridization with the wild-type genome, using the Tn5-containing fragment as a probe, identified a 16 kb *Kpn*I fragment, which was isolated from the gel and cloned. Comparison of restriction analysis data of the cloned 16 kb *Kpn*I fragment with hybridization data obtained from probing the genomic *R. leguminosarum* bv. *viciae* *glnD* region verified that the cloned fragment was native and originated from this organism. The Tn5-containing *Eco*RI fragment cloned initially was shown to map at one end of the *Kpn*I clone (Fig. 1).

Appropriate subclones of the *glnD*-containing region on the 16 kb *Kpn*I fragment were generated and sequenced. A 4087 bp DNA sequence starting at a *Sac*II site located upstream of *glnD* and ending at the *Kpn*I site, marking one boundary of the cloned *Kpn*I fragment (Fig. 1), is available under the GenBank accession number AF155830.

The *R. leguminosarum* bv. *viciae* *glnD* gene was found to be 2904 bp long and encoded a gene product of 968 amino acids with a calculated size of 108.8 kDa and an isoelectric point (pI) of 6.9. The deduced amino acid sequence showed 78% identity and 85% similarity to GlnD of *R. tropici* (O'Connell *et al.*, 1998), and 30–31% identity and 46–47% similarity to both GlnD of *K. pneumoniae* (Edwards & Merrick, 1995) and to the *nfrX* (a *glnD* homologous gene) gene product of *A. vinelandii* (Contreras *et al.*, 1991). The nucleotidyl-transferase-superfamily motif (AVGGYGR-X₂-L-X-P-X-SDIDLL) suggested to be the active site of GlnD and to be involved in forming a so-called phosphate-binding loop (Holm & Sander, 1995) was found to be conserved in all *glnD* gene products compared (position 124–142 in the Rl-GlnD amino acid sequence). The region identified in NfrX which is homologous to a conserved stretch of amino acids in various GS proteins (Contreras *et al.*, 1991) could not be detected in *R. leguminosarum* bv. *viciae* and *R. tropici* GlnD.

Downstream of *glnD*, a second incomplete ORF was identified which shows 91% identity and 95% similarity at the amino acid level to the N-terminus of *R. tropici* MviN and also 42% identity and 61% similarity to the

virulence factors MviN of *Salmonella typhimurium* (SWISS-PROT accession no. P37169) and *E. coli* (GenBank accession no. for the gene product is AAC74153). Therefore this gene was tentatively designated *mviN*. In *R. tropici*, the *mviN* start codon overlaps the *glnD* stop codon (-ATGA-, start/opal stop signal), whereas in *R. leguminosarum* both genes are separated by 52 nucleotides. A transcriptional terminator structure could not be found in this intergenic region but the sequence motif -ACGCATGAGCC-, which contains the overlapping start/opal stop signal -ATGA-, is repeated four times. The sequenced region extends 551 bp upstream of *glnD* but no obvious promoter structure could be detected by comparison to known σ^{70} - or σ^{54} -dependent promoters.

Construction and phenotypic characterization of *R. leguminosarum* bv. *viciae* *glnD* mutants

GlnD mutations described for *A. vinelandii*, *E. coli* and *R. tropici* map very close to the 3'-end of the gene (Contreras *et al.*, 1991; O'Connell *et al.*, 1998; van Heeswijk *et al.*, 1992), which is also true for the Tn5-induced mutant isolated in this study (see above and Fig. 1). This led us to the assumption that mutations in the 5'-region of *glnD* might be lethal and we therefore tried to introduce mutations in the central and 5'-part of the gene by site-directed mutagenesis. A neomycin-resistance cassette was cloned in both orientations into the central *Bam*HI site (located 1418 bp downstream of the *glnD* start codon; Fig. 1) and the mutations could be easily integrated into the VF39 genome via homologous recombination. Correct homogenotization was verified by hybridization (for details see Methods) and the mutant strains were designated VF39-BB1 and VF39-BB3.

We also tried to construct a *R. leguminosarum* bv. *viciae* *glnD* deletion/insertion mutant by replacing an internal 1041 bp *Hind*III fragment with a *Hind*III neomycin-resistance gene cassette (Fig. 1). Several attempts to homogenotize this construct failed (for details see Methods), which supports the hypothesis that mutations close to the 5'-end of the *glnD* gene might be lethal.

Both mutant strains VF39-BB1 and VF39-BB3 were tested for their growth on RMM containing various nitrogen sources. As expected, none of the mutants could grow on nitrate. VF39-BB3 grew normally on ammonium and glutamine, whereas VF39-BB1 showed reduced growth rates with these nitrogen sources and even on rich medium (TY) its generation time was doubled (with and without neomycin) as compared to wild-type. This defect is probably not caused by a polar effect of the resistance cassette on the downstream *mviN*-like gene since the mutation can be complemented by the original *Kpn*I fragment, which contains the complete *glnD* and only the 5'-part of *mviN* (Fig. 1). Moreover, the *mviN* gene in *R. tropici* was found to be involved in motility (O'Connell *et al.*, 1998). We therefore assume that the reduced growth of VF39-BB1

Fig. 2. Autoradiograph of the P_{II} uridylylation assay. Strains VF39 wild-type (lanes 1, 2 and 3), *glnD* mutant VF39-BB1 (lanes 4, 5 and 6), *glnD* mutant VF39-BB3 (lanes 7, 8 and 9), LPR1105 (lane 10) and *glnB* mutant B11 (lanes 11 and 12) were grown in minimal medium containing glutamate and incubated with radioactively labelled UTP for 2 h. Then, either water (lanes 1, 4, 7, 10 and 11), 2-oxoglutarate (lanes 2, 5, 8 and 12) or glutamine (lanes 3, 6 and 9) was added.

is due to an antisense transcript initiated at the neomycin-resistance promoter and interfering at least partially with the *glnD* mRNA 5'-region, which gives further evidence for the assumption that the N-terminal part of GlnD might be essential for viability.

The *glnD* insertion mutants were also tested for their symbiotic performance on *V. hirsuta* plants. VF39-BB3 induced nodules which could not be distinguished from those elicited by the wild-type strain. Also plant growth and appearance were normal and nitrogenase activity was unaffected, which is in agreement with data published for the *R. tropici* *glnD* mutant (O'Connell *et al.*, 1998). In contrast, mutant VF39-BB1 was significantly altered in its nodulation ability: only 8% of the plants were nodulated and the number of nodules per plant was reduced to 30% of the number formed by the wild-type strain. Nitrogen fixation activity, however, appeared to be unaffected as those few nodules fixed normally. Recovery of bacteria from the nodules confirmed that they still contained the *glnD* mutation. Currently it is unclear whether the low nodulation efficiency of this strain is due to the mutated *glnD* gene per se or a secondary effect of its reduced vitality.

Effect of *glnD* mutations on P_{II} uridylylation and *glnI* expression

Mutations in *glnD* should affect uridylylation of the P_{II} protein. We therefore analysed the ability of the *glnD* insertion mutants VF39-BB1 and VF39-BB3 to uridylylate P_{II} under appropriate conditions (see Methods). As controls, we included the *R. leguminosarum* bv. *viciae* *glnB* mutant described by Amar *et al.* (1994), and the corresponding isogenic wild-type strain LPR1105. As shown in Fig. 2, a single protein with a molecular mass of 11 kDa is labelled in the wild-type control strain (lane 10) and absent in the *glnB* mutant (lanes 11 and 12). The same band is present in VF39 wild-type cells grown in minimal medium plus glutamate (lane 1). Labelling is further increased by addition of 2-oxoglutarate (lane 2) and decreased in the presence of glutamine (lane 3). This is in agreement with the reported sensitivity of P_{II} uridylylation to the

Table 2. Expression of the *glnD*–*gusA* reporter gene fusion on plasmid pAS1299 in *R. leguminosarum* bv. *viciae* under free-living and symbiotic conditions

Values are β -glucuronidase activity in Miller units and represent the mean of three independent measurements; numbers in parentheses give the standard deviations. NT, Not tested.

Strain	Characteristics	TY	RMM- NO ₃ [−]	RMM- NH ₄ ⁺	RMM- Gln	Nodule
VF39(pJP2)*	Wild-type	4.1 (1.7)	7.7 (2.2)	8.2 (2.8)	4.3 (1.4)	2 (0.4)
VF39(pAS1299)	<i>glnD</i> – <i>gusA</i>	414 (12)	427 (15)	403 (16)	465 (10)	157 (29)
VF39-C(pAS1299)	<i>fixC::Nm glnD</i> – <i>gusA</i>	NT	NT	NT	NT	209 (24)

* pJP is the basic replicon of pAS1299.

Fig. 3. Expression of the *R. leguminosarum* bv. *viciae* *glnD*–*gusA* reporter gene fusion in a *V. hirsuta* root nodule, inoculated with VF39 carrying a transcriptional *glnD*–*gusA* reporter gene fusion on a broad-host-range plasmid (pAS1299). Nodules inoculated with the Fix[−] mutant VF39-C (pAS1299) showed the same pattern and intensity of expression (not shown). Nodules inoculated with the wild-type strain VF39 carrying only the promoter probe vector pJP2 (the basic replicon of pAS1299) remained colourless after incubation with 5-bromo-4-chloro-3-indolyl β -D-glucuronide (not shown). The histological zone II corresponds to the infection zone; zone III to the symbiotic zone.

glutamine/2-oxoglutarate ratio (Adler *et al.*, 1975). No labelling at all was observed in the *glnD* mutants VF39-BB1 and VF39-BB3 (lanes 4–9), suggesting that both mutant GlnD proteins had lost their uridylylation activity, leading to a constitutively deuridylylated P_{II} protein.

To analyse the effect of a *glnD* mutation on NtrC activity, we tested activation of the *glnII* gene, encoding GSII, the expression of which is under strict *ntr* control (Patriarca *et al.*, 1992). Plasmid pAR36A carrying a *glnII*–*lacZ* reporter gene fusion was introduced into the VF39 wild-type strain and into mutant VF39-BB3. Recombinant *Rhizobium* strains were first grown in TY rich medium, then incubated in RMM containing either nitrate or ammonium (for details see Methods), and exponential-phase cultures were measured for their β -galactosidase activities in three independent assays. Whereas in wild-type the *glnII* fusion was induced by a factor of about 3.4 in medium containing nitrate (83 ± 7 Miller units in RMM-NH₄⁺ and 284 ± 29 Miller units in RMM-NO₃[−]), this induction was abolished in the *glnD*

mutant strain VF39-BB3 (79 ± 7 and 83 ± 9 , respectively). In contrast, a *R. leguminosarum* bv. *viciae* *glnB* mutant shows constitutive expression of *glnII* as shown by Amar *et al.* (1994). The authors proposed that the *glnB* gene product, P_{II}, plays a negative role in the ability of NtrC to activate transcription, probably because it activates NtrB-mediated dephosphorylation of NtrC-P. This dephosphorylation would not take place in the *glnB* mutant, thus leaving NtrC-P in its active form. Accordingly, in a *glnD* mutant, in which P_{II} obviously exists in its deuridylylated form, NtrC is most probably permanently dephosphorylated and thus no longer able to activate transcription at *ntr*-dependent promoters.

Expression of *glnD* under free-living and symbiotic conditions

To analyse the expression of the *R. leguminosarum* bv. *viciae* *glnD* gene, a transcriptional *glnD*–*gusA* reporter gene fusion was constructed using a *Xho*I site located 630 bp downstream of the *glnD* start codon (Fig. 1).

This fusion, carried on a mobilizable broad-host-range plasmid, was transferred to wild-type VF39 and its expression was tested in rich medium and in minimal medium containing nitrate, ammonium or glutamine as a nitrogen source. It was found that *glnD* is constitutively expressed irrespective of the nitrogen source (Table 2), which is consistent with the presumption that GlnD itself is the nitrogen-sensing component. This most probably occurs by a mechanism that modulates its activity in response to nitrogen availability, as suggested by Jiang *et al.* (1998a). Nitrogen-independent constitutive expression was also shown for the *E. coli glnD* gene (van Heeswijk *et al.*, 1993; Kim *et al.*, 1998), whereas in *R. tropici* expression of a transcriptional *glnD-gusA* fusion could not be detected at all (O'Connell *et al.*, 1998).

Since we had shown that a functional *glnD* gene is not essential for nitrogen fixation (see above), it was interesting to see whether *glnD* is expressed at all in symbiosis or switched off. As demonstrated in Fig. 3, expression of the *glnD-gusA* fusion was found throughout the entire symbiotic zone (zone III) of the root nodule and was also visible in the infection zone (zone II). This could be confirmed by quantitative measurements (Table 2). To test whether the nitrogen status of the bacteroids influences *glnD* expression, a *fixC* mutant, which displays a strict Fix⁻ phenotype, was included in this study and the same pattern of expression was detected (data not shown). This demonstrates that the symbiotic expression of *glnD* is not dependent on nitrogen fixation activity and thus most probably independent of the nitrogen status also in symbiosis.

At present, we can only speculate about the function of *glnD* expression in symbiosis. Since *glnII* (GSII) is switched off in the root nodule, GSI is probably the only glutamine synthetase present in bacteroids (Shatters *et al.*, 1989; Patriarca *et al.*, 1992; Amar *et al.*, 1994; Espin *et al.*, 1994). Therefore, one could hypothesize that GlnD could play a role in the regulation pathway leading to GSI adenylation, i.e. inactivation in symbiosis. However, for *S. meliloti* it was reported that GSI adenylation is not essential for an effective symbiosis (Arcondeguy *et al.*, 1996). On the other hand, it has been shown that *Medicago sativa* plants inoculated with a *S. meliloti* mutant unable to synthesize GlnB (P_{II}) are nitrogen-starved and the same effect was also observed with a mutated *glnB* gene encoding a P_{II} protein that could no longer be uridylylated (Arcondeguy *et al.*, 1997). Therefore, in this system, expression and uridylation of P_{II} by the GlnD protein is required for the establishment of a functional symbiosis. Such nitrogen starvation symptoms were not observed for *V. hirsuta* plants inoculated with the *R. leguminosarum* bv. *viciae glnD* mutant (this work), but in contrast to what was previously published by Amar *et al.* (1994), the *R. leguminosarum* bv. *viciae glnB* mutant shows only a 10% residual nitrogen fixation activity (R. Defez, unpublished results). Therefore, the role of the *R. leguminosarum* bv. *viciae* GlnB and GlnD proteins in symbiosis remains unclear and future work is needed to

elucidate the nitrogen regulation pathway operating under symbiotic conditions.

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