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Inhibition of glutamine synthetase II expression by the product of the *gstI* gene

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Summary

We report the identification of a previously unrecognized gene that is involved in the regulation of the *Rhizobium leguminosarum glnII* (glutamine synthetase II) gene. This gene, which is situated immediately upstream of *glnII*, was identified by means of a deletion/complementation analysis performed in the heterologous background of *Klebsiella pneumoniae*. It has been designated *gstI* (glutamine synthetase translational Inhibitor) because, when a complete version of *gstI* is present, it is possible to detect *glnII*-specific mRNA, but neither GSII activity nor GSII protein. The *gstI* gene encodes a small (63 amino acids) protein, which acts *in cis* or *in trans* with respect to *glnII* and is transcribed divergently with respect to *glnII* from a promoter that was found to be strongly repressed by the nitrogen transcriptional regulator NtrC. A mutated version of GstI lacking the last 14 amino acids completely lost its capacity to repress *glnII* expression. Our results indicate that *gstI* mediates the translation inhibition of *glnII* mRNA and, based on *in silico* analyses, a mechanism for GstI action is proposed.

Introduction

Rhizobium leguminosarum biovar *viciae* (hereafter *R. leguminosarum*) expresses at least two different glutamine synthetase (GS) enzymes: GSI and GSII. GSI, encoded by the *glnA* gene, is a dodecameric enzyme, similar to the GS enzymes present in enteric bacteria (for a review, see Merrick and Edwards, 1995). In *R. leguminosarum*, GSI activity is regulated post-translationally by an adenylylation–deadenylylation mechanism in response to the nitrogen status of the cells (Rossi *et al*., 1989). The *glnA* gene, which is part of the *glnBA* operon, is expressed either from its own −35/−10 promoter, *glnA*p, or from a −24/−12 (σ^{54}-dependent) nitrogen-regulated promoter (*glnBA*p) situated immediately upstream of *glnB* (Chiurazzi and Iaccarino, 1990). GSII, encoded by the *glnII* gene, is a heat-sensitive (relative to GSI) octameric enzyme (Manco *et al*., 1992). The *glnII* gene is transcribed as a monocistronic unit from a single −24/−12 (σ^{54}-dependent) promoter, which is induced when *R. leguminosarum* is grown under nitrogen-limiting conditions (Patriarca *et al*., 1992). Promoter deletion analysis identified a *cis*-acting sequence (between positions −316 and −219 with respect to the initiation site) that is essential to promote *glnII* transcription. The absence of transcription in an *ntrC* mutant strain indicates that this *cis*-acting sequence acts through the binding of either NtrC or other *trans*-acting factor(s) that are absent or inactive in an *ntrC*$^-$ strain (Patriarca *et al*., 1993). Binding of NtrC to this DNA fragment has subsequently been demonstrated by gel retardation assays (Patriarca *et al*., 1994). Based on these data, expression of *glnII* appears to be regulated only at the transcriptional level by a nitrogen regulation (Ntr) system, similar to that acting in enteric bacteria to control the expression of genes involved in nitrogen metabolism (Merrick and Edwards, 1995).

Other observations, however, suggest that *glnII* expression in members of the Rhizobiaceae might also be regulated by an as yet unknown mechanism of post-transcriptional control. A quantitative analysis showed a ratio of 10:1 for the amount of *glnII*-specific transcripts detected in *R. leguminosarum* grown with either glutamate or KNO_3 compared with NH_4Cl as the sole nitrogen source (Patriarca *et al*., 1992). Likewise, β-galactosidase activity values obtained using a *glnII*p–*lacZ* transcriptional fusion indicated that the activity of *glnII*p changes in response to the nitrogen source by a similar ratio (Patriarca *et al*., 1992). In contrast, GSII activity varies more than 200-fold in *R. leguminosarum* grown with glutamate versus NH_4Cl, and it has been proposed that the low level (less than 5 units) of GSII activity in NH_4Cl-grown cells could result from post-translational inactivation of the enzyme (Rossi *et al*., 1989). However, using an anti-GSII antiserum, the GSII protein was not detected in crude extracts of *R. leguminosarum* prepared from cells grown with NH_4Cl as nitrogen source (Manco *et al*., 1992). Thus,

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in *R. leguminosarum*, the *glnII* expression pattern appeared to be different when analysed at the RNA or protein level. The absence of GSII protein was also observed in crude extracts of *Rhizobium etli* grown with NH_4Cl as nitrogen source (Patriarca *et al.*, 1993), whereas significant promoter activity was measured under the same growth conditions (Patriarca *et al.*, 1992). Moreover, in N_2-fixing bacteroids of *Bradyrhizobium japonicum*, specific *glnII* transcripts were detected, but GSII activity was undetectable (Martin *et al.*, 1988). The GSII protein was also not detected in crude extracts of soybean nodules (elicited by *B. japonicum*) using an anti-GSII antiserum (Shatters *et al.*, 1989). The observations on GSII expression in free-living cells and in bacteroids may be correlated, as the addition of NH_4Cl to a bacterial culture may represent (in some respects) a similar situation to that taking place when nitrogenase becomes derepressed in bacteroids during the symbiotic interaction.

We now report the identification of a previously unknown gene involved in the regulation of *glnII* expression. A DNA fragment of 460 bp covering the *glnII* upstream region was identified as the minimal region coding for a *trans*-acting factor able to inhibit *glnII* expression. When this product is expressed, *glnII* transcripts but no GSII activity or GSII protein were detected; hence, we have designated this locus *gstI* (glutamine synthetase translation Inhibitor). Our results argue for an involvement of the GstI protein in *glnII* mRNA post-transcriptional regulation.

Results

Complementation experiments

These studies were initiated as a consequence of a complementation analysis that was originally designed to study the expression of the *R. leguminosarum glnII* gene in a heterologous background, namely a *glnA* (GSI) mutant of *Klebsiella pneumoniae*. Two plasmids containing the *glnII* gene of *R. leguminosarum* on DNA fragments of different sizes (see Fig. 1A) were tested for their ability to suppress the glutamine auxotrophy phenotype of the *K. pneumoniae glnA* mutant strain UNF1827. The latter is unable to grow on glutamate-containing NFDM minimal medium unless supplemented with glutamine (Espin *et al.*, 1982). Complementation was observed with one plasmid, pAR18, but a second plasmid, pAD1, which also included a complete version of the *glnII* gene (Fig. 1A), failed to complement (Fig. 1B). Plasmid pAD1 is a pBR329 derivative (with pMB1 replicon) and is therefore maintained in the cell at about 25–80 copies per chromosome equivalent, whereas pAR18 (which is a pACYC177 derivative with p15A replicon) is maintained at about 10 copies per cell. Moreover, strain UNF1827(pAD1) could grow either on rich medium (TY) or in glutamate-containing NFDM medium when supplemented with glutamine, and the parent wild-type ($glnA^+$) *K. pneumoniae* strain, UNF122, carrying pAD1 could grow on NFDM with glutamate as the sole nitrogen source. These results were unexpected, as

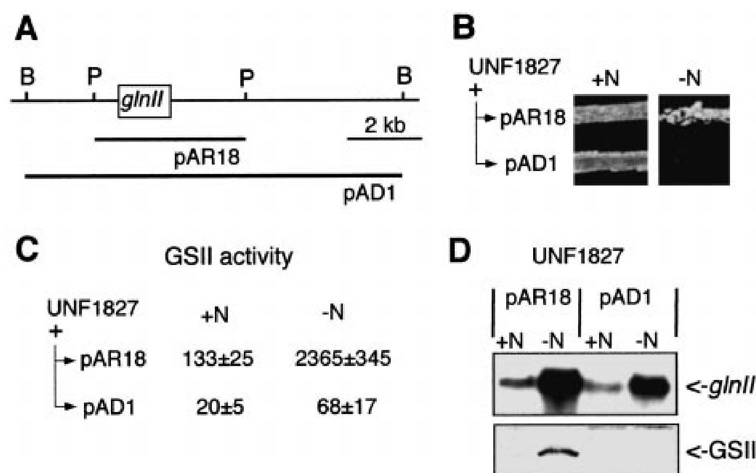


Fig. 1. A. Physical map of the *glnII* DNA region. The insert of plasmids pAD1 and pAR18 is indicated. Restriction enzyme recognition sites are: B, *Bam*HI; P, *Pvu*II.
B. *K. pneumoniae* strain UNF1827 ($glnA^-$) was transformed with either plasmid pAR18 or pAD1, and the resulting strains were grown overnight in TY medium at 37°C. Cells were harvested by centrifugation, washed twice in N-free NFDM, streaked on plates of NFDM containing glutamine plus NH_4Cl (+N, N-excess conditions) or glutamate (−N, N-limiting conditions) as nitrogen source. Growth was observed 24–48 h after incubation at 37°C.
C. GSII activity. Strains UNF1827(pAR18) or UNF1827(pAD1) were grown under N-excess conditions (to 0.6 OD_{590}) and shifted (30 min) to N-limiting conditions. Crude extracts were prepared, and the GSII transferase activity (nmol of γ-glutamylhydroxamate min^{-1} mg^{-1} protein) was determined as described in *Experimental procedures*.
D. Top: Northern blot. RNA was purified from bacteria grown as in (C), and the blot was performed and hybridized as described in *Experimental procedures*. Bottom: crude extracts prepared from cells grown as in (C) were subjected to SDS–PAGE (100 μg in each lane), transferred to a nitrocellulose sheet and probed with affinity-purified antibodies prepared against GSII protein.

it has previously been shown that the *R. leguminosarum glnII* promoter is not only active in *K. pneumoniae*, but is also nitrogen regulated and dependent on specific transcriptional factors (σ^{54} and NtrC) of the host (Patriarca *et al*., 1992).

Glutamine synthetase II activity and immunoblotting experiments

To test GSII activity, cells of strain UNF1827(pAR18) and UNF1827(pAD1) were grown under N-excess conditions and then shifted to N-limiting conditions. Aliquots of cells were harvested, crude extracts were prepared and the GSII transferase activity was determined (Fig. 1C). UNF1827(pAR18) had a GSII activity of around 2300 units, which, like the GSII activity of *R. leguminosarum*, was heat labile. Moreover, GSII activity was strongly regulated by the nitrogen source, being 18-fold higher under N-limiting conditions (30 min after transfer of cells into NFDM–glutamate) than under N-excess conditions (glutamine plus NH_4Cl). In contrast, UNF1827(pAD1) showed low intracellular levels (less than 70 units) of GSII activity under both N-limiting and N-excess conditions.

To test the presence of the GSII protein, we used immunoblot assays with an antiserum raised against purified GSII from *R. leguminosarum* (Manco *et al*., 1992) (Fig. 1D). The GSII protein was detected in a crude extract of strain UNF1827(pAR18) prepared from cells grown under both N-excess and N-limiting conditions. On the contrary, the protein was never detected in a crude extract of strain UNF1827(pAD1), even if prepared from cells grown under N-limiting conditions. In the latter case, no cross-reacting material (also with a higher or a lower molecular weight than GSII) was detected, even when the protein loading was increased 10-fold (up to 1 mg; data not shown), thus excluding the presence of truncated (or altered) versions of GSII protein. Based on these results and data for GSII activity (Fig. 1C), we conclude that, in strain UNF1827(pAD1), GSII protein is not expressed and, consequently, the failure of this strain to grow in NFDM–glutamate medium is simply a result of the absence of GSII.

Transcription of the glnII *gene from pAD1*

To detect the presence of *glnII* mRNA, a Northern blot analysis was performed (Fig. 1D). A ^{32}P-labelled DNA fragment obtained from plasmid pAR23 (*glnII* coding region; Patriarca *et al*., 1992) was used as a probe. RNA was purified from strains UNF1827(pAR18) and UNF1827 (pAD1), which had been grown under N-excess and shifted (30 min) to N-limiting conditions. A single band of about 1.3 kb was detected in RNA isolated from both strains (Fig. 1D).

Although the *glnII* transcripts apparently had the correct size, the 5′ end of the *glnII* mRNA in *K. pneumoniae* was confirmed by RNase protection assays (not shown). RNA samples (10 μg) purified from strains UNF1827, UNF1827(pAR18) and UNF1827(pAD1) were hybridized to a ^{32}P-labelled antisense RNA generated from plasmid pAR28 [a 380 bp DNA fragment extending from position −219(*Hin*dIII) to +161(*Eco*RI) with respect to the *glnII* transcription start site (see Fig. 4D) previously identified in *R. leguminosarum* (Patriarca *et al*., 1992)]. A protected fragment of 161 nucleotides was observed with RNA from both UNF1827(pAR18) and UNF1827(pAD1). No protected fragments were observed when RNA from strain UNF1827 was used or when a ^{32}P-labelled sense RNA generated from plasmid pAR28 (*Eco*RI-digested) was used as probe (data not shown).

Thus, we conclude that the transcription start is identical to that occurring in the homologous background and that the undetectable level of GSII protein in UNF1827(pAD1) is not caused by the absence of *glnII* transcription under the growth conditions tested.

A trans*-acting factor is encoded by pAD1*

To test the effect of pAD1 on the expression of *glnII* from pAR18, UNF1827 was co-transformed with both plasmids (Fig. 2). The resulting strain, UNF1827(pAR18/pAD1), was unable to grow on NFDM–glutamate in the absence of glutamine (Fig. 2A) and, although *glnII*-specific transcripts were present, both GSII activity and GSII protein were almost undetectable (data not shown).

To exclude a possible effect of pAD1 on *glnII* transcription, a β-galactosidase assay was performed using plasmid pAR35, which carries a *glnII*p–*lacZ* transcriptional fusion (Patriarca *et al*., 1992). The parental *K. pneumoniae* strain UNF122 was transformed with pMP220 (vector alone; Spaink *et al*., 1987) or with pAR35 either alone or together with pAD1, and the resultant strains were assayed for β-galactosidase activity. UNF122(pAR35) had an activity of between 600 and 700 units when grown under N-limiting conditions, but a much lower level of activity (47 ± 3.2 units) when grown in N-excess (Table 1). The control strain UNF122(pMP220) had only low levels of activity (between 10 and 40 units) under all conditions tested. Therefore, as shown previously (Patriarca *et al*., 1992), the *glnII* promoter is active and regulated by the nitrogen conditions of growth. Moreover, as judged by the levels of activity measured with strain UNF122 (pAR35/pAD1), *glnII* promoter activity was not affected by the presence of pAD1 in bacteria grown in NFDM–glutamine and was only slightly affected (two times lower) in bacteria grown in NFDM–glutamate. Taken together, these data indicate that a *trans*-acting factor able to inhibit the *glnII* expression is encoded by pAD1.

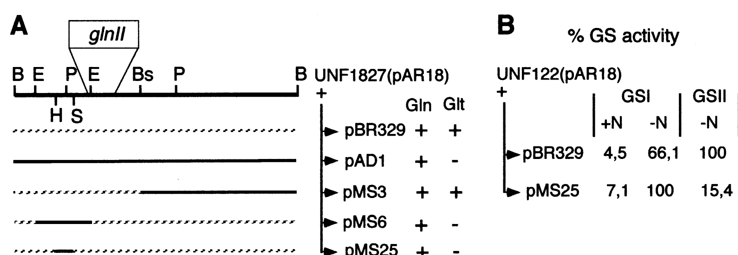


Fig. 2. A. Deletion/complementation analysis of strain UNF1827(*glnA*⁻) of *K. pneumoniae*. Strain UNF1827(pAR18) was transformed with the plasmids represented schematically on the left. Cells from the resulting strains were washed and streaked on NFDM containing either glutamine (Gln) or glutamate (Glt) as nitrogen source.
B. The parental strain UNF122 of *K. pneumoniae* was co-transformed with plasmid pAR18 and either pBR329 (vector alone) or pMS25. The resulting strains were grown under nitrogen-excess (+N) conditions and then shifted for 1 h to N-limiting conditions (–N). The values of GS activity reported are expressed as a percentage of the maximal activity measured (560 units for GSII and 212 units for GSI).

Table 1. Effect of pAD1 on the *glnII* promoter.

Strain	β-Galactosidase activity of cultures grown in		
	Gln	Glt	Gln + NH_4^+
UNF122(pMP220)	13 ± 3	11 ± 4	37 ± 5
UNF122(pAR35)	608 ± 58	671 ± 65	47 ± 3
UNF122(pAR35/pAD1)	744 ± 86	300 ± 47	32 ± 6

Bacteria were assayed for β-galactosidase activity according to the method of Miller (1972). The cells were grown at 37°C to an OD_{590} value of 0.4 in NFDM containing glutamate (Glt; 1 g l^{-1}) or glutamine (Gln + NH_4^+ 0.2 g l^{-1}) plus NH_4Cl (1 g l^{-1}) as nitrogen source. The values reported are the average of least three independent assays, and errors (±) are indicated. Plasmid pAR35 contains a *glnII*p–*lacZ* fusion and has been described elsewhere (Patriarca *et al.*, 1992).

Mapping of the inhibitory locus

To identify the DNA region of pAD1 responsible for the lack of *glnII* expression in strain UNF1827, a deletion analysis of the plasmid was performed. Defined restriction fragments from pAD1 were subcloned into pBR329. The resulting plasmids (Fig. 2A) were tested for their ability to prevent the growth of UNF1827 on a glutamine-free medium (NFDM–glutamate) when co-transformed with pAR18 (which is a pACYC177 derivative). The results of these experiments may be summarized as follows: plasmids carrying the DNA region located downstream of *glnII* (such as pMS3) showed no effect on the ability of pAR18 to complement the *glnA* mutation of UNF1827, allowing us to exclude the presence of an inhibitor of *glnII* expression in this region. Plasmids carrying the *glnII* upstream region (such as pMS6), including the region around the *Pvu*II restriction site, prevent the growth of UNF1827(pAR18) on NFDM–glutamate. Finally, plasmid pMS25 (460 bp *Sph*I–*Hin*dIII DNA fragment) was able to inhibit the expression of *glnII* from pAR18. Furthermore, in the wild-type *K. pneumoniae* strain UNF122, the induction of GSII from pAR18, but not the induction of the endogenous GSI, was strongly prevented (> 80% inhibition) by the presence of pMS25 (Fig. 2B). We conclude that the DNA region immediately upstream of the *glnII* promoter codes for the inhibitor of *glnII* expression. This locus was termed *gstI* (glutamine synthetase translation Inhibitor).

Sequence and expression analysis of the gstI *locus*

To characterize the *gstI* locus, we determined the DNA sequence of the insert in plasmid pMS25 (Fig. 3), and sequence analysis of all six reading frames identified seven complete open reading frames (ORFs) with more than 30 codons. Transcriptional (Fig. 4A and C) and translational (Fig. 4B) analyses were then performed. RNase protection assays were used to test for the presence of specific transcripts, the direction of transcription and the transcriptional start site(s). RNA was prepared from both the *R. leguminosarum* wild type (strain LPR1105) and its *glnII*⁻ derivative (strain LPR1163) grown in N-excess (a chemically defined medium; RMM supplemented with glutamine and NH_4Cl). RNA samples were hybridized with ^{32}P-labelled RNA generated from plasmid pAR27 (Fig. 4D), containing a 275 bp DNA fragment spanning between positions −494 (*Pvu*II) and −219 (*Hin*dIII) with respect to the previously identified *glnII* transcriptional start site (Patriarca *et al.*, 1992). Riboprobes named P_1 and P_2 were synthesized from pAR27 digested with *Bam*HI or *Eco*RI, and used as templates for T7 and SP6 polymerase-mediated transcription respectively. When RNA from the parent strain was hybridized with probe P_1, three protected fragments of about 180 nucleotides were observed (Fig. 4A), whereas no protected fragments were observed using either RNA from the *glnII*⁻ strain or with probe P_2. These data indicate that *gstI* is transcribed in the opposite direction to *glnII* (Fig. 4D) and suggest that the promoter region is located at least 180 bp upstream of the *Pvu*II restriction site (see Fig. 3B).

The presence of a promoter region upstream of these putative transcription start sites was then investigated. The parental *R. leguminosarum* strain LPR1105 was

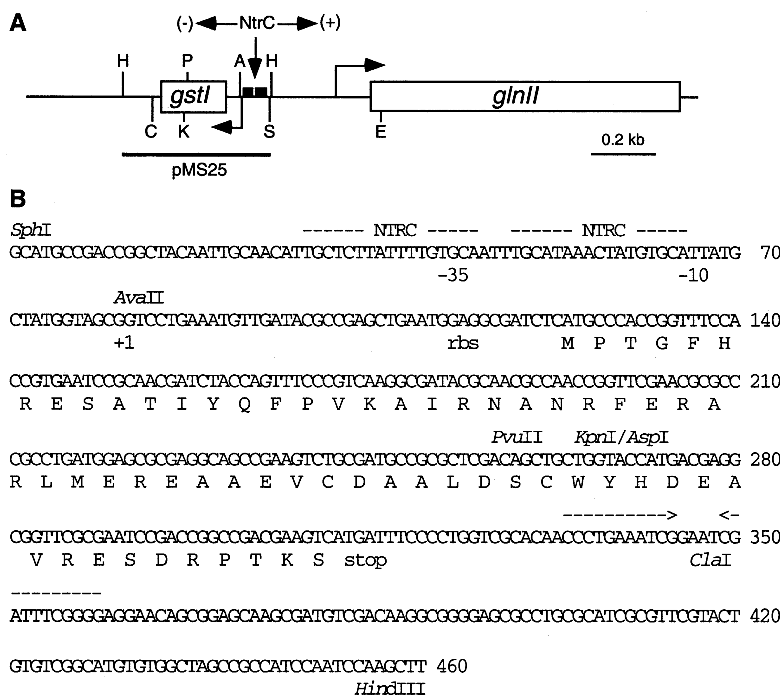
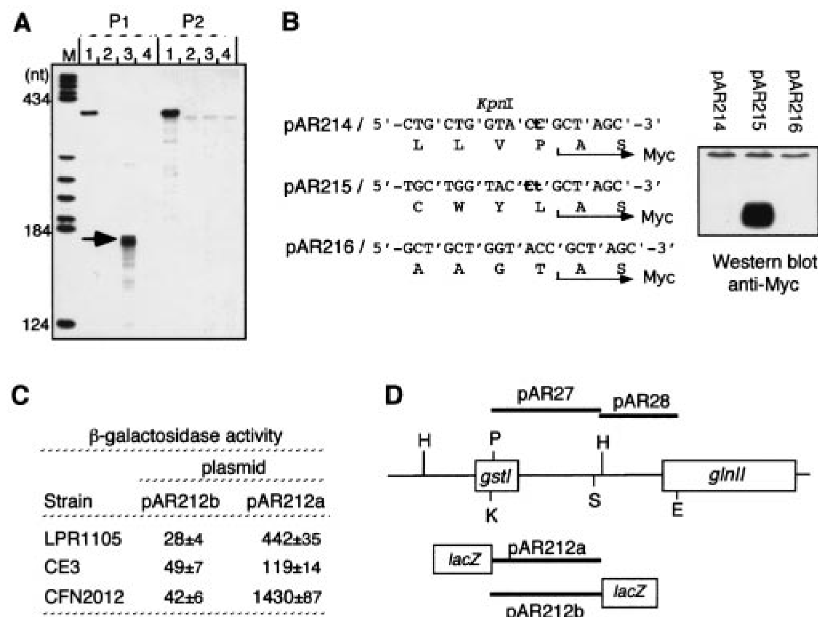


Fig. 3. A. Physical map of the *glnII* upstream DNA region. Restriction enzyme recognition sites are: A, *Ava*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII, K, *Kpn*I, P, *Pvu*II, S, *Sph*I. The insert of plasmid pSM25 (a 460 bp *Sph*I−*Hin*dIII fragment) is indicated.

B. DNA sequence of the *gstI* locus. The nucleotide sequence of the insert in pMS25 is shown. The translated sequence of GstI is shown in a single-letter code based on its presumed start codon. Stop codon is indicated by *stop*, and useful restriction enzyme recognition sites are indicated. The 5′ of *gstI* mRNA, a putative ribosome binding site (*rbs*), a putative Rho-independent transcriptional terminator (→ ←) and two NtrC binding sites are indicated. These sequence data have been submitted to DDBJ/EMBL/GenBank databases.



β-galactosidase activity		
	plasmid	
Strain	pAR212b	pAR212a
LPR1105	28±4	442±35
CE3	49±7	119±14
CFN2012	42±6	1430±87

Fig. 4. A. RNase protection analysis. The insert of the plasmid pAR27, a 275 bp *Hin*dIII−*Pvu*II fragment cloned in pGEM7Zf+, used to prepare the ^{32}P-labelled riboprobes (P_1 and P_2) is indicated (D). Riboprobes were annealed with RNA purified from either the wild-type strain LPR1105 of *R. leguminosarum* (lane 3) or a *glnII*$^-$ derivative (lane 4). Probes were annealed in the absence of RNA and treated with RNases (lane 2). Riboprobes alone (lanes 1) and DNA molecular weight markers (lane M) are also shown.

B. The nucleotide and the deduced amino acid sequence around the *Kpn*I site of plasmids pAR214, pAR215 and pAR216 are shown. Strain DH5α of *E. coli* was transformed with these plasmids and, from the resulting strains, crude extracts were prepared. SDS−PAGE and immunoblot staining were performed with anti-Myc monoclonal antibodies (see *Experimental procedures*).

C. The DNA fragments cloned into plasmids pAR212a and pAR212b (opposite orientation) are indicated in (D). Bacteria were assayed for β-galactosidase activity according to the method of Miller (1972). The cells were grown at 30°C in RMM containing glutamate (1 g l^{-1}) as nitrogen source to an OD_{590} of 0.4 and tested for β-galactosidase activity. The values reported are the average of least three independent assays, and errors (±) are indicated.

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transformed with plasmids carrying *lacZ* transcriptional fusions, pAR212a or pAR212b (see Fig. 4D), and the vector pMP220. Only low levels of activity (between 10 and 55 units) were detected with LPR1105(pAR212b) or LPR1105(pMP220), whereas LPR1105(pAR212a) had significant β-galactosidase activity (442 ± 35 units) (Fig. 4C). As two well-characterized NtrC binding sites are located in the promoter region (see Fig. 3), the promoter activity was also tested in a *R. etli ntrC* mutant, strain CFN2012. The promoter activity measured was 12-fold higher in CFN2012 (*ntrC*$^{-}$) compared with the wild-type *R. etli* strain CE3 (Fig. 4C).

Finally, to determine whether the *gstI* locus is translated, we constructed *gstI*–Myc fusions in all three frames at the *Kpn*I site (see Fig. 3 and *Experimental procedures*). DNA coding for a modified version of the double 9E10 epitope of Myc protein was amplified using plasmid pBSCmyc as a template and synthetic oligonucleotides as primers, and three amplified DNA fragments were cloned into *Kpn*I–*Cla*I-digested plasmid pAR206 (see Fig. 4B). The resulting plasmids (pAR214, pAR215 and pAR216) were used to transform *Escherichia coli* strain DH5α and assayed for the presence of Myc cross-reacting material (Fig. 4B). Bacteria carrying pAR215 expressed a fusion protein, whereas the strains harbouring the other two fusions (plasmids pAR214 and pAR216) showed no specific signal. These data indicate that the *gstI* locus does encode a protein and, by sequence analysis of the three fusions around the *Kpn*I site (Fig. 4B), it was predicted that the inhibitory locus *gstI* codes for a 63 amino acid (molecular weight 7331) protein (Fig. 3B). This protein was called GstI.

Construction of a gstI *mutation*

To assess whether the predicted GstI protein is responsible for the inhibition of *glnII* expression, two out-of-frame mutations were constructed (see *Experimental procedures*) and tested for their ability to inhibit *glnII* expression. In both cases (a fill-in reaction or the removal of 3′ overhangs), the modification of the *Kpn*I–*Asp*718 restriction site (Fig. 5) was predicted to disrupt the reading frame of *gstI* such that no intact GstI protein would be present. The wild-type and mutated versions of *gstI* were cloned into the same vector, pBR329, using the same restriction enzymes (*Sph*I–*Hin*dIII) and, consequently, in the same orientation to avoid possible variations resulting from a different context (such as the presence of promoters or regulatory DNA sequence). In the case of plasmid pAR219 (fill-in after digestion with *Asp*718), a new stop codon is predicted 10 bp downstream of the *Asp*718 site (Fig. 5A). In plasmid pAR220, the removal of 3′ overhangs after digestion with *Kpn*I introduced a frameshift, and the predicted ORF showed no stop codons (at least up to the *Hin*dIII cloning site) downstream of the mutated *Kpn*I site (see Fig. 3B). However, the predicted transcriptional terminator site of *gstI*, located between the *Cla*I and the *Hin*dIII restriction sites (see Fig. 3B), would be maintained and, thus, a truncated protein of a similar length to GstI was expected.

Plasmids pAD1, pBR329 (vector alone) and pMS25 (carrying the wild-type version of *gstI*) were used as controls. All the plasmids were used to transform UNF1827 (pAR18), and the resulting strains were tested for their ability to grow on complete and minimal medium. As shown in Fig. 5B, strain UNF1827 co-transformed with pAR18 plus either pBR329, pAR219 or pAR220 could grow on all media, whereas UNF1827(pAR18/pMS25) could not grow on NFDM–glutamate. Very small colonies were observed (after 48 h at 37°C) in the case of UNF1827(pAR18/pAD1) but, in that situation, both plasmids carry *glnII*, whereas only one of them (pAD1) carries a copy of *gstI*. All the strains tested could grow on NFDM agar supplemented with glutamine or on rich TY agar medium. We conclude that the last 14 amino acids of GstI are essential for its function.

Discussion

In this paper, we have identified a novel post-transcriptional

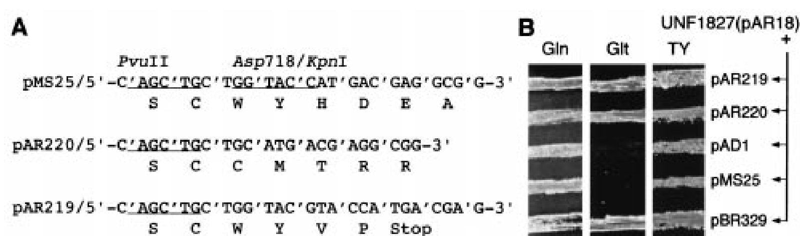

Fig. 5. Complementation assay of the *K. pneumoniae* UNF1827(*glnA*$^{-}$) mutant.A. The nucleotide sequence of the DNA region around the *Kpn*I–*Asp*718 site of the *gstI* gene and the predicted amino acid sequence in each of the different plasmids are shown.
B. Competent cells of strain UNF1827(pAR18) were prepared and transformed with plasmids pBR329, pAD1, pMS25, pAR219 and pAR220. Co-transformed cells were grown in complete medium (TY), harvested by centrifugation, washed twice with N-free NFDM medium and streaked onto TY agar or NFDM agar containing either glutamate (Glt) or glutamine (Gln) as sole nitrogen source. The plates were incubated at 37°C for 2 days. Identical results were obtained with five different transformants from each plasmid.

mechanism for the regulation of glutamine synthetase II expression in *R. leguminosarum* biovar *viciae*. This study developed from the initial observation that certain plasmids carrying the *R. leguminosarum glnII* gene were unable to suppress the glutamine auxotrophy of a *K. pneumoniae glnA* mutant. In a series of experiments, we demonstrated that this phenomenon results from a post-transcriptional inhibition of *glnII* expression mediated by the product of a 189 bp ORF, designated *gstI*, that is transcribed in the opposite direction to *glnII*. Identification of the transcription start site for *gstI* (Fig. 3B) suggests that the gene has a −35/−10-like promoter (5′-GTGCAA-N_{17}-CATTAT-3′) that shares some similarity with the consensus *E. coli* σ^{70} promoter sequence (5′-TTGACA-N_{15-17}TATAAT-3′) (Hawley and McClure, 1983). A consensus for vegetative σ^{70}-like promoters of *Rhizobium* has not yet been defined, but the promoter sequences identified so far do not conform with high fidelity to the *E. coli* consensus (Luka *et al.*, 1996). Two NtrC binding sites, previously characterized by gel retardation experiments and responsible for activation of the σ^{54}-dependent *glnII* promoter (Patriarca *et al.*, 1992; 1994), overlap the −35/−10 region of *gstI*p (Fig. 3). Accordingly, *gstI* transcription is derepressed in an *ntrC* mutant (Fig. 4C). A similar σ^{70}-like promoter that is also negatively regulated by NtrC is found upstream of the ORF1−*ntrBC* operon of *R. etli* (Patriarca *et al.*, 1993; Martino *et al.*, 1996). Taken together, these data indicate that binding of the NtrC protein to the *gstI* upstream region will repress expression of *gstI* and simultaneously activate expression of *glnII* (see Fig. 3A). We conclude from these observations that expression of *R. leguminosarum glnII* is regulated in response to the intracellular nitrogen status at two levels. When cells are nitrogen limited, *glnII* is transcriptionally activated by phosphorylated NtrC and, when the nitrogen status rises, not only does transcriptional activation cease as a result of dephosphorylation of NtrC but, at the same time, *gstI* repression is relieved, and GstI acts to inhibit translation of *glnII* mRNA. Hence, GstI facilitates a sensitive and rapid reduction in GSII expression in response to increasing nitrogen status.

A translational analysis allowed us to determine that *gstI* codes for a small protein of 63 amino acids (Fig. 3B), and the role of GstI in translation inhibition was supported by the fact that two out-of-frame mutant derivatives of GstI completely lost their ability to repress the *glnII* expression (Fig. 5).

In an attempt to shed some light on the possible mode of action of GstI, we used a variety of sequence alignment algorithms, such as those implemented in the programs BLASTP or FASTA, to find protein(s) published in the PDB, PRODOM or GenBank/EMBL databases with sequence similarity to the GstI protein. These searches failed to identify any significant matches (25% identity or more over the full-length protein), so we then used the TOPITS

Fig. 6. Secondary structure of the *glnII* mRNA 5′ UTR predicted by the MFOLD (http://www.ibc.wustl.edu/~zuker/rna) program (dG = −26.1 kcal mol^{-1}). The anticodon triplets for glutamine (CUG and UUG) and the start codon (AUG) are indicated.

'threading' method (Rost, 1995) as a means of identifying remotely homologous proteins based on their secondary structure. Using this approach, the threading tool of program SWISS PDB VIEWER and the SWISSPROT automatic modelling procedure (Guex and Peitsch, 1997), it was predicted that GstI could be folded to match either of two domains of the *E. coli* glutaminyl-tRNA synthetase (GlnRS). However, the best fit (95% of the bond lengths, bond angles and torsion angles in the range of values expected for a naturally folded protein) involved the C-terminal part of GlnRS, the anticodon-binding domain (i.e. the region that in GlnRS makes contact with the anticodon loop of the $tRNA^{gln}$). In this context, it is notable that modelling of the *glnII* 5′ untranslated leader using the program MFOLD (http://www.ibc.wustl.edu/~zuker/rna) suggests that this region could adopt a secondary structure involving two loops, both of which would contain the anticodon sequence UUG for glutamine (see Fig. 6). We performed an RNA protection experiment using labelled *glnII* leader transcripts (prepared *in vitro*) encompassing the predicted stem−loops and crude extract prepared from either bacteria overexpressing GstI or bacteria carrying the vector alone. The RNase T_1 cleavage pattern in the absence of GstI differed from the cleavage pattern when GstI was present (data not shown), thus suggesting that GstI can interact (directly or indirectly) with the 5′ untranslated region (UTR) of *glnII* transcripts.

The most frequent mechanism used in bacteria to repress translation appears to be one that, either by modification of the RNA structure or by steric hindrance, affects the interaction of the rRNA and the Shine−Dalgarno sequence of the target mRNA. This is the case for T4 RegA (Kang *et al.*, 1995), TRAP (Antson *et al.*, 1999), CspE (Bae *et al.*, 1999), ThrS (Sacerdot *et al.*, 1998), SecA (Dolan and Oliver, 1991), PyrR (Switzer *et al.*, 1999) and BtuB (Ravnum and Andersson, 1997). Perhaps of particular relevance is the observation that the threonyl-tRNA synthetase from *E. coli* represses translation of its own mRNA by binding to an operator region located upstream

from the ribosome binding site (Romby *et al.*, 1996). This operator contains two stem–loop structures, which mimic the anticodon arm of *E. coli* $tRNA^{thr}$, and it has been suggested (Romby *et al.*, 1996) that the two anticodon-like domains of the operator bind symmetrically to the two $tRNA^{thr}$ anticodon recognition sites (one per subunit) of the dimeric threonyl-tRNA synthetase. Moreover, the presence of striking similarities between the structures formed by leader transcripts involved in attenuation and the structures of some tRNAs was pointed out by Ames *et al.* (1983). Hence, although the molecular mechanism by which GstI inhibits *glnII* expression is presently unknown, it seems reasonable to speculate that GstI could inhibit the translation of *glnII* mRNA by binding to the *glnII* 5′ UTR and that the substrate for GstI may be a molecule with similar features to the anticodon loop of $tRNA^{gln}$.

Experimental procedures

Bacterial strains and media

Strains of *R. leguminosarum* were grown at 30°C on either TYR rich medium (Beringer, 1974) or on the chemically defined RMM medium (Hooykaas *et al.*, 1977). Strains of *K. pneumoniae* were grown on either TY rich medium or on NFDM (a chemically defined minimal medium) medium (Dixon *et al.*, 1977). *E. coli* strains were grown on TY medium. Antibiotics used were ($\mu g\ ml^{-1}$): tetracycline (5); chloramphenicol (30); kanamycin (30); rifampicin (100); nalidixic acid (20); ampicillin (100); and carbenicillin (250). Nitrogen sources used were ($mg\ ml^{-1}$): glutamine (0.002, 0.2 or 1); glutamate (1); or NH_4Cl (1).

Preparation of crude extracts and glutamine synthetase assay

Bacteria were harvested by centrifugation, resuspended in extraction buffer (10 mM imidazole–HCl buffer, pH 7.2, 1 mM $MnCl_2$, 1 mM β-mercaptoethanol and 5 mM EDTA) and disrupted by sonic oscillation at 0°C. After centrifugation (12 000 *g* for 10 min at 4°C), the supernatant was used to determine both protein concentration and GS activity (γ-glutamyl transferase assay) as described previously (Manco *et al.*, 1992).

Immunoblot experiments

Bacteria were harvested while in the exponential phase by centrifugation at 4°C, washed with 0.9% NaCl and resuspended in 10 mM imidazole–HCl buffer, pH 7.2, containing 10 mM $MgCl_2$. The cells were disrupted by sonic oscillation at 0°C, and the cell debris was removed by centrifugation at 27 000 *g* for 15 min. The samples (between 0.1 and 1 mg) were boiled for 5 min and electrophoresed in 15% SDS–PAGE. Immunoblots were made as described elsewhere (Manco *et al.*, 1992). Molecular weight protein standards, obtained from Sigma, included bovine albumin (M_r 67 000), egg albumin (43 000), carbonic anhydrase (30 000) and trypsin inhibitor (20 100).

RNA biochemistry

RNA was extracted from *K. pneumoniae* and *R. leguminosarum*, and RNase protection assays were performed as described elsewhere (Patriarca *et al.*, 1993), using an *in vitro* transcription system (Promega). Riboprobes were synthesized with [α-^{32}P]-UTP (Amersham). To recognize *glnII* transcripts, plasmid pAR28 was constructed by ligation of a 380 bp *Hin*dIII–*Eco*RI fragment [spanning from position −219 (*Hin*dIII) to +161 (*Eco*RI) with respect to the *glnII* transcription start site] to *Hin*dIII–*Eco*RI-digested pGEM7Zf(+) (Fig. 4D) and used as template for the synthesis of riboprobes. To produce antisense RNA, plasmid pAR28 was digested with *Hin*dIII and used as template for T7 polymerase-mediated transcription. To produce the sense RNA probe, plasmid pAR28 was digested with *Eco*RI and used as template for SP6 polymerase-mediated transcription. The riboprobes used to test *gstI* transcription (Fig. 4A) were synthesized using DNA from plasmid pAR27 [a 275 bp DNA fragment spanning from position −494 (*Pvu*II) to −219 (*Hin*dIII) with respect to the *glnII* transcription start site] as template (see Fig. 4C). To obtain probe P_1, pAR27 was digested with *Bam*HI and used as template for T7 polymerase-mediated transcription; to obtain probe P_2, pAR27 was digested with *Eco*RI for SP6 polymerase-mediated transcription.

For Northern blot analysis (Fig. 1D), RNA prepared from *K. pneumoniae* was precipitated with ethanol and resuspended in running buffer (20 mM MOPS, 10 mM NaAc, pH 5.3, and 1 mM EDTA) containing 50% formamide and 2.2 M formaldehyde, heated for 10 min at 65°C and cooled on ice. Samples were separated by electrophoresis on 1.5% denaturing agarose gel in running buffer. The RNA was then transferred onto a nitrocellulose filter in 20× SSC (1× SSC is 0.15 M NaCl and 0.15 M sodium citrate, pH 7.0), and the filters were hybridized as described previously (Patriarca *et al.*, 1992).

DNA manipulations, plasmids and sequence

Standard methods were used for DNA preparation and manipulation (Sambrook *et al.*, 1989). DNA sequence was determined with a Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham) using the T7 and SP6 promoter primers and appropriate synthetic oligonucleotide primers. The radioactive nucleotides used for sequencing were [α-^{33}P]-dNTP (Amersham).

Construction of GstI–Myc fusions

Polymerase chain reaction (PCR) amplification experiments were performed using DNA from plasmid pBSCmyc as template, three different forward oligonucleotides and one reverse primer. Mycfor1: 5′-ATGGTACCTGCTAGCGGTGGATCTGGTGGA-3′; Mycfor2: 5′-ATGGTACCTTGCTAGCGGTGGATCTGGTGGA-3′; Mycfor3: 5′-ATGGTACCGCTAGCGGTGGATCTGGTGGA-3′; and Mycrev: 5′-ATATCGATAGCTCCACCGCGGTGGCGGCCG-3′. The resulting DNA products were separated by electrophoresis, eluted, digested with *Kpn*I and *Cla*I restriction enzymes and cloned into *Kpn*I–*Cla*I-digested pAR205. The ligation mixtures were used to

transform *E. coli* DH5α (Woodcock *et al*., 1989). Three colonies of each clone were isolated, the plasmid DNA prepared, and the DNA sequence of the insert was confirmed. Bacteria were grown in TY medium, and crude extracts were prepared by sonic oscillation. Protein extracts (100 μg) were run on SDS–PAGE gel, and Western blot analysis was performed using anti-Myc monoclonal antibodies (Santa Cruz Biotech) and revealed using the ECL Western blotting system (Amersham).

Construction of out-of-frame GstI *mutation*

Plasmid pAR205 was obtained by cloning the *Hin*dIII–*Sph*I DNA fragment from plasmid pMS6 (Fig. 2A) into *Hin*dIII–*Sph*I-digested pGEM7Zf(+). Plasmid pMS25 was obtained by cloning the *Hin*dIII–*Sph*I DNA fragment from plasmid pAR205 into *Hin*dIII–*Sph*I-digested pBR329. Plasmid pAR219 was obtained by a two-step procedure: first, plasmid pAR217 was obtained digesting plasmid pAR205 with *Asp*718, followed by a treatment with the Klenow fragment of DNA polymerase I (Promega) to fill the 5′-protruding ends with dNTPs and religation; secondly, by cloning the *Hin*dIII–*Sph*I DNA fragment from plasmid pAR217 into *Hin*dIII–*Sph*I-digested pBR329. Plasmid pAR220 was obtained by a two-step procedure: first, plasmid pAR218 was obtained by digesting plasmid pAR205 with *Kpn*I, followed by treatment with T4-DNA polymerase (Boehringer) to convert the 3′-protruding ends to blunt ends and religation; secondly, by cloning the *Hin*dIII–*Sph*I DNA fragment from plasmid pAR218 into *Hin*dIII–*Sph*I-digested pBR329.

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