Nucleotide sequence and characterization of the Rhodobacter sphaeroides glnB and glnA genes

V. Zinchenko, Y. Churin, V. Shestopalov and S. Shestakov

Department of Genetics, Biology Division, Moscow State University, Moscow, 119899, Russia

The glnA gene of Rhodobacter sphaeroides encoding glutamine synthetase (GS) has been cloned and sequenced. Molecular analysis revealed that there is a glnB gene upstream of glnA, in a single glnBA operon. A putative glnAp1-type promoter sequence, a consensus ntrC gene product binding site and a consensus upstream activator sequence were detected upstream of the glnB gene. The deduced amino acid sequences of the GS and GlnB proteins of R. sphaeroides showed strong homology with the same proteins from other Gram-negative bacteria. The sequence of the glnA gene isolated from glutamine auxotroph GlnB3 was also determined. The glnA83 mutation was shown to result in premature termination of GS synthesis and formation of a 17 kDa C-truncated GS which could be complemented by a 5’-truncated glnA gene which encodes a 30 kDa N-truncated GS. This phenomenon is characteristic for interallelic complementation.

Keywords: Rhodobacter sphaeroides, glutamine synthetase, glnA, glnB

INTRODUCTION

The purple non-sulphur photosynthetic bacterium Rhodobacter sphaeroides fixes nitrogen microaerobically or anaerobically when the level of intracellular fixed nitrogen is limited. Glutamine synthetase (GS: EC 6.3.1.2) plays a key role in nitrogen assimilation. In enteric bacteria, the gene glnA encoding GS is a member of the glnA-ntrBC (glnA1G) operon and is controlled by a complex nitrogen-regulatory system (ntr) that coordinately regulates a number of genes involved in nitrogen metabolism (Gussin et al., 1986; Leonardo & Goldberg, 1980; Pabel et al., 1982; Reitzer & Magasanik, 1985). For transcription, activation of glnA, the ntrA (glnF) and ntrC (glnG) gene products is required.

In Rhodobacter capsulatus, closely related to R. sphaeroides, the glnA gene is a member of the glnBA operon and is regulated in a manner fundamentally different from that in the Enterobacteriaceae (Kranz et al., 1990). Gene nifR, the ntrA equivalent in R. capsulatus, is not required for activation of glnBA. R. capsulatus gene nifR1, which was shown to be homologous to ntrC and which is required for transcription of nitrogen fixation genes, was responsible for approximately 50% of the glnBA transcription. The glnB gene and the 5’-terminal region of the glnA gene from R. capsulatus have been sequenced and a consensus ntrA1-binding site was not found (Kranz et al., 1990). It is possible that some genes, such as glnA, commonly referred to as ntr or nitrogen-regulated genes in the Enterobacteriaceae, are only partially nitrogen-sensitive in photosynthetic bacteria.

In this paper we report the cloning and sequencing of R. sphaeroides glnB and glnA genes, and their organization as an operon unit. The role of several characteristic sequences that have been identified upstream of the glnBA operon in regulation of glnBA expression are discussed.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids utilized in this study are listed in Table 1.

Media and growth conditions. Escherichia coli strains were grown either in complex LB medium or in minimal A medium (Miller, 1972) with appropriate supplements. R. sphaeroides strains were grown in Ormerod medium (Ormerod et al., 1961) containing 30 mM sodium DL-malate and 10 mM glutamine as sole nitrogen source. For growth tests, glutamine was replaced by 10 mM (NH₄)₂SO₄ or 10 mM glutamate. For determination of enzyme activity, R. sphaeroides was grown under anaerobic conditions and illuminated with a bank of three 60W in-
transformation into were constructed. Chromosomal R. sphaeroides 2R in the lambda vector L47.1 (Loenen et al., 1984). After thermal induction, the plasmid p276-derivatives with mini-Mu insertions were mobilized into Mu-immune E. coli strain M8820rif Mu by the conjugative plasmid RP1-7. Transconjugants were selected on medium supplemented with rifampicin and kanamycin. The plasmid p276-derivatives were then transferred into E. coli strain C600 by transformation.

**Construction of plasmid cointegrates in vivo.** It has been shown previously that cointegrates between pBR325 and RP1-derivatives are formed by reciprocal recombination and may be selected by using strains in which the plasmids based on ColEl replicon are not maintained (Zinchenko et al., 1984). Plasmids p171, p300, p276 and p276-derivatives with mini-Mu insertions were mobilized from E. coli strain C600 by the conjugative plasmid RP1-7 into E. coli strain C600 containing the pBR325 derivative p276 containing the 5' truncated glnA gene of R. sphaeroides was used as a hybridization probe against the lambda banks.

**Bacterial mating.** This was done as described previously (Zinchenko et al., 1984). Plasmid pAS8-121Δ3, which is not able to replicate in R. sphaeroides (Dubeikovsky & Kameneva, 1984), was used as a conjugative mobilizing plasmid.

**Mini-Mu mutagenesis.** Plasmid p276 was introduced by transformation into E. coli strain POI11734, which contains both a Mu::rbs prophage and the mini-Mu dillI1734 (Castilho et al., 1984). After thermal induction, the plasmid p276-derivatives

### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties*</th>
<th>Source or reference</th>
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<tr>
<td>C600</td>
<td>thi-1 thr-1 leuB6</td>
<td>Maniatis et al. (1982)</td>
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<td>P3478</td>
<td>tly polA1</td>
<td>Saano &amp; Zinchenko (1987)</td>
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<tr>
<td>P0111734</td>
<td>MC1040 (Mu::rbs) with Mu dillI1734 lac*</td>
<td>Castilho et al. (1984)</td>
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<tr>
<td>M8820 Mu</td>
<td>M8820 with Mu</td>
<td>Castilho et al. (1984)</td>
</tr>
<tr>
<td>M8820rif Mu</td>
<td>As M8820 Mu but rifampicin resistant</td>
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<tr>
<td>P678-54</td>
<td>thr ara leuB1 supE lacY minA minB</td>
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<td>ET8894</td>
<td>(rha-glnA-ntrBC) 1703::Mu::rbs2</td>
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<td>Ap Km IcI Tra'</td>
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<td>pBR325</td>
<td>Ap Tc Cm</td>
<td>Bolivar (1978)</td>
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<td>Cm Km glnA (R. capsulatus)</td>
<td>Scolnik et al. (1983)</td>
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<td>IncQ Su Sm Km Mob', broad host-range vector</td>
<td>Zinchenko et al. (1985)</td>
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<td>p110ES</td>
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<td>p300</td>
<td>Ap Cm glnA (R. sphaeroides)</td>
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* IcI, immunity to colicin E1; Inc, plasmid incompatibility group; Ap, ampicillin resistance; Cm, chloramphenicol resistance; Km, kanamycin resistance; Sm, streptomycin resistance; Su, sulphonamide resistance; Tc, tetracycline resistance; Tra', encodes transfer functions; Mob', mobilizable plasmid. The species origin of cloned genes is shown in parentheses.

candescent lamps. Antibiotics were used at the following concentrations (µg ml⁻¹): ampicillin, 150; chloramphenicol, 25; rifampicin, 50; tetracycline, 20 for E. coli and 1 for R. sphaeroides. The concentrations of kanamycin, streptomycin and for all strains were 50 µg ml⁻¹. The concentration of sulphanilamide (sulfathiazole-Na) was 200 µg ml⁻¹.

**Cloning of the glnA region.** Two gene banks of R. sphaeroides 2R in the lambda vector L47.1 (Loenen & Brammar, 1980) were constructed. Chromosomal R. sphaeroides DNA was completely digested with EcoRI or partially with Sau3A, then ligated to lambda L47.1 digested with EcoRI or BamHI respectively, and packed in lambda particles in vitro as described by Hohn (1979). Plasmid pJV27, containing the R. capsulatus glnA gene, was used as a hybridization probe against the lambda banks.

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Routine molecular biology techniques. Plasmid DNA isolation, restriction endonuclease digestion, agarose gel electrophoresis, ligation, nick-translation and Southern blot hybridization were performed using established techniques (Maniatis et al., 1982). DNA sequences were obtained by dideoxy sequencing using Sequenase (USB). The problem of
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**Fig. 1.** Physical map of the cloned *R. sphaeroides* DNA in recombinant phages and plasmids and complementation analysis of the mutant Gln83. The upper line indicates the DNA region in which *glnB* and *glnA* genes are located. The positions of genes shown are based on sequence analysis. Phages and plasmids are described in the text. Fragments of lambda phage L47.1 are indicated by double lines. Cloning vectors are shown in brackets. The capacity of the different DNA fragments to complement strain Gln83 for prototrophic growth, for restoration of GS activity and for restoration of nitrogenase activity to be repressed by ammonium ions is shown to the right of the figure: +, complementation; -, no complementation. B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; P, PvuII; S, SmaI; Sa, Sau3A. Only relevant Sau3A sites are shown.

**Fig. 2.** Location of mini-Mu d11734 insertion sites in the 4.79 kb BamHI-HindIII fragment of p276 and complementation analysis of the mutant Gln83. Numbers refer to the distance in kb from the BamHI site. The arrows show the direction of transcription of the lacZ gene in mini-Mu insertions. Other abbreviations are the same as in Fig. 1.

Band compressions in the sequencing gel was overcome by making use of deaza-dGTP (Pharmacia LKB). Minicells were used to visualize labelled proteins synthesized by various plasmids as described by Allibert *et al.* (1987).

**Enzyme assays.** Nitrogenase activity was determined by measurement of acetylene reduction as described by Willison & Vignais (1982). GS was determined by the y-glutamyltransferase assay (Shapiro & Stadtman, 1970). The protein content of
whole cells was measured by the Lowry method after lysis of cells with 0.2 M NaOH for 1 min in a boiling water bath.

RESULTS
Cloning and localization of the glnA region
Two recombinant phages, E91 and E101, containing different EcoRI fragments of 3.4 and 4.4 kb, and one recombinant phage, S215, containing a Sau3A fragment of about 12.6 kb, were isolated from R. sphaeroides gene banks, as described in Methods. Restriction and hybridization analyses revealed that the Sau3A fragment completely overlaps the 4.4 kb EcoRI fragment and partially overlaps the 3.4 kb EcoRI fragment (Fig. 1).

To localize the glnA gene within the cloned fragments, different deletion and insertional derivatives of these recombinant plasmids were generated and their ability to restore the wild phenotype of R. sphaeroides glutamine-dependent mutant Gln83 was tested. This mutant with a lesion in the glnA gene has a complex phenotype: absence of GS activity, derepressed nitrogenase synthesis and nitrogenase activity in the presence of ammonium ions (Zinchenko et al., 1991).

For deletion analysis, the 3.4 and 4.4 kb EcoRI fragments of recombinant phages E91 and E101 respectively, and the 10.9 kb HindIII fragment of the recombinant phage S215 and its subfragments were recloned into the multicopy broad host range vector pVZ2209 (Fig. 1); the resultant plasmids were transferred by mobilization into R. sphaeroides Gln83 and E. coli ET8894. As shown in Fig. 1, the 1.0 kb EcoRI–PvuII subfragment (plIOEP) was the minimal fragment tested that was sufficient for the restoration of the wild phenotype of the mutant Gln83. Neither plasmid restored the prototrophic phenotype of E. coli ET8894 (data not shown).

The molecular mass of the R. sphaeroides GS subunit, determined by SDS-gel electrophoresis is 50 kDa (Engelgardt & Klemme, 1982). Therefore, the R. sphaeroides glnA gene should encompass no less than 1.35 kb. Thus, these data indicate that the complementing 3.4 kb EcoRI and 1.0 EcoRI–PvuII fragments could comprise a truncated glnA gene.

For insertional analysis, the phage mini-Mu dIII1734 was used. The instability of mini-Mu insertional derivatives of recombinant plasmids based on pVZ209 was revealed in preliminary experiments. Therefore, for insertional analysis, the 3.4 kb EcoRI fragment from plasmid p152, and the 4.79 kb BamHI–HindIII and 3.95 kb BglII–HindIII subfragments from plasmid p202 were recloned into the vector pBR325 resulting in plasmids p171, p276 and p300 (Fig. 1). Although plasmids with ColE1-like replicons are not maintained in R. sphaeroides, the inheritance of these plasmids may be obtained by cointegration with mobilizing plasmids (Zinchenko et al., 1984). Cointegrates between plasmids p171, p276 and p300 and the conjugative plasmid RP1-7 were made, as described in Methods, and were introduced into strain Gln83. As shown in Fig. 1, mutant Gln83 was complemented by p276, but not by p171 or p300. In further mini-Mu insertional mutagenesis experiments, only plasmid p276 was used. From 110 mini-Mu insertions in p276, ten were chosen for further study. Others were either located in the vector or were duplicates of the ten selected. Each of these ten mini-Mu inserts was mapped by digestion with EcoRI, HindIII and BamHI. The results of the mapping and complementation analysis (Fig. 2) indicate that the glnA gene is located within the 2.2 kb region between the insertions at 0.5 and 2.7 kb.

Sequence of the glnA region
The fact that the 3.4 and 4.4 kb EcoRI fragments of the R. sphaeroides genome identified by hybridization with the R. capsulatus glnA gene are linked suggested that their joint EcoRI sites lie within the glnA gene. A DNA fragment of 2460 bp overlapping this EcoRI site was sequenced on both strands (Fig. 3). Two open reading frames (ORFs) were found in this sequence. ORF1, encoding a 112 amino acid polypeptide of 12067 Da, was assigned to glnB according to its similarity with other eubacterial GlnB amino acid sequences. The homology of the R. sphaeroides glnB gene product ranged from 64.3% identity with E. coli GlnB (Son & Rhee, 1987) to 84.8% with R. capsulatus GlnB (Kranz et al., 1990). Following the termination codon of ORFglnB, there is no inverted repeat structure which could play a role in transcription termination.

ORF2, distal to glnB, showed significant homology with prokaryote GS amino acid sequences. The homology of the R. sphaeroides glnA gene product ranged from 52.7% for the GS of Anabaena sp. strain 7120 (Turner et al., 1983) to 90.0% for the N-terminal region of R. capsulatus GS (Kranz et al., 1990). ORFglnA encodes a 467 amino acid polypeptide of 51961 Da.

A possible rho-independent termination signal was found close to the 3' end of ORFglnA (Fig. 3). Both glnB and glnA ORFs are preceded by a putative ribosome-binding site AAAGGG.

Codon usage frequencies for ORFglnB and ORFglnA reflect a G+C content, characteristic for R. sphaeroides.
(67%; Marmur & Doty, 1962), possessing strong bias toward the use of codons in which G and C predominate. In the *R. sphaeroides* *glnA* and *glnB* genes, G and C are used almost everywhere in the third position, except tyrosine, in which there is no obvious preference between TAT and TAC. Both *glnB* and *glnA* exhibit a G+C content of 63.9% and 63.1% respectively. The G+C content for the intercistronic regions varies from 57.3% (position 419–568) to 65.9% (position 905–989).

The absence of complementing activity of p300, based on pBR325 and containing the distal part of *glnB* and the total sequence of *glnA* (Figs. 1 and 3), indicates the lack of suitable promoters both in the vector plasmid and in the *glnB*-*glnA* intergenic region.

Sequence analysis of *glnA* confirmed the *EcoRI* site inside the *glnA* gene (Fig. 3) and that the plasmids p152 and p110EP, which are able to complement the mutant Gln83, contain a truncated *glnA* gene. This truncated gene contains only the 3'-terminal region of *glnA* without its 397 bp 5'-terminal region. The proximal portion of the 3'-terminal region (downstream of *EcoRI*) contains several ATG codons (at positions 1491, 1578, 1590, 1599 and 1605) encoding methionine. All of these ATG codons are preceded by sequences similar to ribosome-binding sites (Fig. 3).

It is possible that one of these ATG codons may be used as a transcriptional start codon in the presence of a suitable promoter. Such a promoter in the plasmids p152 and p110EP, based on the RSF1010 replicon, may be the promoter of the *suII* gene. Plasmid p171, based on pBR325 and containing a 3.4 kb *EcoRI* fragment similar to p152, lacks a suitable promoter.

One of the putative ORFs, corresponding to the longest possible translation, begins with ATG at position 1491 and ends with TGA at position 2391, and was designated ORF3 (Fig. 3). The molecular mass of the deduced amino acid sequence of ORF3 is 33426 Da.

The 4.4 kb *EcoRI* fragment of the plasmid p152 contains ORF3 and downstream nucleotide sequences, while the 1.0 kb *EcoRI*-*PvuII* fragment of plasmid p110EP contains a truncated ORF3 without the last 4 bp and stop codon since the *PvuII* cleavage site lies at a distance of 4 bp upstream of the end of ORF3. However, in the resultant plasmid p110EP, restoration of the *PvuII* site was accompanied by elimination of the last TGC codon and formation of a new TGA codon, according to the nucleotide sequence of plasmid RSF1010 around the *PvuII* site (Scholz et al., 1989).

### Identification of the plasmid p110EP-encoded proteins

The synthesis of plasmid-encoded proteins was studied in a mini-cell system. One novel polypeptide with apparent molecular mass of 30 kDa was synthesized from plasmid p110EP (as compared with the vector plasmid pVZ209), (Fig. 4). The data obtained indicate that this protein is encoded by ORF3, since this would encode a protein with a predicted molecular mass of 33426 Da. This result, taken in conjunction with complementation and sequence analyses (see above), suggests that the 30 kDa protein is the C-terminal portion of GS.

Gel electrophoresis under non-denaturing conditions of total protein from *R. sphaeroides* wild-type and Gln83 cells with plasmids p110EP or p152, followed by activity staining revealed that the molecular masses (about 600 kDa) of the GS from these strains are indistinguishable (data not shown).

### Analysis of the glnBA leader region

Several characteristic sequences have been identified upstream of the *glnB* gene (Fig. 3). The sequence GCACN₅TAGGGC, position 444 to 458, possesses 80% homology with the repressor binding site GCACN₅TGGTGC for the *ntrC* gene product (Dixon, 1984). Expression of the *glnA* gene in the *Enterobacteriaceae* is regulated at tandem promoters (Dixon, 1984; Reitzer & Magasanik, 1985). In *E. coli*, the upstream *glnApl* promoter has a sequence similar to the −35 and −10 RNA polymerase-binding consensus sequence, and is repressed by the *ntrC* gene product and requires CAP (Reitzer & Magasanik, 1985). The repressor binding site for the *ntrC* gene product overlaps the −35 RNA polymerase contact site and the transcriptional start site of

![Fig. 4. Expression of the 5'-truncated glnA gene in E. coli minicells. Proteins were labelled with [35S]methionine. Crude extracts were then analysed by SDS-PAGE, and the gel was autoradiographed. The minicell-producing strain P678-54 contained the following plasmids: lane 1, p110EP; lane 2, pVZ209. Molecular mass is indicated in kDa.](image)
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glnAp1. The downstream promoter glnAp2 resembles the NtrA-dependent promoters with conserved residues at −24 and −12 with respect to the transcriptional start site. In R. sphaeroides, the glnB leader region consensus sequence TTGCAC-N$_2$GGTCC at position 442 to 476 possesses a high degree of homology with the E. coli glnAp1 promoter sequence (−35)TTGCAC-N$_{18}$ (−10)TTCCAT. The proposed −35 consensus sequence of R. sphaeroides is overlapped, like glnAp1 in E. coli, with the ntrC gene binding site.

The R. sphaeroides sequence TGTTG-N$_6$CCGCA, localized at position 226 to 241, possesses 80% homology to the consensus upstream activator sequence (UAS) TGTYR-N$_6$YRACA of Klebsiella pneumoniae (Cannon et al., 1991; Gussin et al., 1986). The motif TCTGCCAGA proposed earlier may be involved in binding the capsulatus ntrC gene product (Preker et al., 1992); however, no sequence resembling this 9 bp motif was found upstream of glnB and glnA in R. sphaeroides.

Analysis of the previously sequenced R. capsulatus glnB leader region revealed the absence of an ntrA consensus promoter (Kranz et al., 1990). Our analysis of this region revealed characteristic sequences similar to E. coli −35 of glnAp1, ntrC-binding site and UAS (data not shown).

Cloning and sequencing the glnA83 mutant allele

Fragments of approximately 3–4 kb were isolated after separation of EcoRI-digested chromosomal DNA from R. sphaeroides strain Gln83 on a 0.7% agarose gel and ligated into EcoRI-digested pTZ18R. Recombinant plasmids containing fragments of glnA83 were isolated by hybridization with the 3′ and 4−4 kb EcoRI fragments of p152 and p229, respectively, as glnA probes.

Sequence analysis revealed that the mutation resulted in the substitution of G for A at position 1469 and in the replacement of GlnA83 was 17,527 Da.

DISCUSSION

In Escherichia coli and Klebsiella pneumoniae, the glnA gene is a member of the glnAntrBC operon (Leonardo & Goldberg, 1980; Pahel et al., 1982), whereas glnB is not linked with these genes and is located in a different region of the chromosome (Magasanik, 1982). In a number of micro-organisms – Rhodobacter capsulatus, Rhizobium leguminosarum, Bradyrhizobium japonicum and Azospirillum brasilense – the glnA gene is distal to glnB (Kranz et al., 1990; Colonna-Romano et al., 1987; Carlson et al., 1985; de Zamaroczy et al., 1990). In addition, R. capsulatus glnB and glnA genes belong to one operon, glnBA (Kranz et al., 1990).

The absence of expression of the glnA gene in plasmid p300, containing a DNA fragment with a deletion of the glnB 5′-terminus (Figs. 1 and 3) indicates that the R. sphaeroides glnA and glnB genes are organized in a glnBA operon. The absence of a recognizable terminator structure between glnA and glnB supports this conclusion. Therefore, unlike the Enterobacteriaceae, purple bacteria and probably some other micro-organisms in which glnB and glnA genes are tightly linked may show coordinated synthesis of glnB and glnA gene products as one of the regulatory mechanisms of nitrogen metabolism.

Analysis of the upstream regions of the R. sphaeroides and R. capsulatus glnB coding regions revealed similar characteristic sequences which may take part in glnBA operon regulation. In the Enterobacteriaceae, the glnA gene is transcribed from tandem promoters, in which the upstream promoter glnAp1 is negatively regulated and the downstream promoter glnAp2 is positively regulated by the products of the ntr system (Dixon, 1984; Reitzer & Magasanik, 1985). In R. sphaeroides and R. capsulatus, the glnBA operon leader regions contain only one promoter element, similar to glnAp1. As in glnAp1, the −35 consensus of this promoter is overlapped by the binding site of the ntrC gene product. The absence of the NtrA-dependent promoter with conserved residues at −24 and −12 in the upstream region of glnBA operon is in agreement with the data obtained on R. capsulatus, showing the glnBA operon to be NtrA (NifR4)-independent (Kranz et al., 1990). The absence of the canonical ntr-regulated promoter is a good explanation for the lack of expression of R. capsulatus (Sculnik et al., 1983) and R. sphaeroides glnA genes in E. coli cells. In purple bacteria, synthesis of GS is repressed when the cells are grown in media containing excess ammonia, and is derepressed in ammonia-limited conditions (Caballero et al., 1985; Johansson & Gest, 1976; Sakhno et al., 1981). Transcription of the glnBA operon is activated by the ntrC (nifR1) gene product (Kranz et al., 1990; V. Zinchenco, unpublished). Based on these data, the existence of a second, positively regulated promoter may be proposed.

The functional role of the sequences possessing high degree of homology with the UAS (nifA-motif) of K. pneumoniae and localized in the glnBA operon upstream region remains unclear. Consistent with a DNA-looping model, the nifA gene product binds at the UAS and contacts the sigma$^{34}$-RNA-polymerase complex at the −24 to −12 region by looping-out of the intervening DNA (Buck et al., 1987). However, no sequence similar to the −24 to −12 consensus sequence for ntrA-dependent promoters was identified upstream of the R. sphaeroides glnB gene.

The glnA83 mutation was shown to result in premature termination of GS synthesis and formation of a 17 kDa C-truncated GS which could be complemented by a 5′-truncated glnA gene encoding a 30 kDa N-truncated GS. These data indicate that the N- and C-terminal fragments of R. sphaeroides GS, which possess a molecular mass essentially equal to the mass of the monomer, can reassemble to form the dodecamer with restoration of the active sites. This phenomenon is characteristic for interallelic complementation.

In connection with the results obtained, it is possible to note that limited proteolysis of E. coli GS results in the inactivation of the enzyme, while the nicked protein
remains dodecameric (Dautry-Varsat et al., 1979; Lei et al., 1979). The proteolysis-sensitive site is known to be the central loop region of GS (Almassy et al., 1986), the same region in which the glnA83 mutation is located in R. sphaeroides GS.

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