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glnD and *mviN* Are Genes of an Essential Operon in *Sinorhizobium meliloti*

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To evaluate the role of uridylyl-transferase, the *Sinorhizobium meliloti glnD* gene was isolated by heterologous complementation in *Azotobacter vinelandii*. The *glnD* gene is cotranscribed with a gene homologous to *Salmonella mviN*. *glnD1*::Ω or *mviN1*::Ω mutants could not be isolated by a powerful sucrose counterselection procedure unless a complementing cosmid was provided, indicating that *glnD* and *mviN* are members of an indispensable operon in *S. meliloti*.

Sinorhizobium meliloti forms a symbiosis with *Medicago sativa* (alfalfa) in which nitrogen is fixed by the bacteria and released to the plant in exchange for photosynthates. After infection of plants, bacterial cells differentiate into bacteroids contained within plant membrane-enclosed organelles, symbiosomes, located within root nodules. Establishment of a successful symbiosis involves a shift in bacterial metabolism from assimilation of ammonia to export of nitrogenous compounds to the host plant. Therefore, ascertaining the features regulating this particular metabolic switch may provide valuable insight into symbiotic nitrogen fixation. As in many other bacteria, *S. meliloti* assimilates ammonia through the glutamine synthetase (GS)/glutamate synthase cycle. Unusually, members of the *Rhizobiaceae* carry three genes encoding isoforms of GS at separate loci (8). The major enzyme, GSI, is similar to GS of the enteric bacteria and is susceptible to posttranslational adenylylation, which reduces the rate of ammonia assimilation in vivo (1). As in enteric bacteria, a specialized protein called P_{II} regulates the level of GSI adenylylation (2). In *Escherichia coli*, a model organism for studies on nitrogen regulation, the interactions of P_{II} with its targets depend on the uridylylation state of P_{II}, which responds to intracellular concentrations of the key metabolites glutamine and α-ketoglutarate (for current reviews, see references 10 and 12). Depletion of glutamine is sensed by the GlnD protein (9), which carries uridylyl-transferase/uridylyl-removing (UTase) activities, resulting in uridylylation of P_{II} (10).

The role of P_{II} in *S. meliloti* was previously examined by construction of two alleles of the corresponding *glnB* gene: Δ*glnB10*, a nonpolar null mutation, and *glnBP5*, a second allele that encodes a protein altered at the site of uridylylation. With respect to symbiosis, P_{II} was required to efficiently transfer fixed nitrogen to the plant but not for nitrogenase expression (2). Predictions were that *glnD* mutants would exhibit phenotypes similar to *glnBP5* mutants; however, this is complicated by the occurrence of multiple P_{II}-like proteins in *S. meliloti* (D. Kahn and P. Rudnick, unpublished data) as in many other organisms (reviewed in reference 12). Therefore, the focus of this work was to directly address the role of the nitrogen-sensing UTase in the regulation of P_{II} and its homologues in *S. meliloti*. We report here the cloning, sequencing, and mutagenesis of the *S. meliloti glnD* gene as well as evidence that this region of the chromosome is essential.

Cloning and sequence analysis of the *glnD* region of the chromosome. To isolate a library clone encoding a functional UTase, a pLAFR3 genomic library was mobilized into two Nif$^-$ *glnD* strains of *Azotobacter vinelandii*, MV17 (16) and MV71 (5) (Table 1). Several cosmids were isolated that complemented MV17 and MV71 by restoring their ability to fix nitrogen on N-free Burk's agar medium (11). One complementing cosmid, pTA30, contained a single central *Hin*dIII site within the insert. One half of the insert of pTA30 was cloned as a *Hin*dIII fragment into pBluescriptII KS+ to form plasmid pPR604. This 13-kb fragment was then cloned into the broad-host-range vector pJRD215 to form pPR608 and mobilized into *Azotobacter vinelandii* strain MV71 by triparental mating. Neither this plasmid nor pPR601, containing the remaining portion, complemented this strain for growth on N_2 (Fig. 1). Indeed, sequencing of the *Hin*dIII junction revealed that this restriction site cleaves the *glnD* gene. The sequence of a 5.2-kb *Pst*I fragment containing the *Hin*dIII site was determined, showing that the *S. meliloti glnD* gene is located between *mutS*, encoding DNA mismatch repair enzymes in many organisms, and a gene homologous to *mviN* from *Salmonella enterica* serovar Typhimurium (hereafter called *mviN*) (4). Sequence similarities of MutS, GlnD, and MviN are, respectively, 40, 32, and 32% amino acid identity with the corresponding proteins from *E. coli*. An ATGA sequence overlap between the *glnD* TGA stop codon and the *mviN* ATG start codon suggests that *glnD* and *mviN* are translationally coupled and part of the same operon.

Complementation of an *A. vinelandii glnD* mutant with *S. meliloti glnD*. The 5.2-kb *Pst*I fragment of pPR602, containing partial open reading frames for *mutS* and *mviN* and the complete *glnD* gene, was subcloned into pJRD215 to give plasmid pPR611 (Fig. 1). This plasmid was conjugated into *A. vinelandii glnD* strain MV71. Transconjugants harboring pPR611 had their

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Phenotype or characteristic	Reference
A. vinelandii			
MV17	*nfrX16*::Tn*5*[a]	Nif sup? (fast growing)	16
MV71	*glnD1*::Ω *glnY1*	Nif GS[c]	5
S. meliloti			
GMI708	Strain 2011 derivative	Rif[r]	3
GMI3105	*glnA1*	GSI unadenylylatable	1
Plasmids			
pBluescriptII KS+		Amp[r]; cloning vector	Stratagene
pGEM-T Easy		Amp[r]; cloning vector self-ligated at *Eco*RV	Promega
pHP45Ω		Amp[r] Sp[r]; carries Ω interposon	14
pJRD215		IncQ broad-host-range vector; Kan[r] Sm[r]	7
pJQ200KS		Gm[r]; *sacB* mobilizable cloning vector	15
pLAFR3		IncP cosmid; Tet[r]	13
pPR601		Derivative of pTA30 with *Hin*dIII fragment deleted	This study
pPR602		pBluescript KS+ with 5.2-kb *Pst*I fragment	This study
pPR604		pBluescript KS+ with 13-kb *Hin*dIII fragment	This study
pPR608		pJRD215 with 13-kb *Hin*dIII fragment	This study
pPR611		pJRD215 with 5.2-kb *Cla*I/*Sac*I fragment; *glnD*$^+$	This study
pPR612		pGEM-T Easy carrying 5.2-kb *Pst*I fragment	This study
pPR613		pPR612 with Ω inserted at *Hin*dIII; *glnD1*::Ω	This study
pPR614		pJQ200KS carrying *glnD1*::Ω	This study
pPR615		pBluescriptII KS+ with 3.6-kb *Hin*dIII/*Sac*I fragment	This study
pPR617		pPR615 with Ω inserted at *Pst*I blunt end; *mviN1*::Ω	This study
pPR618		pJQ200KS with *mviN1*::Ω	This study
pTA30		Library clone with *mutS glnD mviN*	This study

[a] *nfrX* was the previous designation for *A. vinelandii glnD* (6).

ability to grow on N-free medium restored, indicating that *S. meliloti glnD* was the only gene required by this strain to restore growth on this medium and that *glnD* encodes a functional UTase.

Mutagenesis of *glnD*. To examine its role, the *glnD* gene was mutagenized by insertion of an Ω element from pHP45Ω conferring resistance to spectinomycin into the central *Hin*dIII site of pPR612, creating a polar mutation in *glnD* (pPR613). The *Pst*I fragment from pPR613, containing the mutation, was cloned into pJQ200KS, a vector carrying gentamicin resistance and conferring sensitivity to sucrose (*sacB*), to form plasmid pPR614 (15). pPR614 cannot be replicated in *S. meliloti* and is a suicide vector. This plasmid was mobilized into wild-type *S. meliloti* GMI708 by conjugation followed by selection on spectinomycin, which will select for strains in which homologous recombination at the target locus has occurred. A single Sp[r] colony was propagated overnight without antibiotics to facilitate excision of vector sequences and replacement of the wild-type allele with *glnD1*::Ω by homologous recombination (Fig. 2). The overnight culture was plated on medium containing spectinomycin and sucrose. Surprisingly, 88% of the Suc[r] Sp[r] isolates were also Gm[r], indicating that the vector had not excised. To examine the structure of the chromosome targeted by the mutagenesis, primers were designed to amplify a 400-bp product from wild-type *glnD* and a 2.4-kb product from *glnD1*::Ω (Fig. 3, lanes 1 to 3). Long-range PCR analysis of the Suc[r] Sp[r] Gm[r] isolates (lanes 6 to 8) generated products identical to Suc[s] Sp[r] Gm[r] isolates, which have an integrated copy of pPR614 (lanes 4 and 5). This indicates a strong selective pressure driving Suc[r] by mutation in *sacB* rather than by recombination that would excise *glnD*. The remaining 12% Suc[r] Sp[r] Gm[s] clones also retained both mutant and wild-type copies of *glnD* (Fig. 3, lane 9), similar to the above-described isolates. In an experiment reported by Quandt and Hynes in which a construct carrying a similar amount of homologous DNA flanking the cassette was used, only 0 to 2% Gm[r] colonies were isolated following selection on sucrose and spectinomycin compared to 88% for this experiment (15). Our inability to isolate allelic replacement null mutants of *glnD* prompted us to investigate further the possibility that *glnD*, or downstream genes occurring in the same transcriptional unit, may be essential.

In the next experiment, a single clone was selected on gentamicin after conjugation of pPR614. This isolate was then grown overnight without antibiotics and plated on sucrose as the sole selective pressure. Figure 2 illustrates possible events stemming from integration of pPR614 and outcomes. In this experiment, we predicted that resolution of the single crossover would occur at similar frequencies on either side of the

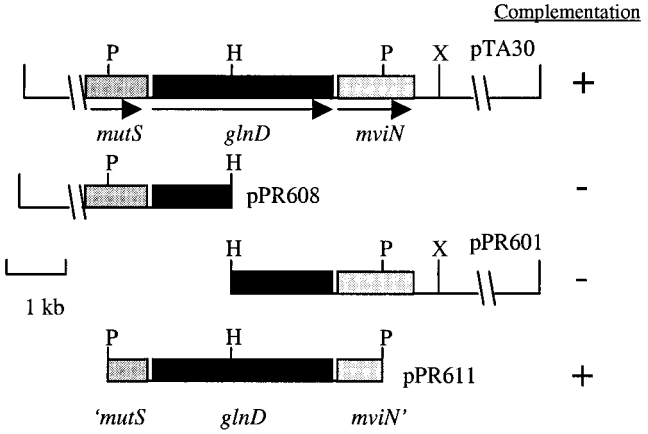

FIG. 1. Restriction map of the cloned *glnD* region of the chromosome. P, *Pst*I; H, *Hin*dIII; X, *Xho*I. The column on the right indicates whether the corresponding plasmid complements *A. vinelandii glnD*.

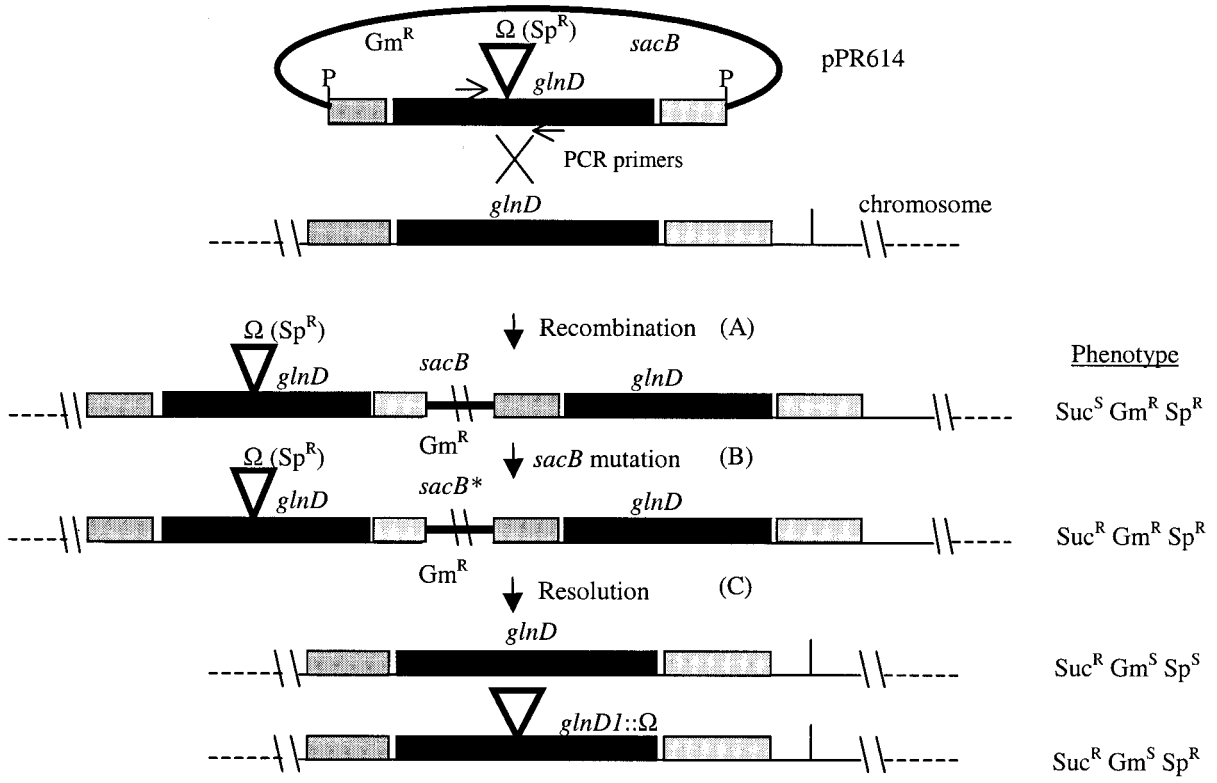

FIG. 2. Schematic representation of events following conjugation of plasmid pPR614 into *S. meliloti*. (A) Following homologous recombination, the corresponding strain would contain one copy of each of *glnD*, *glnD1*::Ω, *sacB*, and the gentamicin resistance gene, *aacC1*, giving the indicated phenotype. (B) Loss of *sacB* (*sacB** indicates a mutated form which does not confer sensitivity to sucrose) by mutation would allow both copies of *glnD* to reside on the chromosome along with *aacC1*. (C) Resolution of the single recombination resulting in excision of the vector might have one of the two possible outcomes illustrated. The second, resulting in the loss of wild-type *glnD*, was never observed without another source of the target gene. The locations of the PCR primers used to generate the products seen in Fig. 3 are shown for reference.

cassette if no pressure was applied for maintenance of the wild-type target gene. For example, considering an equal amount of flanking DNA on either side of the cassette, sucrose-resistant isolates should arise at a frequency of 50% Suc^r Sp^s, 50% Suc^r Sp^r, and 0% Suc^r Gm^r. Very few, if any, should be Gm^r, having occurred by presumptive mutations in *sacB*. However, in contrast to this prediction, between 95 and 99% of the Suc^r isolates were Sp^s, indicating preferential loss of *glnD1*::Ω and restoration of the wild-type copy by recombination. Of the approximate 5% Sp^r isolates, all were Gm^r, indicating retention of vector sequences (Table 2). These results demonstrate strong selective pressure to maintain the wild-type locus.

In other organisms, GlnD regulates the activity of GS through P_{II}, which also seems to be the case for *S. meliloti*, since cells that encode a uridylylation-deficient P_{II} are unable to deadenylylate GSI (2). Interestingly, *A. vinelandii glnD* null mutations are lethal in the absence of a suppressor mutation (5, 6) because GS is constitutively inactivated by adenylylation; GS is an essential enzyme in this organism probably because the organism lacks other means to assimilate ammonia, as well as a transport mechanism for glutamine (17). We therefore tested the possibility that *S. meliloti glnD* mutants could not be isolated because of a requirement for glutamine, either by supplementing all media with 0.4% glutamine, a concentration sufficient to allow growth of GS mutants (data not shown) or by using the *glnA1* strain GMI3105, in which GSI is insensitive to adenylylation (Table 2) (1). In both cases, Suc^r Sp^r Gm^s colonies could not be isolated which lacked the wild-type copy of *glnD*, further supporting an essential role for the *glnD* locus.

mviN mutagenesis. In parallel, an *mviN* mutagenesis construct, pPR617, was made by insertion of a *Sma*I-digested Ω element into the single blunt-ended *Pst*I site of *mviN*, carried

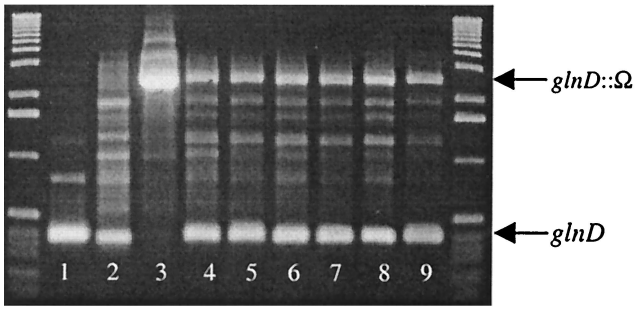

FIG. 3. Long-range PCR amplification of a central fragment of *glnD* or *glnD1*::Ω from various sources during mutagenesis. A single isolate was grown overnight without antibiotics and plated on Luria-Bertani medium plus 5% sucrose and spectinomycin (100 μg/ml). Isolates were then screened for resistance to gentamicin. PCR products were amplified from single colonies unless otherwise indicated. Lane 1, pPR602 (plasmid DNA); lane 2, GMI708; lane 3, pPR614 (plasmid DNA); lanes 4 and 5, Suc^s Sp^r Gm^r isolates; lanes 6 to 8, Suc^r Sp^r Gm^r isolates; lane 9, rare Suc^r Sp^r Gm^s isolate which unexpectedly retained wild-type *glnD*. Unlabeled lanes contain the 1-kb DNA ladder (Life Technologies).

TABLE 2. Gene replacement analysis of the *glnD-mviN* region of the *S. meliloti* chromosome

Strain	Plasmid(s)	Primary selection[a]	Secondary selection[b]	Fraction[c] Suc^r Sp^r	Fraction[d] of Suc^r Sp^r that are Gm^r
GMI708	pPR614 (*glnD1*::Ω)	Gm	Suc	5/100	5/5
GMI3105	pPR614	Gm	Suc	6/100	6/6
GMI708	pPR618 (*mviN1*::Ω)	Gm	Suc	7/100	7/7
Mutagenesis of strains harboring complementing plasmids					
GMI708	pPR614, pPR611 ($glnD^+$)	Gm, Km	Suc, Km	58/100	58/58
GMI708	pPR614, pTA30 ($glnD^+$ $mviN^+$)	Gm, Tet	Suc, Tet	33/100	0/33
GMI3105	pPR614, pPR611	Gm, Km	Suc, Km	22/100	22/22
GMI3105	pPR614, pTA30	Gm, Tet	Suc, Tet	31/69	1/31
GMI708	pPR618, pPR611	Gm, Km	Suc, Km	94/100	33/94
GMI708	pPR618, pTA30	Gm, Tet	Suc, Tet	24/99	0/24
GMI3105	pPR618, pPR611	Gm, Km	Suc, Km	65/100	65/65
GMI3105	pPR618, pTA30	Gm, Tet	Suc, Tet	10/76	0/10

[a] Indicates original selection of transconjugants.
[b] Selection after growth of transconjugants overnight in the absence of antibiotics.
[c] Represents fraction of Suc^r isolates that have retained Sp^r from *glnD1*::Ω or *mviN1*::Ω.
[d] Fraction of Sp^r isolates that have retained Gm^r encoded on the vector.

on plasmid pPR615. The entire 4.6-kb *Xho*I fragment containing *mviN1*::Ω was subcloned into pJQ200KS to form plasmid pPR618. This plasmid was used exactly as pPR614 was (Fig. 2) except for inactivation of *mviN*. Surprisingly, there was a strong bias towards maintenance of wild-type *mviN* as well (Table 2).

glnD and *mviN* mutants can be readily isolated with a cosmid carrying multiple genes in *trans*. To support our claim that the *glnD-mviN* operon may encode essential gene products in *S. meliloti*, complementing plasmids were introduced into a strain carrying either pPR614 or pPR618 integrated on the chromosome. For these experiments, plasmid pPR611 and cosmid pTA30 were used as a source of the wild-type gene(s). The mutagenesis was carried out as described above with the exception that all plates and cultures were supplemented with the appropriate antibiotic for maintenance of the complementing plasmid. Mutagenesis of *glnD* carrying pPR611 gave no isolates that were Suc^r Sp^r Gm^s, indicating that the mutation in *glnD* has polar effects on essential linked genes and that *glnD* and *mviN* belong to the same operon (Table 2). In contrast, *glnD* or *mviN* could be easily replaced by gene replacement if pTA30 was in the background. For the *glnD* mutagenesis, approximately 33% of the Suc^r isolates were Sp^r and Gm^s and 0% of the Sp^r isolates were Gm^r. This is as predicted when there is little or no selective pressure to maintain the wild-type target gene. Mutagenesis of *mviN* in the presence of pTA30 gave similar results: 45% of the Suc^r isolates were Sp^r Gm^s and 1% were Sp^r Gm^r. We conclude that *glnD* and *mviN* are members of an essential operon in *S. meliloti.*

Nucleotide sequence accession number. The sequence of the 5.2-kb *Pst*I fragment containing the *Hin*dIII site was deposited in GenBank under accession no. AF227730.

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