Close Linkage of Genes Encoding Glutamine Synthetases I and II in *Frankia alni* CpI1

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Frankia alni CpI1 has two glutamine synthetases (GSs), GSI and GSII. The GSI gene (*glnA*) was isolated from a cosmid library of *F. alni* CpI1 DNA by heterologous probing with *glnA* from *Streptomyces coelicolor*. The *glnA* gene was shown to be located upstream of the GSII gene (*glnII*) by DNA-DNA hybridization. The nucleotide sequences of the 1,422-bp CpI1 *glnA* gene and of the 449-bp intervening region between *glnA* and *glnII* were determined, and the *glnA* amino acid sequence was deduced. In common with GSIs from other organisms, CpI1 GSI contains five conserved regions near the active site and a conserved tyrosine at the adenylation site. *F. alni* CpI1 *glnA* complemented the glutamine growth requirement of the *Escherichia coli* *glnA* deletion strain YMC11 but only when expressed from an *E. coli* *lac* promoter. While the functional significance of maintaining two GSs adjacent to one another remains unclear, this arrangement in *F. alni* provides support for the recently proposed origin of GSI and GSII as resulting from a gene duplication early in the evolution of life.

Frankia sp. strains are of interest for the roles they play as N₂-fixing root nodule symbionts of actinorhizal plants. Globally, such plants are important inhabitants of marginal soils and provide substantial input of new nitrogen, particularly in temperate forested regions (5). Little is known about the physiology or genetics of nitrogen metabolism in *Frankia* strains, since they have only recently been isolated and their growth is uniformly slow.

The central enzyme in nitrogen metabolism is glutamine synthetase (GS). In most bacteria, GS has two essential functions: it is involved in the primary assimilation of ammonia, and it provides the only means by which glutamine is made for protein synthesis. A product of N₂ fixation by nitrogenase is ammonia, and GS is therefore proximate to nitrogenase in mediating the assimilation of nitrogen in N₂-fixing cells.

Frankia spp. have two GSs, GSI and GSII (13), as do members of the bacterial families *Rhizobiaceae* (10, 15, 16, 39) and *Streptomycetaceae* (4, 23). *Frankia alni* CpI1 GSI is a dodecameric enzyme, with a subunit molecular weight (determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) of about 59,000 (13). It is subject to adenylation of individual subunits and is feedback inhibited by metabolites derived from glutamine (13, 44). Physiologically, GSI appears to be produced at similar levels under all conditions of growth examined (44, 47).

The *F. alni* CpI1 GSII has been studied biochemically and physiologically (13, 44), and the nucleotide sequence of the gene coding for it has been determined (32). It is an octamer of identical subunits and apparently is not adenylylated. Relative to GSI, GSII is heat labile. In CpI1 cells growing in combined nitrogen-free medium or in media containing amino acids degraded to glutamate, total GS activity increases severalfold, with GSII accounting for over 90% of

the total activity (44, 47). In cells growing on ammonia or on amino acids degraded to ammonia, neither GSII activity nor the polypeptide itself is detectable (26, 38, 44, 47). GSII is thus an inducible enzyme that is made in response to perceived nitrogen limitation.

The amino acid sequence of CpI1 GSII is similar not only to those of GSIIIs from other prokaryotes, but also to those of GS isoforms widely distributed in the eukaryotic cytosol and organelles (8, 39). For this reason, GSIIIs as a group have been used to estimate evolutionary distances (29), and GSI and GSII have been recently proposed to have originated from a gene duplication that occurred well before the divergence of eukaryotes from prokaryotes (22).

Here we report the cloning and nucleotide sequence of *F. alni* CpI1 *glnA*, the amino acid sequence of GSI, and the expression of CpI1 *glnA* in *Escherichia coli*. In addition, we show that *glnA* is located 449 bp upstream of *glnII*, an observation which has not previously been reported for organisms having multiple GSs and which may bear on the proposal that GSI and GSII are derived from an ancient gene duplication.

The bacterial strains and plasmids used, and their relevant characteristics, are shown in Table 1. *E. coli* XL1-Blue, DH5 α , and YMC11 were grown on Luria-Bertani medium, Terrific Broth, or M9 minimal medium containing 0.2% glucose (35); *E. coli* cells containing plasmids were grown in Terrific Broth with the appropriate antibiotic. *F. alni* CpI1 (*Frankia* catalog no. HFP070101 [24]), was grown on a succinate-ammonium-based medium at 30°C as previously described (38). With the acceptance of *alni* as a species designation within the genus *Frankia*, *Frankia* sp. strain CpI1 is now referred to as *F. alni* CpI1 (14).

Restriction enzyme digestions, subcloning procedures, plasmid extractions, and gel electrophoresis were carried out by standard methods (35). *E. coli* DH5 α and XL1-Blue cells were transformed either by electroporation (40) or by using frozen competent cells (18). DNA sequencing was performed by the method of Sanger et al. (36), with the TASequence version 2.0 sequencing kit (United States Biochemical Corporation, Cleveland, Ohio) using 7-deaza-2'-dGTP and a gel running temperature of 75°C to alleviate

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TABLE 1. Bacterial strains, phages, and plasmids

Strain, phage, or plasmid	Relevant characteristics	Source or reference
Strains		
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80dlacZ Δ M15 Δ (lacZYA-argF) U169deoR <i>recA1 endA1</i>	17
XL1-Blue	<i>hsdR17</i> ($r_K^- m_K^+$) <i>supE44</i> λ^- <i>thi-1</i> <i>gyrA96</i> <i>relA1</i>	6
	<i>endA hsdR17</i> ($r_K^- m_K^+$) <i>supE44</i> <i>thi-1</i> λ^- <i>recA1</i> <i>gyrA96</i>	
	<i>relA1 lac</i> [F' <i>proAB</i> ⁺ <i>lacI</i> ⁺ <i>lacZ</i> Δ M15 Tn10 (Tet ^r)]	
YMC11	<i>thi endA hsr</i> Δ <i>lacU169</i> <i>hutC</i> _{Klebs} Δ (<i>glnG-glnA</i>)	3
<i>F. alni</i> Cp11	From <i>Comptonia peregrina</i> , wild type	7
Phage and plasmids		
VCSM13	Helper phage	Stratagene
pBluescript II	pUC18-derived cloning vector	Stratagene
pKK223-2	Expression vector	Pharmacia
pFG1	Cosmid clone with <i>glnA</i> and <i>glnII</i> in pLAFR3	This study
pFG1-2.0S1	2.2-kb <i>SalI</i> subclone, orientation 1, in pBluescript II KS ⁺	This study
pFG1-2.0S2	2.2-kb <i>SalI</i> subclone, orientation 2, in pBluescript II KS ⁺	This study
pFG1-1.8Sm1	1.8-kb <i>SmaI</i> subclone, orientation 1, in pBluescript II KS ⁺	This study
pFG1-1.8Sm2	1.8-kb <i>SmaI</i> subclone, orientation 2, in pBluescript II KS ⁺	This study
pFG1-9P	9.0-kb subclone with entire <i>glnA</i> plus upstream region	This study
pKK- <i>glnA</i>	<i>glnA</i> expression clone in pKK223-2	This study
pFR121a	<i>glnII</i> expression clone	32
pFR101	4.5-kb <i>KpnI</i> clone with <i>glnII</i> and 3' end of <i>glnA</i>	31

band compressions and secondary structure formation due to the high G+C content of *Frankia* DNA. Single-stranded DNA for sequencing was obtained by the modified single-strand rescue protocol (Stratagene, La Jolla, Calif.). The IBI-MacVector program was used for analyzing DNA sequences.

Cloning *glnA* and location of *glnII*. Cosmids containing the Cp11 *glnA* gene were identified by probing nylon filters containing a dot blotted pLAFR3 cosmid library of Cp11 DNA with the ³²P-labeled, 2.3-kb *SstI* fragment from pSF203

containing the *Streptomyces coelicolor glnA* gene (46) (Fig. 1A). Each dot in Fig. 1 corresponds to the combined DNA from 12 cosmid clones contained in the master library that had been combined and dot blotted onto a nylon filter. Six dots showed strong hybridization to the *S. coelicolor glnA* probe. One cosmid designated pFG1 was chosen for further analysis after screening of the 12 clones represented in one of the positive dots by another round of dot blot hybridization.

The proximity of Cp11 *glnA* to *glnII* was found by probing the same filter with a ³²P-labeled 0.85-kb *SmaI-SalI* fragment of pFR109, which is an internal fragment of *F. alni* Cp11 *glnII* (Fig. 1B) (32). The blots gave a similar hybridization pattern with *glnA* or *glnII*, with two additional dots (F1 and G5) on the homologous blot of *glnII* (Fig. 1B), suggesting the proximity of *glnA* and *glnII* on the genome of Cp11.

A Southern analysis of pFG1 digested with *Bam*HI, *Kpn*I, *Sal*I, and *Sst*I and probed with *S. coelicolor glnA* showed a hybridization pattern almost identical to that of *Frankia* chromosomal DNA digested with the same restriction enzymes but probed with *Bradyrhizobium japonicum glnII* (data not shown). These results confirmed that *glnA* and *glnII* were closely linked on the *F. alni* Cp11 chromosome and also that pFG1 contained DNA that originated from Cp11.

DNA sequence analysis of *glnA*. For sequencing, a 5.0-kb *Pst*I-*Sst*I fragment of pFG1 that hybridized with the *S. coelicolor glnA* probe was subcloned into pBluescript II KS⁺ and designated pFG1-5.0P. From this subclone, a 2.0-kb *SalI* fragment containing the 5' end of *glnA* was cloned in both orientations into the pBluescript II KS⁺ vector to give pFG1-2.0S1 and pFG1-2.0S2. Nested deletions of these two subclones were used for sequence analysis. The 1.8-kb *SmaI* fragment of pFR101, which contains *glnII* as well as 1,100 bp of *glnA* in pUC19 (31), was cloned in both orientations into the pBluescript II KS⁺ vector to give pFG1-1.8Sm1 and pFG1-1.8Sm2. Nested deletions for sequencing were obtained with the Promega Erase-a-Base system (Promega Corp., Madison, Wis.).

FIG. 1. Dot blot hybridization of the *F. alni* Cp11 genomic library primary screening filter. The filters were probed with *S. coelicolor glnA* (A) and *F. alni* Cp11 *glnII* (B).

lational start shown was chosen on the bases of sequence alignments with *glnA* genes from other organisms and the observation that no other potential GTG or ATG start codons that are preceded by a potential ribosomal binding site are in the region. The start site is preceded by a Shine-Dalgarno sequence (5'-TGGAGG-3') which begins 10 bp before the ATG start site. This sequence is complementary to the sequence 3'-ACCUC-5' found at the 3' end of *Frankia* sp. strain ORS020606 16S rRNA (28). The *glnA* transcript appears to be monocistronic with a possible rho-independent transcriptional termination stem-and-loop structure at nucleotide positions 1652 to 1659 and 1666 to 1673 (Fig. 3). The open reading frame encodes a protein of 474 amino acids with a combined molecular weight of 53,978. This molecular weight is in reasonably good agreement with the size of the *Frankia* GSI subunit of about 59,000 estimated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13).

The G+C content of the *glnA* DNA sequence is 64 mol%, which is close to the overall G+C content of *Frankia* strains of 68.4 to 72.1 mol% (2, 14) and the G+C content of GSII of

FIG. 3. Nucleotide sequence of the *glnA* gene of *F. alni* Cp11 with upstream regions and the intervening sequence between *glnA* and *glnII*. The derived amino acid sequence is given beneath the nucleotide sequence. Features indicated include the putative Shine-Dalgarno sequences (underlined) and *glnA* transcriptional termination loops (underlined). The five conserved regions (I to V) and the adenylylation region of the *Frankia* GS are underscored and labeled. The putative adenylylation site at amino acid position Tyr-402 is in boldface. The beginning of *glnII* is shown with the amino acids indicated.

FIG. 4. Comparison of amino acid sequences of the five conserved regions and the adenylylation site of GSs from *F. alni* CpI1 (Fa), *S. coelicolor* (Sc) (46), *Anabaena* sp. strain 7120 (An) (45), *B. japonicum* (Bj) (8), *S. typhimurium* (St) (20), and *Bacillus subtilis* (Bs) (41). The amino acids are indicated by the single-letter code, and the positions of the first amino acids in each region are indicated. Identical residues are boxed.

68.7 mol% (32). As in many actinomycete genes, the codon usage is extremely biased at the third position, with a G or C residue occurring 92% of the time in CpI1 *glnA*.

The amino acid sequences of five regions that have been associated with the active site in type I GSs are shown compared with those of the analogous regions of CpI1 GSI in Fig. 4. X-ray-crystallographic studies of GSI from *Salmonella typhimurium* have shown that region I from the N-terminal domain of one subunit interacts with regions II to V of the C-terminal domain of an adjacent subunit to form the active site (1). Regions II and IV are β strands associated with two Mn^{2+} cations required for catalytic activity. Region II has the highly conserved amino acid motif [E V (X_{3-4}) Q (X_1) E] (19), and the CpI1 GSI region II has an exact match to this motif. Region IV has been implicated in the binding of glutamate because of some similarity to the bovine and chicken glutamate dehydrogenase substrate-binding sites of DRGASIV (11, 27, 30, 43). *F. alni* CpI1 GSII was found to have a close match for this motif (DRGASVRI) (32). The CpI1 GSI and other GS regions IV shown in Fig. 4 do not closely match this motif, but CpI1 GSI has matches at positions 2 and 4 and functionally similar amino acid substitutions at the third and fifth positions (NRSACCRI). The adenylylation region of CpI1 GSI also has strong homology to the other adenylylation regions. The adenylylation site of *Frankia* GSI is most likely Tyr-402, which is analogous to

the adenylylation site determined for *S. typhimurium* GS at position Tyr-397. In all conserved regions, CpI1 GSI is most similar to *S. coelicolor* GSI among the GSs shown in Fig. 4.

The 449-bp intervening region has some features of potential regulatory significance, including an 11-bp perfect inverted repeat spaced 211 bp apart (Fig. 3). The G+C content of the entire intervening region is 68 mol%, but the A and T residues are heavily clustered within four regions. A search for possible open reading frames in the intervening region revealed six possible translation initiation codons (five GTGs and one ATG), only one of which was preceded by a potential ribosome binding site. However, the putative peptide encoded would have been only 10 amino acids long.

Complementation of an *E. coli glnA* deletion strain. A plasmid (pFG1-9P) containing the entire *Frankia glnA* structural gene and about 500 bp of the upstream region was introduced into *E. coli* YMC11 (3) but failed to complement the glutamine auxotrophy of YMC11. Thus, polymerase chain reaction (PCR) amplification of *glnA* was done with primers (5'-GGGAATTCTGGAGGTCGAATGTTACAAAAGCC3'; 5'-GGGAATTCTGGCGGCCGACGGGAATCTCCACCG3') that created *EcoRI* restriction sites at the ends of the amplified DNA (37). The PCR was carried out with a PCR amplification kit (Perkin-Elmer Cetus, Danbury, Conn.) according to the manufacturer's recommended protocol, with CpI1 chromosomal DNA for amplification. The

TABLE 2. Sensitivity of cloned *F. alni* GSI and GSII to heat treatment

Extract source	Heat treatment (min) ^a	Sp act ^b
<i>E. coli</i> YMC11	0	0.018
	5	0.016
<i>E. coli</i> YMC11(pFR121a)	0	0.586
	5	0.008
<i>E. coli</i> YMC11(pKK- <i>glnA</i>)	0	0.200
	5	0.373
<i>E. coli</i> XL1-Blue	0	0.126
	5	0.145

^a Heat inactivation was done at 57°C. *E. coli* strains were grown in Terrific Broth to an optical density at 600 nm of 1.5 to 2.0. *E. coli* strains containing plasmids were induced with 0.048 µg of IPTG per ml. Crude extracts were prepared by sonicating in 20 mM imidazole-HCl buffer (pH 6.8)–2 mM MnCl₂–1% glycerol as described previously (32). Protein concentration was determined by the method of Lowry et al. (25).

^b Specific activity is given in micromoles of γ-glutamyl hydroxamate formed per minute per milligram of protein at 30°C.

1.6-kb PCR product was digested with *Eco*RI, ligated into the *Eco*RI site of pKK223-3, electroporated into *E. coli* YMC11, and plated onto M9 minimal plates containing 100 µg of ampicillin per ml, 0.048 µg of IPTG (isopropyl-β-D-thiogalactopyranoside) per ml, and 1 µg of thiamine per ml. Colonies growing on these plates were shown to contain a plasmid designated pKK-*glnA* consisting of the pKK223-2 vector with a *Frankia glnA* insert of 1.6 kb. This transcriptional fusion placed *Frankia glnA* under the control of the *E. coli lac* promoter and provided an *E. coli* Shine-Dalgarno sequence starting –10 bp upstream of the *glnA* ATG start codon.

Frankia GSI is stable at 57°C, unlike GSII, which is inactivated at this temperature (13). Crude extracts of YMC11 containing pKK-*glnA* or pFR121a (a plasmid expressing CpII GSII [32]) were assayed by the γ-glutamyl-transferase method as described previously (13) (Table 2). Extracts of YMC11(pKK-*glnA*) containing GSI retained GS activity at 57°C. As expected, YMC11(pFR121a) containing GSII lost almost all GS activity after 5 min. Extracts from the *glnA* deletion strain YMC11 showed essentially no GS activity while crude extracts from the positive control *E. coli* XL1-Blue showed wild-type GS activity. These results indicate that the GS being expressed from the pKK-*glnA* plasmid is *Frankia* GSI as characterized previously.

We conclude that the *F. alni* CpII *glnA* gene has been cloned through heterologous hybridization with the *S. coelicolor glnA* on the basis of the following evidence: (i) the original cosmid clone pFG1 and *Frankia* chromosomal DNA had identical restriction patterns when probed with *S. coelicolor glnA*, thus confirming that the characterized gene originated from CpII; (ii) *F. alni* CpII *glnA* has a high degree of sequence similarity to the phylogenetically close *S. coelicolor glnA* as well as other *glnA* sequences from both gram-positive and gram-negative bacteria, at both the nucleotide and amino acid sequence levels, particularly in the conserved regions forming the active site; and (iii) CpII *glnA* complements the *glnA* deletion mutation of *E. coli* YMC11 when expressed from an *E. coli lac* promoter, and heat-stable GS activity (γ-glutamyltransferase activity) is produced in transformed YMC11.

Like *F. alni* CpII *glnII*, CpII *glnA* is not expressed from its own promoter when cloned into *E. coli*. A similar observation has been made with other heterologous *glnA* genes in *E. coli*, including the *glnA* genes from *S. coelicolor*

(46) and *B. japonicum* (9). In fact, most euactinomycete promoters are not recognized in *E. coli* unless they resemble typical low-G+C-content *E. coli* promoters. Such sequences have been referred to as streptomycete-*E. coli*-like promoter sequences (21, 42).

CpII *glnA* is linked to *glnII*, which is located 449 bp downstream in the *Frankia* chromosome and is transcribed in the same direction. Kumada et al. (22) have recently speculated that extant GS genes have evolved from a gene duplication that occurred long before the divergence of prokaryotes and eukaryotes. They, and others (29), also suggested that GS genes behave as apparent molecular clocks for tracing the course of evolutionary history. In other organisms that have both GSI and GSII and that have been examined, including *Agrobacterium tumefaciens* (33, 34), *B. japonicum* (9), *Rhizobium meliloti* (12), *Streptomyces viridochromogenes* (4), and *Streptomyces hygroscopicus* (23), the genes for GSI and GSII are not linked. Their proximity in *F. alni* supports the argument for an ancient gene duplication, although the reason why they should have remained together during the evolution of *F. alni* is unclear but may reflect some unidentified functional dependence.

In culture, GSI is made under all conditions tested, and GSII is made under conditions that signal N limitation (13, 44, 47), but apparently neither is expressed in symbiosis (26). The transcriptional control of *Frankia glnII* may thus have similarities to the nitrogen-regulated transcriptional control of GSII in gram-negative bacteria, with additional levels of control in symbiosis. This hypothesis awaits support by additional information on the regulatory regions preceding both *glnA* and *glnII*.

Nucleotide sequence accession number. The nucleotide sequence of the *glnA* gene of *F. alni* CpII and the intervening sequence between *glnA* and *glnII* have been deposited with GenBank; accession numbers are L10631 and L10632, respectively.

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