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Tight linkage of *glnA* and a putative regulatory gene in *Rhizobium leguminosarum*

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ABSTRACT

Rhizobium leguminosarum, biovar viceae, strain RCC1001 contains two glutamine synthetase activities, GSI and GSII. We report here the identification of glnA, the structural gene for GSI. A 2 kb fragment of DNA was shown to complement the Gln$^-$ phenotype of Klebsiella pneumoniae glnA mutant strains. DNA sequence analysis revealed an open reading frame (ORF) of 469 codons specifying a polypeptide of 52,040 daltons. Its deduced amino acid sequence was found to be highly homologous to other glutamine synthetase sequences. This ORF was expressed in Escherichia coli minicells and the corresponding polypeptide reacted with an antiserum raised against GSI. Upstream of glnA we found an ORF of 111 codons (ORF111) preceded by the consensus sequence for an ntrA-dependent promoter. Minicells experiments showed a protein band, with a molecular weight in good agreement with that (10,469) deduced from the nucleotide sequence. On the basis of homology studies we discuss the possibility that the product of ORF111 is equivalent to the P_{II} protein of E.coli and plays a similar role in regulation of nitrogen metabolism.

INTRODUCTION

Rhizobium bacteria use ammonia for growth in the free-living state, but in the Rhizobium-legume symbiosis the nitrogen fixing bacteroids export all ammonia produced in the nitrogen fixing process to the plant fraction of the symbiotic nodule (1). Thus, the enzymes for ammonia assimilation need to be regulated differently in the two bacterial states. Another peculiarity of Rhizobiaceae is the presence of two glutamine synthetases (GS; EC 6.3.1.2): GSI, similar to the GS of enteric bacteria, regulated by adenylylation and relatively heat stable; and GSII, heat labile and not known to be modified after translation (2,3,4,5,6). Biochemical studies (7) have shown that GS plays a central role in the regulation of nitrogen metabolism, while genetical studies (8,9) indicate that regulation of the glnA gene in Enterobacteriaceae is very complex. A coordinated nitrogen control system similar to that of enteric bacteria (10) has not been described in Rhizobium spp..

We started a study of the GS activities of R.leguminosarum biovar vicese, strain RCC1001, in order to better understand nitrogen assimilation and its regulation in this species (11). In this paper we report the DNA sequence of glnA, the structural gene for GSI, and the comparison of the deduced polypeptide sequence with other known sequences. We also report the sequence of an open reading frame upstream of glnA coding for a protein which appears to play a regulatory role, probably equivalent to that of the P_{II} protein, which is involved in the regulation of GS adenylylation and glnA expression in Escherichia coli (12,13).

MATERIALS AND METHODS

Strains, plasmids and media

Abbreviations used are: Ap (Ampicillin), Km (kanamycin), Cm (chloramphenicol), Tet (tetraciclin).

Strains used were E.coli HB101 (14), JM83 (15), DS998 (16); Klebsiella pneumoniae wild type (17), UNF1827 and UNF1838 (18); R.leguminosarum strain LPR1105, a rifampicin resistant derivative of RCC1001 (19); Agrobacterium tumefaciens strain LBA2715 containing the R.leguminosarum symbiotic plasmid (pSym) (20).

Plasmids used were: pMMB34 (21); pSVB20, pSVB23, pSVB24, pSVB25 (pUC8 derivatives, Ap^r; W.A. et al., in preparation); pACYC184 (22); p7D9 (11), Gln^+, Km^r, containing 27 kb of R.leguminosarum DNA inserted in the cosmid vector pMMB34; pMG10 (11), Gln^+, Cm^r, containing 6.5 kb of R.leguminosarum DNA and 3.4 kb of pMMB34 DNA inserted in the vector pACYC184.

Media used were: TY (14); PA (14) supplemented with 0,1% glucose; minimal citrate (23). When needed, glutamine was used at a concentration of 200 ug/ml.

DNA manipulation

Most procedures used were according to Maniatis et al.(14). The rapid isolation of plasmid DNA was performed as described (24). Plasmid DNA was prepared according to Davis et al.(25). pSym DNA was prepared from strain LBA2715 as described (26). Hybridization experiments were all carried out at 60°C. DNA sequencing was performed with minor modifications (W.A. et al., in preparation) of the method of Maxam and Gilbert (27).

Protein synthesis in minicells

Strain DS998 was transformed with specific plasmids and grown in minimal

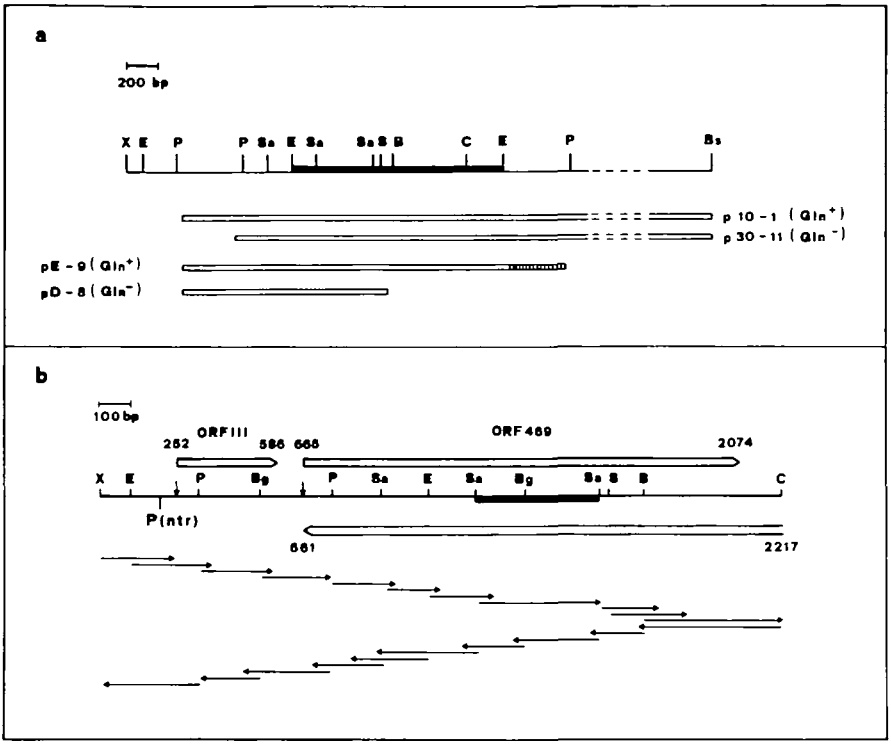

Figure 1. (a): restriction map of the glnA region and the DNA left in different Bal31 deletions. The black bar indicate the location of the EcoRI fragment cross-hybridizing to K.pneumoniae glnA (11). The dotted lines indicate DNA (about 3 kb) omitted from the figure. Only the glnA region-proximal end of the deletions is indicated in the figure. The boundary of p10-1 and p30-11 was confirmed by DNA sequencing (see Fig.2). The boundary of pE-9 is between the EcoRI and PstI restriction sites (striped bar); that of pD-8 is between the SmaI and BamHI sites. (b): sequencing strategy and location of the thre ORFs deduced by computer analysis. Vertical arrows indicate putative ribosome binding sites. $P_{(ntr)}$ shows the position of a sequence homologous to the consensus for an ntrA-dependent promoter. A black bar indicates the SalI fragment used as a probe in the hybridization experiments of Fig.5.

medium supplemented with thiamine (10 ug/ml), casaminoacids (0,5%) and the appropriate antibiotic. Minicells were purified (28), divided into aliquots and stored at -80°C. After preincubation for 30 min, incorporation of either ^{35}S-methionine, ^{3}H-valine or ^{3}H-leucine was performed for 60 min (28). Immunoprecipitation was performed as described (29).

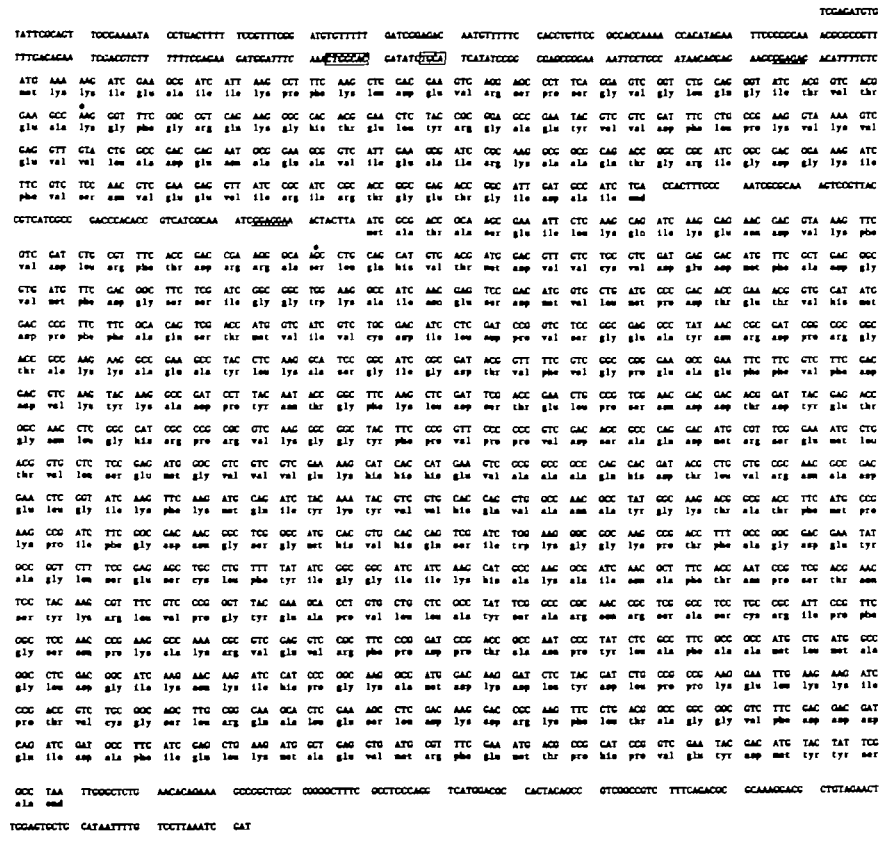

Figure 2. DNA sequence and deduced amino acid sequence of ORF111 and ORF469 from the XhoI site to the ClaI site of Fig.1. A consensus sequence for an ntrA-dependent promoter is boxed, while putative ribosome binding sites are underlined. An asterisk (*) shows the left boundary of p10-1 and p30-11 deletions.

RESULTS

Localization of the glnA region on pMG10.

We have previously shown (11) that cosmid p7D9 (see Materials and Methods) complements the Gln$^-$ phenotype of K.pneumoniae strains UNF1827 (glnA) and UNF1838 (Δ glnA-ntrC). A subclone of p7D9, pMG10, complementing both strains, is used in this paper. In Fig.1a we show part of pMG10 DNA containing the 1.3 kb fragment cross-hybridizing to K.pneumoniae glnA (11).

We generated deletions of pMG10 by digestion at the single XhoI site shown in Fig.1a and treatment with Bal31 nuclease. After ligation and transformation into strains UNF1827 and UNF1838 we analyzed 24 clones, 7 of

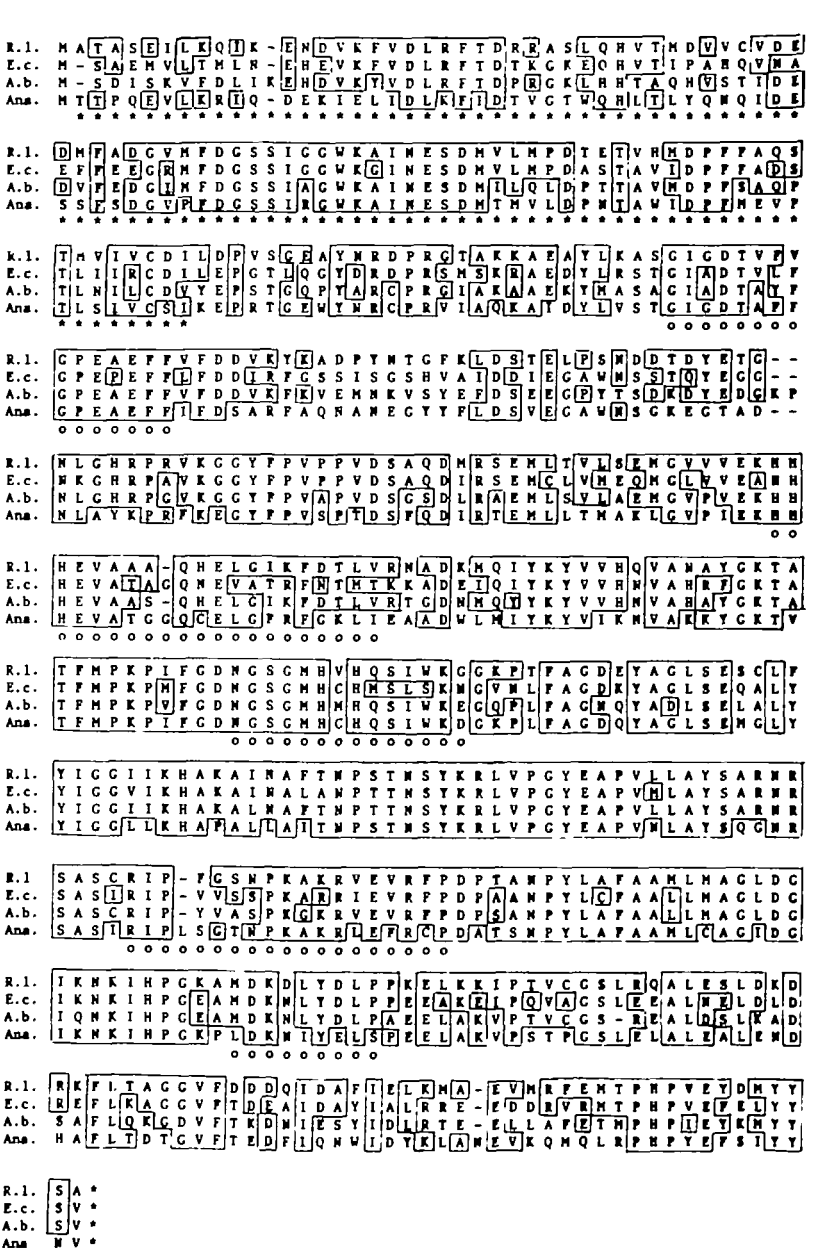

Figure 3. Homology of the deduced R.leguminosarum GSI sequence to other GS sequences. R.l.= R.leguminosarum; E.c.= E.coli; A.b.= A.brasilense; Ana.= Anabaena. Sequences assumed to correspond to the N-domain and C-domain (33) are indicated by (*) and (o) respectively.

which were Gln^+ and 17 were Gln^-. Restriction analysis showed that the XhoI site was deleted in all clones. We searched for the presence of the surrounding sites in order to construct a restriction map of all clones and the Gln^+(p10-1) and Gln^-(p30-11) clones shown in Fig.1a were chosen because their end-points, confirmed by sequencing (see below Fig.2), are the closest definition of the left-side border of the glnA region.

We used clone p10-1 to generate a set of new deletions by digestion at the single BstEII site and treatment with Bal31 nuclease. Restriction analysis of 40 clones carrying deletions showed that the EcoRI site to the right of ClaI is present in all Gln^+ clones and absent in all Gln^- clones. In Fig.1a a Gln^+ clone (pE-9) and a Gln^- clone (pD-8) are shown. R.leguminosarum DNA left in pE-9 is 2 kb long and therefore about 2 kb of DNA are sufficient to suppress the Gln^- phenotype of strains UNF1827 and UNF1838.

DNA sequencing of the glnA region.

The sequence of R.leguminosarum DNA from the XhoI site to the ClaI site indicated in Fig.1 is presented in Fig.2. A computer search for open reading frames longer than 100 codons resulted in the three ORFs shown in Fig.1b. A sequence with good homology to an ntrA-dependent promoter (9) is found at position 174-190 followed by a putative ribosome binding site at position 235-240 and an ORF of 111 codons (ORF111). At the end of this ORF there is no obvious sequence suggesting rho-independent transcription termination and at position 650-655 there is a new putative ribosome binding site followed by an ORF of 469 codons (ORF469). Downstream of this, we do not see any sequence suggesting rho-independent transcription termination. On the opposite strand another ORF of 517 codons is present (see Fig.1b) starting with an Arg codon at position 2215 and ending at position 662.

DNA sequencing of appropriate subclones shows that p10-1 is deleted up to nucleotide 347 and that p30-11 is deleted up to nucleotide 750, as indicated in Fig.2.

We compared the deduced aminoacid sequence of the ORF111 product with the protein sequences present in the January 1986 data bank using the Micro-Genie program (30) and found no significant homology. However, it was pointed out to us by dr. M. Merrick that codons 47 to 57 of ORF111 are strikingly similar to the sequence of a peptide of the P_{II} protein of E.coli (12):

P_{II}: -Gly-Ala-Glu-Tyr-Met-Val-Asp-Phe-Leu-Pro-Lys-
ORF111: -Gly-Ala-Glu-Tyr-Val-Val-Asp-Phe-Leu-Pro-Lys-

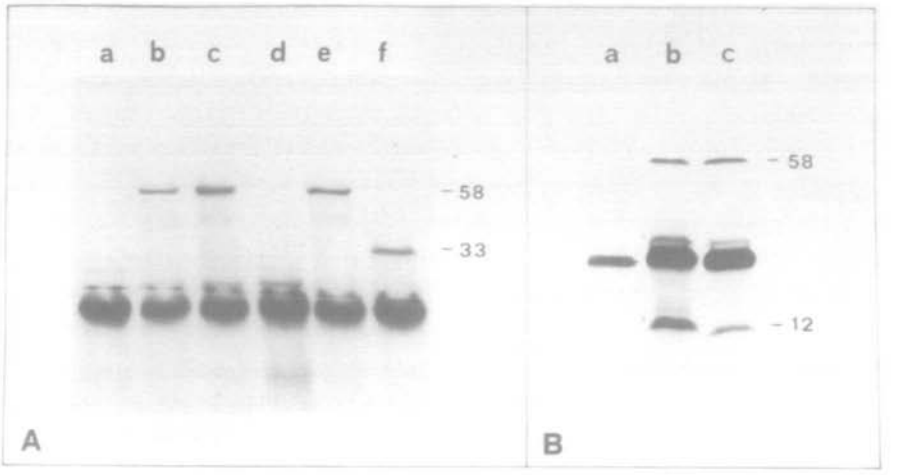

Figure 4. SDS-PAGE of minicells extracts containing different plasmids. Panel A: ^{35}S-methionine labeling of minicells containing: (a): vector pACYC184; (b): pMG10; (c): p10-1; (d): p30-11; (e): pE-9; (f): pD-8. Panel B ^{3}H-valine (a and b) or ^{3}H-leucine (c) labeling of: (a): pACYC184; (b) and (c): pMG10. The 58, 33 and 12 markers are explained in the text. Lanes (a) show the CAT band of pACYC184 present also in the other lanes. Molecular weights of insert-specific bands were interpolated from the molecular weight of commercially available ^{14}C-standards. The experiments shown were performed with 10% (Panel A) and 20% (Panel B) polyacrylamide.

Since the complete amino acid sequence of the P_{II} protein of E.coli is not published, we could not compare other portions of the two proteins. The molecular weight of the P_{II} protein of E.coli is 11,000 to 13,400 (31), similar to that of the deduced amino acid sequence of ORF111 (10,469). The comparison of the amino acid composition of the P_{II} protein of E.coli (31) and of the ORF111 product shows a striking similarity. We conclude that the product of ORF111 might be equivalent to the E.coli P_{II} protein.

We also searched for homology of the deduced amino acid sequence of ORF469 to other protein sequences present in the data bank and found a significant value only with Anabaena sp. strain 7120 GS. We introduced in the data bank the deduced protein sequences of E.coli (32) and Azospirillum brasilense sp.7 (33) GSs and that of Bradyrhizobium japonicum GSII (6). Homology of ORF469 is 56%, 63% and 68% with Anabaena, E.coli and A.brasilense GSs, respectively, and most of it is in boxes of identical amino acids, as shown in Fig.3, thus suggesting that this ORF codes for R.leguminosarum GSI. Homology with B.japonicum GSII is 15%. The molecular weight of the deduced amino acid sequence of ORF469 is 52,040, in good agreement with that (60,000)

of the GS purified from UNF1827(pMG10) (A. Fuggi and R.D., manuscript in preparation). The molecular weight of the deduced amino acid sequences of Anabaena, E.coli and A.brasilense GSs are 53,265, 51,814 and 51,917 respectively.

A recent paper (34) reports the crystallographic structure of Salmonella typhymurium GS and defines an N-domain formed by 92 N-terminal amino acids and a C-domain formed by 5 stretches of amino acids (123-137; 208-228; 263-276; 343-361 and 392-400), participating in the conformation of the active site. We measured homology on portions of the GS sequences which we assume to be analogous to the above mentioned domains (see Fig.3). We found that N-domain sequences of Anabaena, E.coli and A.brasilense GSs give values of homology to R.leguminosarum GSI slightly lower than those found for the complete protein (49%, 58% and 55% respectively), while C-domain sequences show higher values (72%, 69% and 82% respectively). A more detailed analysis shows that 4 of the C-domain stretches are highly homologous while the 5th one (residues 392-400) is very conserved in R.leguminosarum, E.coli and A.brasilense GSs and different in Anabaena GS.

Using the computer program reported above (30) we analyzed the secondary structure of 110 N-terminal and of 110 C-terminal residues of the 4 protein sequences. No significant homology was found among the secondary structures of the C-terminal sequences while the N-terminal sequences showed homology except in the case of Anabaena (data not shown).

The ORF on the opposite strand starts at least 80 codons upstream of the Arg codon, as shown by DNA sequencing not reported in this paper. Also E.coli and A.brasilense sequences present an extended ORF on the opposite strand, which is absent in the Anabaena sequence. The codons of these three ORFs are on a reading frame coinciding (with opposite polarity) with the reading frame of the respective GSs. Analysis of the deduced amino acid sequence in the data bank showed no significant homology of the R.leguminosarum ORF to other proteins.

Expression of pMG10 and its deletion derivatives in minicells.

In order to demonstrate the expression of the ORFs identified by DNA sequencing, minicells experiments were carried out. Plasmid pMG10 and the deletion derivatives described above were introduced into strain DS998 by transformation and minicells were purified. After labeling with ^{35}S-methionine and SDS-PAGE, we observed, in addition to vector-coded bands, a band specific of the insert of pMG10 (Fig.4A). This band, 58,000 in molecular weight, is also present in the Gln^{+} deletion derivatives p10-1 and pE-9, is absent in the Gln^{-} deletion p30-11, while it is reduced in

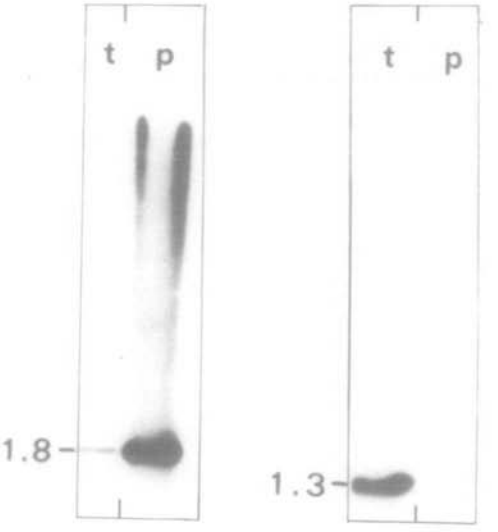

Figure 5. Hybridization of a ^{32}P-pSym DNA fragment (left panel) and ^{32}P-glnA DNA (right panel) to total DNA (lane t) and pSym DNA (lane p). The probe pSym DNA is a 1.8 kb EcoRI fragment isolated from plasmid p1085 (40); glnA DNA is a SalI fragment of pMG10 indicated in Fig.1b and in the text. 5 ug of total DNA and 1 ug of pSym DNA (a plasmid preparation from strain LBA 2715), were digested with EcoRI and used in each experiment. The molecular weight of the hybridizing band is interpolated from a λ -HindIII marker (not shown).

molecular weight (33,000) in the Gln$^-$ deletion pD-8. We conclude that the latter is a truncated polypeptide. Therefore, this experiment shows that the insert of pMG10 codes for a protein, 58,000 in molecular weight, that the direction of transcription is left to right as indicate in Fig.1b and that this protein is necessary for the Gln$^+$ phenotype either in strain UNF1827 or UNF1838. The additional band, approximately 28,000 in molecular weight (lanes b,c and d), appears to be coded by the DNA deleted in pE-9 and it is not relevant to this study.

A protein A-Sepharose 4CL column was treated with a polyclonal antiserum raised in rabbit against pure GSI obtained from strain UNF1827 (p7D9) (A.Fuggi and R.D., manuscript in preparation). This column retained the 58,000 molecular weight protein produced in ^{35}S-methionine labeled minicells. After elution with the appropriate buffer (see Materials and Methods), SDS-PAGE showed a single band comigrating with that of pMG10 (data not shown). We conclude that this band is GSI, coded by ORF469, which we call glnA.

In the experiment of Fig.4A we could not see any band corresponding to the size predicted from ORF111. Since this ORF contains only one methionine at its amino-terminal end (Fig.2) we repeated a minicell experiment using either ^{3}H-valine or ^{3}H-leucine and found a band, 12,000 to 14,000 in molecular weight, in addition to the 58,000 band (Fig.4B). These experiments demonstrate that ORF111 and glnA are expressed in minicells, while the ORF present on the opposite strand is not expressed.

The glnA region is not located on the symbiotic plasmid

A SalI fragment of pMG10, shown in Fig.1b, was used as a probe and hybridized to EcoRI digests of R.leguminosarum total and pSym DNA. We observed hybridization only to total DNA, as shown in Fig.5. If, instead, the probe originates from R.leguminosarum pSym DNA, there is hybridization to both DNAs. Therefore the insert of pMG10 is not located on pSym. The size of the hybridizing fragment is that (1.3 kb) of the EcoRI fragment of pMG10 shown in Fig.1a. Since total DNA was digested with EcoRI, this experiment shows colinearity between the EcoRI restriction sites of the insert of pMG10 and those of total DNA.

DISCUSSION

Free-living Rhizobium bacteria assimilate ammonia for growth, but the nitrogen fixing bacteroids export to the plant fraction of the symbiotic nodule all ammonia produced. That is, when nitrogenase activity becomes derepressed ammonia assimilation is blocked, probably by repression and inhibition of the GSs present in the nodule (2,4). At the same time a nodule-specific plant GS is derepressed (35). The study of regulation of R.leguminosarum glnA is important to understand the physiology of ammonia utilization in the symbiosis.

We report in this paper the sequence of the glnA gene and of a contiguous gene which, at the amino acid level, shows homology to the P_{II} protein of E.coli (31). These two genes are not located on the symbiotic plasmid (pSym) as shown in Fig.5.

The DNA of pMG10 required to suppress the Gln^{-} phenotype of UNF1827 and of UNF1838 has been localized with the help of Bal31 deletions and restricted to about 2 kb. This DNA region overlaps with the fragment cross-hybridizing to K.pneumoniae glnA DNA (11) as indicate in Fig.1a. The DNA sequence of this region, reported in Fig.2, contains an ORF at position 665 to 2071 (ORF469), the deduced amino acid sequence of which is highly homologous to that of E.coli, Anabaena and A.brasilense GSs (Fig.3), and

poorly homologous to B.japonicum GSII. Expression of ORF469 in minicells (Fig.4) reveals the presence of a protein, 58,000 in molecular weight, that specifically reacts with an antiserum against R.leguminosarum GSI. The molecular weight of GSI is 60,000 (A.Fuggi and R.D., in preparation), while the deduced molecular weight of the protein encoded by the ORF at position 665-2071 is 52,040. We conclude that ORF469 corresponds to glnA, the structural gene for GSI.

R.leguminosarum GSI can be adenylylated in vivo, at least in K.pneumoniae, because pure GSI, or GSI activity in crude extracts of UNF1827(pMG10), is partially adenylylated (A.Fuggi and M.G., unpublished results). Therefore, R.leguminosarum, E.coli and A.brasilense GSs can be adenylylated, while Anabaena GS cannot (36). We compared the 5 amino acid stretches of the C-domain (34) in the four protein sequences of Fig.3 and found Anabaena-specific differences only in the 5^{th} stretch (residues 392 to 400, containing the Tyr target of adenylylation). An analysis of the secondary structure of the four proteins in this region, using the Micro-Genie computer program (30) shows that these are all different and therefore the correlation between lack of adenylylation and secondary structure around the target Tyr, previously proposed (32), is not confirmed. Anabaena-specific differences by this type of analysis were only found in the N-domain (34). If this is related to the lack of adenylylation should be confirmed by independent evidence.

The ORF on the opposite strand of glnA does not show a band in minicell experiments and we found no significant homology of its deduced amino acid sequence to other sequences in the data bank. An extended ORF is present also on the opposite strand of E.coli and A.brasilense glnA DNA, but not in the case of Anabaena. As shown under Results the reading frame of these three ORFs is coincident, but with opposite polarity, with that of GS. Although this is certainly striking and suggests a functional or evolutionary role (37) we did not investigate its significance further on.

Plasmid pMG10 codes not only for GSI, but also for a protein expressed in minicells with a molecular weight from 12,000 to 14,000. This molecular weight is in good agreement with that (10,469) of the deduced amino acid sequence of ORF111 (nucleotides 251 to 584). As indicated under Results the product of ORF111 might be equivalent to the E.coli P_{II} protein. If so, while glnB, the supposed structural gene for P_{II}, is unlinked to glnA in K.pneumoniae (38) and in E.coli (10) it might be contiguous to it in R.leguminosarum. A glnB-like gene appears to be contiguous to glnA also in

A.brasilense and B.japonicum. In fact, 350 bp upstream of A.brasilense glnA (33) the published sequence starts with 75 bp coding for 25 residues, 20 of which are identical to residues at the -COOH end of the ORF111 product. In the case of B.japonicum (5), 336 bp upstream of glnA the published sequence starts with 180 bp coding for 60 residues, 45 of which are identical to residues at the -COOH end of the ORF111 product.

We previously reported evidence (39) that DNA in the region coding for ORF111 is responsible for inhibition of the growth observed in the presence of nitrate. In fact, when UNF1827 carrying either p7D9 or pMG10 is grown in nitrate, there was no complementation of the Gln^- phenotype. Although still obscure, this phenomenon suggests that GSI might be repressed or adenylylated when K.pneumoniae containing pMG10 is grown in nitrate. UNF1827(p10-1) grows in nitrate indicating that the DNA deleted in pMG10 to generate p10-1 is required for the nitrate effect. This observation suggests a regulatory role, or an interference with a K.pneumoniae regulatory circuit, caused by the product of ORF111.

In Fig. 2 we show a sequence with good homology to an ntrA-dependent promoter at position 174-190. It is tempting to conclude that glnA is transcribed from this promoter in pMG10, since R.leguminosarum DNA in this plasmid is inserted with opposite orientation to the Tet promoter of the pACYC184 vector. However, the putative ntrA-dependent promoter is deleted in the p10-1 (Gln^+) clone (Fig.1a) and therefore a promoter more proximal to glnA might exist. Indeed, at position 564-597 we find a sequence homologous to the promoter sequence of B.japonicum glnA (5). Experiments are in progress to identify transcription initiation(s) in this region.

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