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Molecular Cloning, Sequencing, and Expression of the Glutamine Synthetase II (*glnII*) Gene from the Actinomycete Root Nodule Symbiont *Frankia* sp. Strain CpI1

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In common with other plant symbionts, *Frankia* spp., the actinomycete N_2-fixing symbionts of certain nonleguminous woody plants, synthesize two glutamine synthetases, GSI and GSII. DNA encoding the *Bradyrhizobium japonicum* gene for GSII (*glnII*) hybridized to DNA from three *Frankia* strains. *B. japonicum glnII* was used as a probe to clone the *glnII* gene from a size-selected *Kpn*I library of *Frankia* strain CpI1 DNA. The region corresponding to the *Frankia* sp. strain CpI1 *glnII* gene was sequenced, and the amino acid sequence was compared with that of the GS gene from the pea and *glnII* from *B. japonicum*. The *Frankia glnII* gene product has a high degree of similarity with both GSII from *B. japonicum* and GS from pea, although the sequence was about equally similar to both the bacterial and eucaryotic proteins. The *Frankia glnII* gene was also capable of complementing an *Escherichia coli* Δ*glnA* mutant when transcribed from the vector *lac* promoter, but not when transcribed from the *Frankia* promoter. GSII produced in *E. coli* was heat labile, like the enzyme produced in *Frankia* sp. strain CpI1 but unlike the wild-type *E. coli* enzyme.

In bacteria, glutamine synthetase (GS) plays dual roles in providing glutamine for biosynthesis and in assimilating ammonia. *Escherichia coli* GS is regulated at the transcriptional level by the Ntr (nitrogen regulation) system and at the posttranslational level through an adenylylation cascade in which each of 12 identical subunits is reversibly adenylylated (34). As the enzyme is progressively adenylylated, it becomes increasingly sensitive to feedback inhibition by metabolites downstream from glutamine (1). This pattern of GS regulation is repeated in most members of the family *Enterobacteriaceae* and in many other bacteria.

Several exceptions to the enteric model of GS structure and regulation have been reported. For example, GSs in *Anabaena* and *Bacillus* spp. are not adenylylated (16, 31, 49), and short-term regulation occurs through feedback inhibition by metabolites that originate from glutamine and, in the case of *Bacillus* spp., by glutamine itself. A novel hexameric enzyme from *Bacteroides fragilis* has little DNA or protein sequence similarity with other GSs and lacks an adenylylation site (19).

Multiple GSs have been found in several bacteria. Two similar GSs found in the thermophile *Bacillus caldolyticus* are regulated in a complementary fashion (51, 52). Two GSs in plant symbionts from the families *Rhizobiaceae* (including *Rhizobium*, *Bradyrhizobium*, *Agrobacterium*, and *Phyllobacterium* spp.) and *Frankiaceae* are dissimilar and are regulated in response to the nitrogen source (14, 17). In members of the *Rhizobiaceae*, total GS activity is modulated by the differential transcription of *glnA* (GSI) and *glnII* (GSII) (12, 15, 35). Like the enteric GS, GSI is regulated by adenylylation, but it is expressed constitutively (12). GSII resembles eucaryotic GSs; it is an octamer of identical subunits and *glnII* is expressed from an Ntr-like promoter during nitrogen limitation (15, 26, 35). In the plant symbiotic actinomycete *Frankia* sp. strain CpI1, the pattern of GS regulation resembles that found in members of the *Rhizobiaceae*. Both GSI and GSII are produced; GSI is a classic GS in the sense that it is regulated by adenylylation, and GSII is biochemically similar to rhizobial GSIIs (17). GSII appears to be specifically synthesized in response to nitrogen starvation or in response to growth on a poor nitrogen source, such as glutamate, in culture, whereas GSI is constitutively present under all conditions tested (48).

To date, no role has been assigned to GSII in either group of organisms that would help explain its apparent persistence in plant symbionts. We show here that GSII from *Frankia* sp. strain CpI1 is similar in its DNA and protein sequence to the GS from *Pisum sativum* (pea) and GSII from *Bradyrhizobium* spp. Our results indicate that GSII is common among *Frankia* strains and, together with companion papers (4, 22), show that GSII is generally widespread among actinomycetes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All *Frankia* strains were grown as described previously, except that Ai17 and HFPCcI3 were grown on propionate- rather than succinate-containing medium (29). *E. coli* strains were grown on Luria medium (27) with 0.2% glucose or on M9 minimal medium (25). Ampicillin was added to a final concentration of 100 μg/ml.

Preparation of DNA. Plasmid DNA from *E. coli* strains was prepared by the boiling lysis method (20). DNA from *Frankia* strains was prepared by a modification of the method of Ligon and Nakas (24) or by a modification of the method of Stauffer et al. (43).

Restriction endonuclease analysis, electrophoresis of DNA, and hybridization conditions. Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and used as recommended by the manufacturer. DNA was fractionated on 0.4% agarose (genomic digests) or 0.8% agarose (plasmids) and transferred to nitro-

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TABLE 1. Plasmids, *Frankia* strains, and *E. coli* strains used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Frankia spp.		
Ai17	Isolated from alder (*Alnus incana* subsp. *rugosa*)	6
CpI1	Isolated from sweet fern (*Comptonia peregrina*)	9
HFPCcI3	Isolated from casuarina (*Casuarina cunninghamiana*)	54
E. coli		
JM105	*supE endA sbcB15 hsdR4 rpsL thi* Δ(*lac-proAB*) (F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	53
YMC11	*thi endA hsr* Δ*lacU169 hutC*$_{Klebs}$[a] Δ(*glnG-glnA*)	3
Plasmids		
pBJ196A	Ap^r; *B. japonicum glnII* in pBR322	10
pFR101	Ap^r; *Frankia* CpI1 *glnII* in pUC19	This study
pFR121a	Ap^r; *Frankia* CpI1 *glnII* with truncated 5′ regions in pUC19	This study
pUC18, pUC19		30

[a] *Klebsiella hutC* gene.

cellulose filters (Millipore Corp., Bedford, Mass.) by the method of Southern (42). Hybridization was done by standard methods with approximately 10^7 cpm of [α-^{32}P]dCTP-labeled probe per ml of hybridization solution (25).

Genomic size-selected library construction. A size-selected library was made from *Frankia* sp. strain CpI1 DNA by excising the region of agarose gels corresponding to the 4.6-kilobase-pair (kbp) band of the *Kpn*I restriction enzyme digest of CpI1 DNA. DNA was isolated by the freeze-squeeze method (46) followed by phenol-chloroform extraction and ethanol precipitation. The fragments were ligated (21) into alkaline phosphatase-treated pUC19 that had been digested with *Kpn*I. Competent *E. coli* JM105 cells were prepared and transformed by the method of Dagert and Ehrlich (13) with cells washed in 100 mM $MgCl_2$ prior to calcium chloride treatment. Insert-bearing clones were screened by hybridization with the insert from pBJ196A carrying the *Bradyrhizobium glnII* gene. Screening was performed on dot blots of DNA from individual clones grown in 96-well microdilution plates, lysed with 1 M NaOH, transferred by dot blot vacuum manifold to nitrocellulose membranes, and treated by the method of Landers (23).

Subcloning. DNA restriction fragments were fractionated in 1.0% low-melting-point agarose (NuSieve; FMC Corp., Rockland, Maine) and ligated in agarose to appropriately digested vector (45).

Complementation of *E. coli* Δ*glnA*. To express the *glnII* gene, a construct was made by deleting the region 5′ to the start of the putative *Frankia glnII* gene on the 1.6-kbp *Sal*I fragment (44). This construct was directionally subcloned into the *Hin*dIII and *Sal*I sites of pUC18 and pUC19. The resulting transformants were checked for the presence of the construct by restriction digest. The expression plasmid was termed pFR121a in pUC19 and pFR121b in pUC18. Approximately 50 bp of *Frankia* DNA upstream of the start of similarity with *Bradyrhizobium glnII* is present in pFR121a and pFR121b along with the entire *Frankia glnII* region.

DNA sequence analysis. DNA sequencing was done by the dideoxynucleoside triphosphate chain termination method of Sanger et al. (37), with [α-^{35}S]dATPαS as label and templates prepared from subclones made in pUC plasmids. Sequencing reactions were done with T7 DNA polymerase by following the protocol supplied by the manufacturer (Pharmacia, Inc., Piscataway, N.J.). Since *Frankia* DNA has about a 70% G+C content (2), sequencing reactions were done with 7-deaza-2′-dGTP and sometimes 7-deaza-2′-dITP to relieve band compressions. *E. coli* JM105 was used as the recipient strain. Software for analyzing the DNA sequences included the IBI-Pustell program for the IBM and IBI-MacVector program for the Apple Macintosh computer (IBI, New Haven, Conn.).

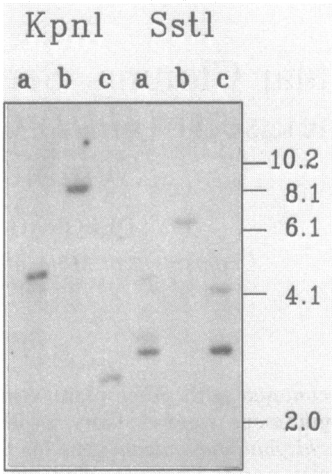

FIG. 1. Southern blot of *Frankia* sp. strain CpI1, CcI3, and Ai17 DNA hybridized to [α-^{32}P]dCTP-labeled *B. japonicum glnII* DNA. Lanes a, b, and c correspond to CpI1, CcI3, and Ai17 DNA, respectively.

GS assay. GS was assayed by the γ-glutamyltransferase method (5), modified as described previously (17). Heat lability of the enzyme in *E. coli* extracts was tested at 60°C as described previously (48). Protein was determined by the Bradford dye-binding assay (8).

RESULTS

Cloning of the *Frankia glnII* gene. Significant sequence similarity between *Bradyrhizobium japonicum glnII* DNA and *Frankia* DNA was observed by Southern blot analysis (Fig. 1). Total genomic DNA from *Frankia* sp. strains CpI1,

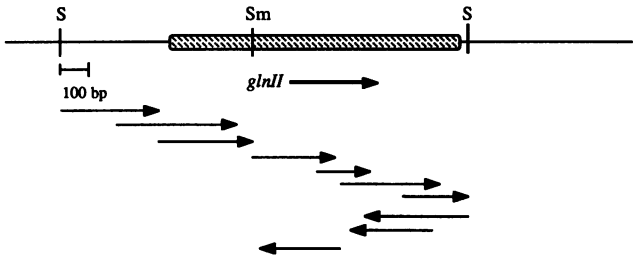

FIG. 2. Physical map and sequencing strategy for the *Frankia* sp. strain CpI1 *glnII* region. The boxed area represents the *glnII* gene. The arrows represent the templates and direction of sequencing. Restriction endonuclease site abbreviations: S, *Sal*I; Sm, *Sma*I.

```
                10                  30                  50          60          70          80          90          100
CCCAGGCCGA TCACGAAGTC GCACCGTTTC GTGCAGGACC AACCACGGGG AGTATTGA GTG AGT TAT CAG GCC GAG TAC ATC TGG ATC GAC GGC ACC GAG CCC
                                                                  (start) Ser Tyr Gln Ala Glu Tyr Ile Trp Ile Asp Gly Thr Glu Pro

110         120         130         140         150         160         170         180         190         200
GAG CCT TTG ATG CGT AGC AAG ACG CGC ATC ATC AAG GAC GGC AAG GAG CCG GAG ATC TGG GGC TTC GAC GGC TCG AGC ACC AAC CAG GCG CCG GGA TCG
Glu Pro Leu Met Arg Ser Lys Thr Arg Ile Ile Lys Asp Gly Lys Glu Pro Glu Ile Trp Gly Phe Asp Gly Ser Ser Thr Asn Gln Ala Pro Gly Ser

        210         220         230         240         250         260         270         280         290         300
AAC TCG GAC TGC GTG CTG CGT CCC GTC TTC GAG ACG CCC GAC CCG ATC CGG GGA GGC GAC AAC CGC CTG GTG CTG TGC GAG GTC CAG CTG ACC GAC TTC
Asn Ser Asp Cys Val Leu Arg Pro Val Phe Glu Thr Pro Asp Pro Ile Arg Gly Gly Asp Asn Arg Leu Val Leu Cys Glu Val Gln Leu Thr Asp Phe

        310         320         330         340         350         360         370         380         390         400
ACG CCA CCG ACG AAC ACC CGG GCG GCC GCC CTC GGT GTC GCC GAG CGG TAC GCC GAC ATG TCG CCC ATG TTC GGC ATC GAG CAG GAG TAC ACC TTC TTC
Thr Pro Pro Thr Asn Thr Arg Ala Ala Ala Leu Gly Val Ala Glu Arg Tyr Ala Asp Met Ser Pro Met Phe Gly Ile Glu Gln Glu Tyr Thr Phe Phe

        410         420         430         440         450         460         470         480         490
AAG GAC GGC CGC CCG TAC GGC TGG CCG GAG GTC GGC TAC CCC GCG CCG CAG GGT CCG TAC TAC TGC GGC GTC GGC GGC TCG AAG ATG CCC GGT CGC CAG
Lys Asp Gly Arg Pro Tyr Gly Trp Pro Glu Val Gly Tyr Pro Ala Pro Gln Gly Pro Tyr Tyr Cys Gly Val Gly Gly Ser Lys Met Pro Gly Arg Gln

500         510         520         530         540         550         560         570         580         590
ATC GTC GAG CGG CAC ACC CAG GCA TGC CTC GAC GCC GGC CTC GCC ATC GAG GGC ACC AAC GCC GAG GTC ATG ATG GGC CAG TGG GAG TTC CAG ATC GGC
Ile Val Glu Arg His Thr Gln Ala Cys Leu Asp Ala Gly Leu Ala Ile Glu Gly Thr Asn Ala Glu Val Met Met Gly Gln Trp Glu Phe Gln Ile Gly

600         610         620         630         640         650         660         670         680         690
GTC CTG CCC GCG CCC GCC ATC GGC GAC CAG ATC TGG TTG GGC CGC TGG CTG CTC CAC CGG ATC GCC GAG GAC TAC GGC GTC GAG GTG TCC TTC GCC GCG
Val Leu Pro Ala Pro Ala Ile Gly Asp Gln Ile Trp Leu Gly Arg Trp Leu Leu His Arg Ile Ala Glu Asp Tyr Gly Val Glu Val Ser Phe Ala Ala

700         710         720         730         740         750         760         770         780         790
AAG CCG ATC CCC GGT GAC TGG AAC GGT GCC GGC GCG CAC ACC AAC TTC TCC ACC AAG CAG ACG ATG GAG GGC TGG GACGCC ATC GTC ACG TGC TGC GAG
Lys Pro Ile Pro Gly Asp Trp Asn Gly Ala Gly Ala His Thr Asn Phe Ser Thr Lys Gln Thr Met Glu Gly Trp Asp Ala Ile Val Thr Cys Cys Glu

800         810         820         830         840         850         860         870         880         890
GCG CTC GGC ACG CGC GTC ACC GAG CAC GTCACC CAC TAC GGG AAG GGC ATC GAG GAC CGG CTG ACC GGC AAG CAC GAG ACC GCC CCC TGG AAC AAG TAC
Ala Leu Gly Thr Arg Val Thr Glu His Val Thr His Tyr Gly Lys Gly Ile Glu Asp Arg Leu Thr Gly Lys His Glu Thr Ala Pro Trp Asn Lys Tyr

900         910         920         930         940         950         960         970         980         990
TCC TGG GGC GCC TCG GAC CGC GGC GCC TCG GTC CGC ATC CCC TGG GCC GTC GAG AAG GCC AAG AAG GGC TGG CTG GAG GAC CGT CGT CCG AAC GCG
Ser Trp Gly Ala Ser Asp Arg Gly Ala Ser Val Arg Ile Pro Trp Ala Val Glu Lys Ala Lys Lys Gly Trp Leu Glu Asp Arg Arg Pro Asn Ala

        1000        1010        1020        1030        1040        1050        1060        1070        1080        1090
AAC ATG GAC CCG TAC CTG GTC ACC GCG CTC ATG ATC GAC ACC TGC TGC AGC GCC CTG GCC GGC GAC AAG CCG ACC CTG TTC GTG CCG TCA CAG ACC ACT
Asn Met Asp Pro Tyr Leu Val Thr Ala Leu Met Ile Asp Thr Cys Cys Ser Ala Leu Ala Gly Asp Lys Pro Thr Leu Phe Val Pro Ser Gln Thr Thr

        1100        1110        1120        1130        1140        1150        1160        1170        1180        1190
CCG GCG CCC GCC GAG GCG AGC GTC TGA GCC TGA CCGACAC GTCTGCCCGGA CCGGTCGGC CGAGCAGTCC GCCGGGCCGG AAACACGCCG GCGCGGCGGA
Pro Ala Pro Ala Glu Ala Ser Val End Ala End
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FIG. 3. DNA sequence of the *Frankia glnII* gene (GenBank accession no. M35050) and inferred protein sequence of GSII. The coding sequence begins with GTG. A putative ribosome-binding site is underscored, as are putative ATP-binding and glutamate-binding sites of the protein sequence (see text). A possible Ntr promoter sequence is overscored.

CcI3 and Ai17 was digested with *Kpn*I and *Sst*I, fractionated by electrophoresis in 0.4% agarose, denatured, and transferred to nitrocellulose membranes. The 2.1-kbp *Sal*I insert of plasmid pBJ196A containing the *B. japonicum glnII* gene was used as a probe. The hybridization conditions used (45% formamide and 40°C) should allow hybridization with about a 40% mismatch. The hybridization patterns of the putative *glnII* gene varied with the strain (Fig. 1). *Kpn*I digests yielded single 4.6-, 8.4-, and 2.5-kbp bands from CpI1, CcI3, and Ai17, respectively. *Sst*I gave single 2.9- and 6.5-kbp bands from CpI1 and CcI3, respectively, and two bands of 4.3 and 2.9 kbp from Ai17. We decided to clone the 4.6-kbp fragment from the *Kpn*I digest of CpI1.

Size-selected libraries were screened for the presence of *glnII* hybridizable DNA. Although conventional colony hybridizations failed to yield any clones hybridizing to *B. japonicum glnII*, several positive clones were readily identified from dot blots of DNA made from individual clones of the size-selected library. One plasmid, designated pFR101, was selected for further study. The insert from pFR101 was about 4.6 kbp and hybridized to the same bands on genomic blots of CpI1 and CcI3, as did the insert from pBJ196A (data not shown).

The putative *Frankia glnII* gene was localized to a 1.6-kbp *Sal*I fragment in the pFR101 insert by hybridization with *glnII* from pBJ196A. The *Sal*I fragment and adjacent regions were further subcloned and sequenced by dideoxynucleotide sequencing by using the strategy shown in Fig. 2; the entire gene was sequenced in the sense direction at least twice, and about 60% of the gene was sequenced in the antisense direction.

The *Frankia glnII* DNA and protein sequences are presented in Fig. 3. The gene is 1,056 bp in length and corresponds to 352 amino acids. Using the sequence of the *Bradyrhizobium glnII* as a guide, we tentatively identified the start of the coding region of *Frankia glnII* as corresponding exactly to the start of the region of sequence similarity with *Bradyrhizobium glnII*. The start codon in this alignment is GTG. The use of this codon for translational initiation is not unusual, since about 18% of characterized streptomycete genes begin with GTG rather than ATG (38). A consensus ribosome-binding sequence (5′-GGAG-3′) began 12 bases 5′ to the GTG start codon. Two TGA stop codons separated by a single alanine codon were found in-frame beginning at position 1057 downstream from the start.

GS requires ATP for its activity. The putative ATP-binding site, Lys-Pro-Ile-Pro-Gly-Asp-Trp-Asn-Gly-Ala-Gly-Ala-His-Thr is shown in Fig. 3; it resembles the consensus ATP-binding site, Lys-X-X-X-X-Gly-X-X-Gly-X-Gly-X-Lys-Thr (where X is any amino acid) found for several ATP-binding proteins (50). This region of GSII is highly conserved with similar regions found in virtually all GS proteins analyzed to date (19). Also indicated in Fig. 3 is a possible substrate-binding site, Asp-Arg-Gly-Ala-Ser-Val, that closely resembles a sequence, Asp-Arg-Gly-Ala-Ser-Ile-Val, near the Lys-27 residue in bovine glutamate dehydrogenase that has been implicated in binding α-ketoglutarate (33, 47). Of interest is the presence of two Arg residues that are conserved in other GSs (19) in and near this sequence; ADP-ribosylation of specific Arg residues inactivates eucaryotic GSs (28), so these residues may play a role in the active site of *Frankia* GSII.

A sequence with similarity to Ntr promoters was identified at position −31 before the start of translation. The putative promoter has the sequence (5′-GTCGCAC.....GTGCA-3′), which is quite similar to the consensus Ntr promoter (5′-CTGGCAC.....TTGCA-3′) identified from enteric organisms and *Bradyrhizobium* spp. (12).

TABLE 2. Summary of codon usage for *Frankia* sp. strain CpI1 *glnII*

Codon	Amino acid	Count (%)	Codon	Amino acid	Count (%)
TTT	Phe	0 (0.0)	ATT	Ile	0 (0.0)
TTC	Phe	10 (2.8)	ATC	Ile	18 (5.1)
TTA	Leu	0 (0.0)	ATA	Ile	0 (0.0)
TTG	Leu	2 (0.6)	ATG	Met	9 (2.5)
TCT	Ser	0 (0.0)	ACT	Thr	1 (0.3)
TCC	Ser	3 (0.8)	ACC	Thr	17 (4.8)
TCA	Ser	1 (0.3)	ACA	Thr	0 (0.0)
TCG	Ser	7 (2.0)	ACG	Thr	7 (2.0)
TAT	Tyr	1 (0.3)	AAT	Asn	0 (0.0)
TAC	Tyr	11 (3.1)	AAC	Asn	10 (2.8)
TAA	End	0 (0.0)	AAA	Lys	0 (0.0)
TAG	End	0 (0.0)	AAG	Lys	14 (4.0)
TGT	Cys	0 (0.0)	AGT	Ser	1 (0.3)
TGC	Cys	8 (2.3)	AGC	Ser	4 (1.1)
TGA	End	1 (0.3)	AGA	Arg	0 (0.0)
TGG	Trp	12 (3.4)	AGG	Arg	0 (0.0)
CTT	Leu	0 (0.0)	GTT	Val	0 (0.0)
CTC	Leu	6 (1.7)	GTC	Val	16 (4.5)
CTA	Leu	0 (0.0)	GTA	Val	0 (0.0)
CTG	Leu	11 (3.1)	GTG	Val	5 (1.4)
CCT	Pro	1 (0.3)	GCT	Ala	0 (0.0)
CCC	Pro	12 (3.4)	GCC	Ala	21 (5.9)
CCA	Pro	1 (0.3)	GCA	Ala	1 (0.3)
CCG	Pro	14 (4.0)	GCG	Ala	11 (3.1)
CAT	His	0 (0.0)	GAT	Asp	0 (0.0)
CAC	His	6 (1.7)	GAC	Asp	20 (5.7)
CAA	Gln	0 (0.0)	GAA	Glu	0 (0.0)
CAG	Gln	12 (3.4)	GAG	Glu	25 (7.1)
CGT	Arg	4 (1.1)	GGT	Gly	5 (1.4)
CGC	Arg	8 (2.3)	GGC	Gly	28 (7.9)
CGA	Arg	0 (0.0)	GGA	Gly	2 (0.6)
CGG	Arg	6 (1.7)	GGG	Gly	1 (0.3)

Codon usage. The coding region for the *Frankia glnII* gene had a G+C content of 68.7%, which agrees with an estimated total G+C content of 68.4 to 72.1% for *Frankia* strains in general (2). The genetic code permits the use of G or C in the third position in codons for all 20 amino acids and in the first position for Arg and Leu; no choice exists in the second position. Forty-one codons were used in the *Frankia glnII* sequence (Table 2). Of all codons used, 94.8% had either a G or C in the third position. All of the Arg codons and 17 of 19 Leu codons began with C. Thus, there is a strong bias toward the use of G and C in the first and third positions, a circumstance which is not unusual for organisms with a high G+C content. In the second position, 49.8% of the codons had either an A or T. The absence of extreme bias in the inferred second position provides strong evidence that the reading frame determined by sequence comparison with *B. japonicum glnII* is correct (7).

Comparison with *Bradyrhizobium* and *P. sativum* GSs. Considerable amino acid sequence similarity was noted between *Frankia* GSII and homologous proteins from *B. japonicum* and *P. sativum* (Fig. 4). The matrix patterns reveal that the regions of highest sequence similarity are in analogous positions in both the pea and bradyrhizobial enzymes. The total numbers of identical amino acids found when the CpI1 *glnII* product was compared with the *B. japonicum* and pea proteins were 155 of 352 (44%) and 167 of 352 (47.4%), respectively. The pea (357 residues) and CpI1 (352 residues) sequences were somewhat longer than the *B.*

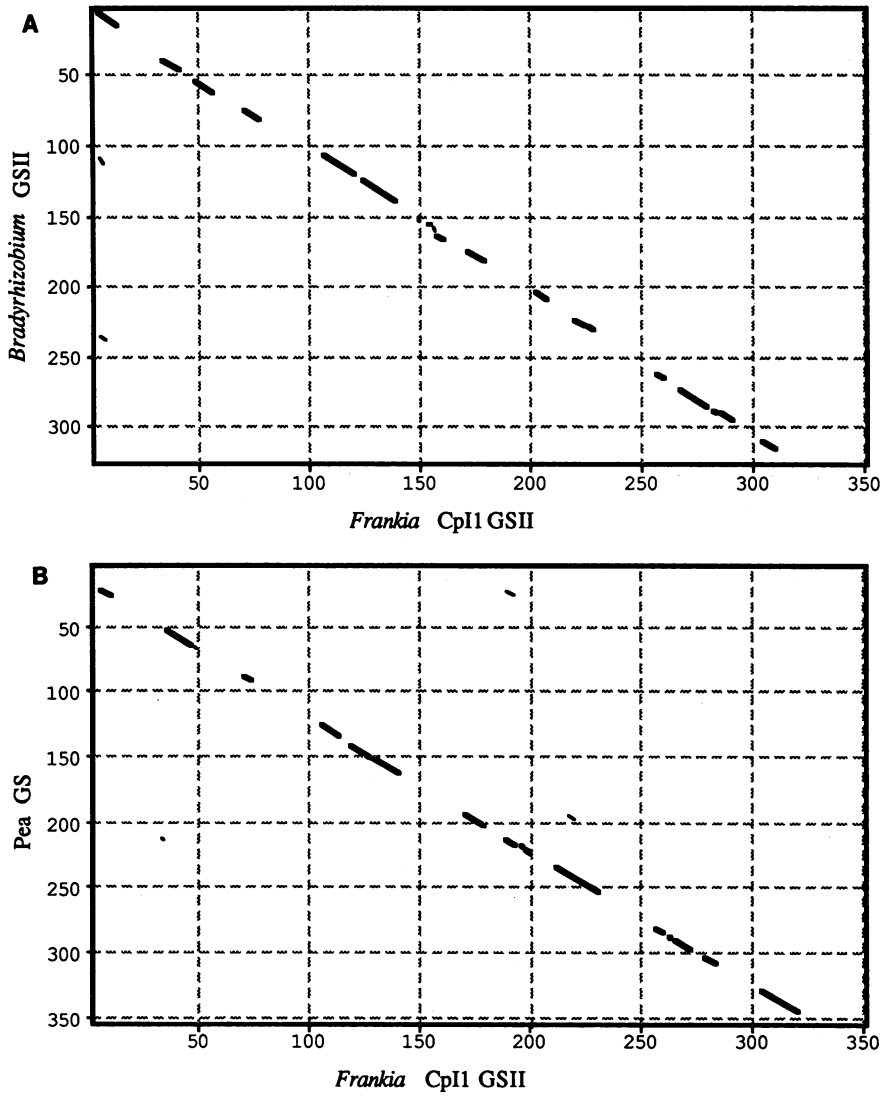

FIG. 4. Protein matix plots showing amino acid similarity between *Frankia* sp. strain CpI1 GSII (*x* axis) and GS from *P. sativum* (A) and GSII from *B. japonicum* (B). Matrices were plotted by Pustell Matrix Analysis (32) with a pam250 scoring mitrix. Parameters: window size, 8; minimum percent score, 60% similarity.

japonicum sequence (329 residues), so recalculating the percent identity when using conserved regions only gave 155 of 316 (49%) and 167 of 320 (52%) for *B. japonicum* and pea, respectively. Basing the calculation on the length of the genes from *B. japonicum* and pea gave 155 of 329 (47.1%) and 167 of 357 (46.8%), respectively. Calculating a percent similarity by categorizing amino acids into functionally similar groups (on the basis of charge and hydropathy) and using only conserved regions gave 188 of 316 (59.5%) and 206 of 320 (64.4%), respectively. Therefore, in any method of calculation the sequences from *B. japonicum* and pea were about equally similar to those from *Frankia* CpI1, with a slightly higher value for pea.

Expression of *Frankia glnII*. Plasmid pFR101 failed to complement the *E. coli* Δ*glnA* strain YMC11. The *glnII* genes from *B. japonicum* and *Rhizobium meliloti* are similarly not expressed from their own promoters in *E. coli* (12, 41). When pFR121a and pFR121b were transformed into *E. coli* YMC11, pFR121a but not pFR121b complemented the *E. coli* Δ*glnA* mutant when grown on M9 medium, indicating that *glnII* expression was being mediated by the *lac* promoter. The γ-glutamyltransferase assay showed that cells transformed with pFR121a had substantial GS activity (Table 3). Unlike wild-type GS activity from *E. coli* K-12, the

TABLE 3. Sensitivity of cloned *Frankia* sp. strain CpI1 GSII to heat treatment

Extract source	Heat treatment[a]	Sp act[b]
E. coli YMC11	−	0
	+	0
E. coli YMC11(pFR121a)	−	2.10
	+	0
E. coli K-12	−	0.243
	+	0.254

[a] Extracts were heated to 60°C for 5 min, allowed to cool to room temperature, and then placed on ice until assay.

[b] Specific activity is given in micromoles of γ-glutamylhydroxamate formed per minute per milligram of protein at 30°C.

activity in YMC11 containing pFR121a was heat labile, thus confirming the presence of GSII. Complementation was strongly enhanced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the medium (data not shown), further indicating that the gene was being transcribed from the *lac* promoter on pUC19.

DISCUSSION

On the basis of the nucleotide sequence analysis, the complementation of the *E. coli* Δ*glnA* strain YMC11, and the characteristics of the enzyme when expressed in *E. coli*, we conclude that the coding region isolated from the *Frankia* size-selected library corresponds to the GSII enzyme that was described previously from *Frankia* strain CpI1 (17). The presence of hybridizing bands in two other *Frankia* strains, one from *Alnus incana* subsp. *rugosa* and the other from *Casuarina cunninghamiana*, strongly implies that GSII is widespread among *Frankia* strains. We have also found hybridizing bands from the related nonsymbiotic soil actinomycete *Geodermatophilus* (18; data not shown), and we note that *glnII* has been cloned and sequenced from at least two *Streptomyces* species (4, 22).The finding of *glnII* sequences in several actinomycetes suggests that GSII is generally widespread among this group of organisms.

The presence of GSII genes in actinomycetes and in members of the *Rhizobiaceae* raises several important questions. The first question concerns the role of GSIIs in general metabolism. Their primary role seems to be that of ammonia assimilation during nitrogen limitation (48). This general role is suggested by in-depth studies on *glnII* expression in *B. japonicum*, *R. meliloti*, and *Agrobacterium tumefaciens*, in which transcriptional control is exerted by the *ntrC* (NR1) gene product of the Ntr system (15, 26, 35); the Ntr system itself is involved in global responses to nitrogen starvation (34). The physiological behavior of GSII in *Frankia* sp. strain CpI1 resembles that of an Ntr-controlled enzyme (48), and the identification of a putative Ntr-like promoter in the *Frankia glnII* gene described above supports the proposal that *Frankia* spp. possess the essentials of an Ntr system. Further characterization of the transcriptional regulation of *glnII* in *Frankia* sp. strain CpI1 is necessary before the extent to which comparisons can be made with the Ntr system in gram-negative organisms becomes clear.

Another question concerns the role of GSII in plant symbioses. The discovery of GSII first in members of the *Rhizobiaceae* and then in *Frankia* spp., which are also plant root nodule symbionts, implied a role in symbiotic function (14, 17). In light of present knowledge, however, the limitation of GSII to plant symbionts seems more apparent than real, perhaps because relatively few studies have been published on the nitrogen metabolism of actinomycetes and related organisms; those that have been published have not indicated the presence of a second glutamine synthetase (39). Therefore, GSII may have no special function in symbiotic organisms aside from assimilating ammonia under nitrogen-poor conditions. Evidence for this view comes from studies with rhizobial and bradyrhizobial mutants defective in GSI and/or GSII synthesis. Different Nod phenotypes have been observed depending on the organism. Mutants of *R. meliloti* 104A14 or *R. meliloti* 1021 with mutations in *glnA* or *glnII*, or double mutants lacking both GSs, are fully functional in symbiosis (15, 41), as are mutants of *R. meliloti* 1021 lacking a third GS encoded by the *glnT* locus (15). *A. tumefaciens* mutants with mutations in either *glnII* or *glnA* are similarly unaffected in their virulence (35).

Double mutants (*glnA*, *glnII*) of *B. japonicum* that are Gln^- are also Nod^- (12), suggesting that *Bradyrhizobium* Gln^- strains may be unable to obtain glutamine from the host, in contrast to *glnA glnII* double mutants of *R. meliloti* 1021, which are Gln^- Fix^+. The latter observation may indicate either that the *glnT* locus is involved in synthesizing glutamine in symbiosis or that *R. meliloti* is able to obtain glutamine from the host plant (15). In any case, GSII in these organisms seems not to be specific for the symbiotic state, but rather to be involved during growth on poor nitrogen sources (12). The enzyme may yet have some less specific role in symbiosis; GSII may reflect a type of nitrogen metabolism that predisposed certain bacteria to form plant symbioses.

Another question that arises concerns the origin of GSII. A proposal suggesting that *glnII*, the gene encoding GSII (10), was transferred to procaryotes from plants has been recently questioned on the basis that the protein sequence similarities between GSII from bacteria and the GSs from plants and from mammals are proportional to the phylogenetic divergence of these major groups (40). The observation that the *Frankia* GSII protein sequence has about as much similarity with the pea GS as with the bradyrhizobial GSII argues that the enzyme is highly conserved among procaryotes and eucaryotes and that a recent gene transfer between bradyrhizobia and frankiae is unlikely. A comparison of the *Frankia* and *Streptomyces* sequences may shed additional light on the relationships between GSII-like proteins.

It may be argued that GSII was a normal part of the ancestral procaryotic ammonia-assimilating enzyme complement and that members of the *Rhizobiaceae* and actinomycetes are among the few bacterial groups to maintain both enzymes. Support for this view is provided by a preliminary finding that *glnA* and *glnII* are separated by about 500 bp in *Frankia* sp. strain CpI1 (D. A. Rochefort, T. J. Hosted, and D. R. Benson, unpublished data). It is unlikely that *glnA* and *glnII* could have come together during the course of evolution. No such linkage has been found in *B. japonicum* (11), *R. meliloti* (15), or *A. tumefaciens* (35, 36).

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