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Genetics

# Physical and genetic characterization of the *glnA–glnG* region of the *Escherichia coli* chromosome

(regulation/nitrogen metabolism/glutamine synthetase/molecular cloning/GlnC phenotype)

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ABSTRACT **We have cloned and characterized a fragment of the *Escherichia coli* chromosome spanning *glnA*, the structural gene for glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2]. The fragment also carries *glnG*, whose product is necessary for regulation of *glnA* expression, and a previously unidentified gene whose function we have not discovered. Transcription of *glnA* and the newly identified gene occurs divergently from a region between the two genes. Transcription of *glnA* proceeds toward *glnG*, which is transcribed in the same direction. A region of DNA between *glnA* and *glnG* contains genetic information whose loss may result in the inability to reduce expression of *glnA* and other operons in response to ammonia (the GlnC phenotype).**

Glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2], the product of the *glnA* gene, plays a central role in the assimilation of ammonia in enteric bacteria. Cellular glutamine synthetase activity is regulated in response to the quality and abundance of the nitrogen source in the growth medium both at the level of enzymatic activity (by covalent modification—adenylylation) and at the biosynthetic level (for review, see ref. 1). Moreover, at least some of the mechanisms that regulate the biosynthesis of glutamine synthetase also regulate the expression of other operons and genes whose products are responsible for the utilization of nitrogenous compounds. One such operon, *hut*, produces the conveniently assayed enzyme histidase (2). We have cloned the *glnA* region of the *Escherichia coli* chromosome. This region also includes the *glnG* gene, whose product is involved in the regulation of glutamine synthetase biosynthesis (3, 4), and a third gene, whose role in nitrogen assimilation is unclear. We have determined the direction of transcription of each of these genes, and we have identified their products in minicells.

## MATERIALS AND METHODS

Standard techniques of molecular cloning were followed (see ref. 5). Restriction endonucleases, bacteriophage T4 DNA ligase, and polynucleotide kinase were obtained from commercial sources. DNA polymerase I was a gift of W. McClure. Bacterial strains used are listed in Table 1. Plasmid pBR322 (8) and phage λ132 (9) were used as vectors in cloning experiments. Derivative plasmids or phage are described in the text and Fig. 1. Media have been described (10). Clones carrying *glnA* were selected on media lacking glutamine. Antibiotic-resistant clones were selected on media containing an appropriate antibiotic at 20 μg/ml. Plasmid DNA was prepared by the method of Clewell and Helinski (11), and DNA from bacteriophage λ derivatives was prepared as described by Maurer *et al.* (12). Minicells were prepared and labeled with [$^{35}$S]methionine as described by Meagher *et al.* (7). Labeled polypeptides were examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (13). $^{14}$C-Labeled molecular weight standards were obtained from New England Nuclear. Assays of glutamine synthetase, histidase, and β-galactosidase have been described (14).

Table 1. Bacterial strains

| Strain | Description | Source or reference |
|---|---|---|
| ET6018 | *araD*, Δ*lacU169*, *strA*, *thi*, *rhaD*, Δ(*glnG–glnA*) | B. Tyler |
| ET6362 | *araD*, Δ*lacU169*, *strA*, *thi*, *rhaD*, *glnF*::Tn*10*, *glnG*::Tn*5* | D. Rothstein |
| GP8013 | *araD*, Δ*lacU169*, *strA*, *thi*, $hutC_{Klebs}$, Δ(*glnG–rha*) | G. Pahel |
| 294 | *pro*, *thi*, *endA*, *hsr* | Backman *et al.* (6) |
| YMC9 | Like 294, but Pro$^+$ Δ*lacU169* | P1 transduction (ET6018 × 294) |
| YMC10 | Like YMC9, but $hutC_{Klebs}$ | P1 transduction (ET8013 × YMC9) |
| YMC11 | Like YMC10, but Δ(*glnG–glnA*) | P1 transduction (ET6018 × YMC10) |
| YMC12 | Like YMC10, but *glnG*::Tn*5* | P1 transduction (ET6362 × YMC10) |
| YMC17 | Like YMC9, but *glnG*::Tn*5* | P1 transduction (ET6362 × YMC9) |
| P678-54 | *thr*, *leu*, *thi*, *supE*, *lacY*, *tonA*, *gal*, *mal*, *xyl*, *ara*, *mtl*, *min* | Meagher *et al.* (7) |

Subscript *Klebs* indicates a gene originating in *Klebsiella aerogenes*. Pro$^+$, proline-independent.

## RESULTS

DNA from *E. coli* strain 294 was digested with restriction endonuclease *Hin*dIII and cloned in the *Hin*dIII site of plasmid pBR322. The host strain for the cloning experiment (YMC11) had a deletion of the *glnA* region. Recipient cells were selected for glutamine prototrophy and resistance to ampicillin. Among the colonies that grew, one harbored a plasmid (named pgln2) carrying 11,500 base pairs (bp) of DNA inserted in the *Hin*dIII site of pBR322. Transfer of this plasmid confirmed that it carried *glnA*.

Using standard techniques, we mapped the locations of cleavage sites in pgln2 for a number of restriction endonucleases, as summarized in Fig. 1. We constructed numerous subclones of the 11,500-bp insert, and we analyzed these subclones to refine our mapping data.

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Abbreviation: bp, base pair(s).

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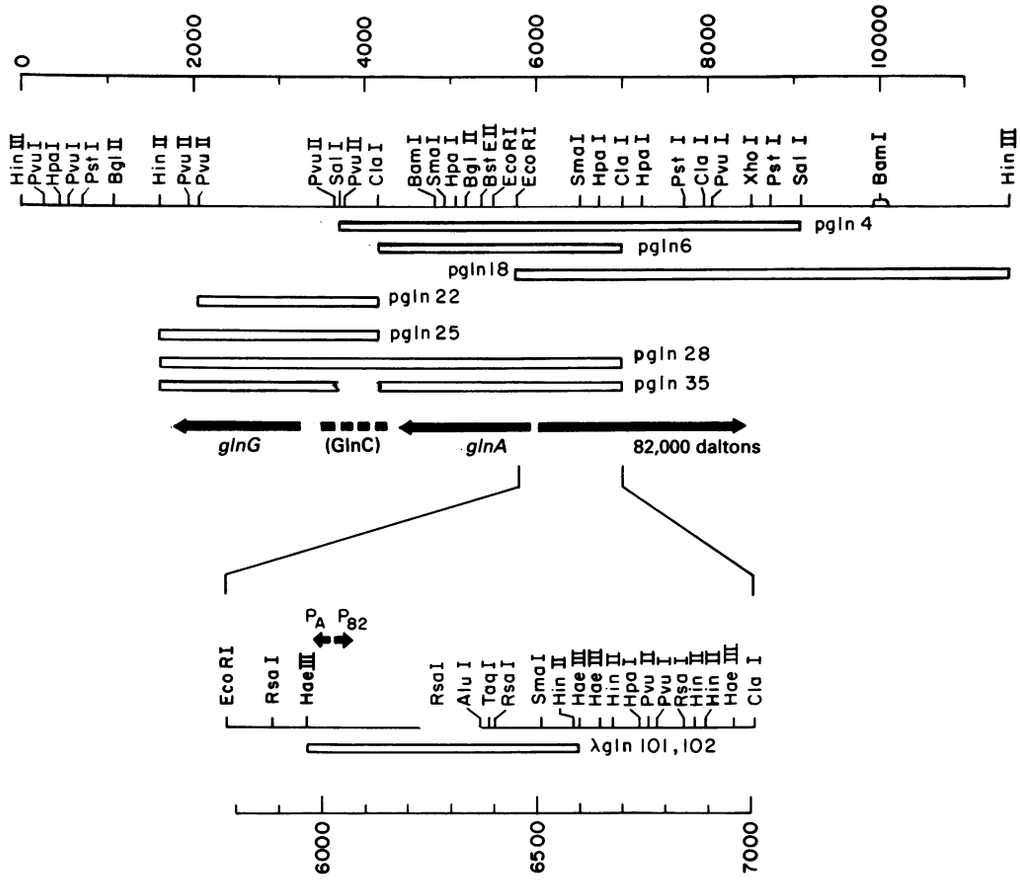


FIG. 1. Under a scale in bp is represented the *E. coli* DNA fragment contained in pgln2. This representation includes the map locations of numerous cleavage sites of various restriction endonucleases. The extreme right end of this region has not been extensively mapped; the location of a *Bam*HI cleavage site is given only approximately. Below this map, several empty bars represent the portions of DNA carried by other plasmids mentioned in the text. These are followed by solid arrows representing the extent and direction of transcription of each of the three genes discussed in the text. On the same level, a broken line indicates the general location of genetic information whose loss results in the GlnC phenotype. In the lower half of this figure, a segment of the region is represented in an enlarged scale and further information on the location of restriction endonuclease cleavage sites is given. Two short, solid arrows show the approximate locations of the promoters for *glnA* ($P_A$) and the gene described in the text ($P_{82}$). An empty bar represents the material carried in λgln101 and λgln102.

By examining the patterns of complementation of our plasmids, we determined the locations of *glnA* and *glnG*. The smallest plasmid carrying all of *glnA* is pgln6, which contains a 2800-bp fragment of *E. coli* specific DNA (from position 4150 to 7010 in Fig. 1). Plasmids containing DNA fragments extending rightward from the *Bam*HI site (position 4810) or leftward from the *Eco*RI site (position 5500) did not complement a chromosomal deletion of *glnA*.

Plasmid pgln6 (Fig.2, lane 4) directs the synthesis of two major polypeptides in minicells, other than those specified by the vector pBR322 (Fig. 2, lane 7). The size of one, between 55,000 and 60,000 daltons, approximates the size of the glutamine synthetase monomer (15). Because the DNA segment carried by pgln6 is not large enough to contain nonoverlapping coding sequences for two polypeptides of this size, we conclude that this polypeptide is the subunit of glutamine synthetase. The size of the other polypeptide specified by pgln6 is about 34,000 daltons. The heavy labeling of this polypeptide suggests that it is a fragment of the heavily labeled 82,000-dalton polypeptide that is encoded just to the right of *glnA* (Fig. 2, lanes 3 and 5). This assignment is strengthened by the lack of sufficient room to encode this polypeptide between the left end of the region (4150) and the *Bam*HI site (4810); as mentioned above, the *Bam*HI site is inside the region necessary for *glnA* expression. Thus, of the 2800 bp of *E. coli* DNA carried by pgln6, the rightmost 930 bp code for the amino terminus of a gene not identified previously (discussed below). Another 1500 bp are required to encode glutamine synthetase, and perhaps 75–100 bp are required for the promoter and control regions of each of the genes. This accounts for most of the 2800 bp in the region. It is unlikely that the *Cla* I site that bounds this region on the left falls within *glnA*, because the glutamine synthetase polypeptide determined by pgln6 in minicells is not detectably shorter than the analogous polypeptide determined by a plasmid carrying a substantially larger region spanning *glnA* (Fig. 2, lanes 2 and 3).

Immediately to the right of *glnA* is a cistron that produces an 82,000-dalton polypeptide (Fig. 1; Fig. 2, lane 5). Despite its proximity to *glnA*, this gene seems not to be implicated in glutamine or nitrogen metabolism. We examined a strain (ET8013) from which the entire *glnA* region, including this gene, had been deleted, and harboring plasmids carrying just *glnA* and *glnG*, or *glnA*, *glnG*, and the gene in question. As shown in Table 2, we can observe no effect of this gene on the expression or adenylylation state of glutamine synthetase.

We subcloned a 635-bp DNA fragment generated by *Hae* III digestion of the region between *glnA* and this gene. The fragment was cloned by using *Hin*dIII linkers in the *Hin*dIII site of the promoter probe vector λ132 (9). Although λ132 carries the *lacZ* gene, lysogens of this phage do not express this gene because it is not located downstream from a promoter. Insertion

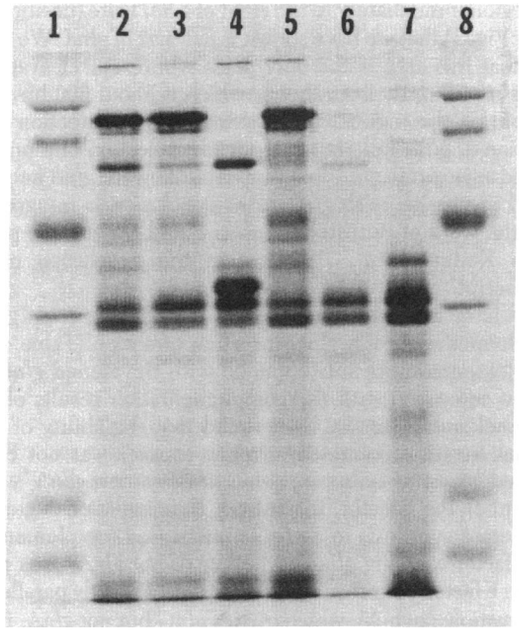


FIG. 2. The minicell-producing strain P678-54 was transformed with various plasmids. Minicells were prepared and labeled with [$^{35}$S]methionine. The polypeptides were resolved by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and visualized by autoradiography. Lanes 1 and 8, marker proteins (92,500, 69,000, 46,000, 30,000, 18,400, and 12,300 daltons); lane 2, pgln2; lane 3, pgln4; lane 4, pgln6; lane 5, pgln 18; lane 6, pgln25; lane 7, pBR322. A heavily labeled 82,000-dalton polypeptide (lanes 2, 3, and 5) is always accompanied by slightly smaller species; we believe this to be a fragment of the 82,000-dalton polypeptide. The *glnG* product (lane 6) is lightly labeled; this seems to be idiosyncratic to this particular minicell preparation, because a repeat of this labeling yielded a more heavily labeled *glnG* product (see Fig. 3).

of a promoter-bearing DNA fragment in the *Hin*dIII site of this phage permits expression of *lacZ* in lysogens. We examined derivatives of λ132 carrying the 635-bp fragment in each orientation; the orientation of the insert was deduced from the relative location of a *Sma* I cleavage site that is near one end of the fragment. From our observations (Table 3), we conclude that the fragment carries two divergently transcribing promoters. The promoter that normally transcribes away from *glnA* is not regulated by the source of nitrogen on which the lysogens are growing or by the product of *glnG*. The promoter that normally transcribes towards *glnA* is regulated in parallel with the *glnA* gene in the host chromosome; we conclude that it is the

Table 2. Lack of effect of a closely linked gene on glutamine synthetase expression

| Strain | Glutamine synthetase expression and adenylylation | |
|---|---|---|
| | Gln | Gln + $NH_3$ |
| ET8013/pgln2 | 1900 (0) | 300 (10) |
| ET8013/pgln28 | 1890 (1) | 350 (11) |

Strain ET8013 has a *glnG–rha* deletion, and therefore lacks *glnA* and the gene under investigation. ET8013 was used as a host for plasmids that did (pgln2) or did not (pgln28) contain the gene. Cells were grown in the presence of the indicated nitrogen sources, and level of expression and degree of adenylylation (indicated in parentheses) were measured. Activity is expressed in nmol/min per mg of protein. Adenylylation number indicates average number of monomers per glutamine synthetase dodecamer that have an adenylyl group.

Table 3. Regulation of promoters from the *glnA* region

| Strain | Glutamine synthetase | | β-Galactosidase | |
|---|---|---|---|---|
| | Gln | Gln + $NH_3$ | Gln | Gln + $NH_3$ |
| YMC9(λgln101) | 700 | 100 | 6840 | 820 |
| YMC9(λgln102) | 870 | 110 | 5540 | 5230 |
| YMC17(λgln101) | 75 | 90 | 260 | 240 |
| YMC17(λgln102) | 70 | 80 | 5720 | 5050 |

A 635-bp fragment of DNA was cloned in λ132 and found to contain a promoter capable of directing β-galactosidase expression in either orientation. The promoter that normally transcribes toward *glnA* is oriented toward *lacZ* in λgln101, and the promoter that transcribes away from *glnA* is oriented toward *lacZ* in λgln102. Lysogens of each phage were made in wild-type (YMC9) or *glnG*::Tn*5* (YMC17) hosts. Expression of glutamine synthetase (from the chromosomal *glnA* gene) and β-galactosidase (from the phage-carried *lacZ* genes) were measured in cells grown in the presence of the indicated nitrogen sources. Activities are expressed in nmol/min per mg of protein.

*glnA* promoter and that transcription of *glnA* is right to left in Fig. 1.

Our smallest plasmid carrying *glnG* is pgln25, which carries material between a *Hin*dII site (position 1600) and a *Cla* I site (position 4150). A similar plasmid, lacking only the leftmost 400 bp of this region (pgln22), did not complement the *glnG* insertion mutation in strain YMC12; in contrast, pgln25 did complement defects in the regulation of glutamine synthetase and histidase biosynthesis in strain YMC12 (Table 4).

In minicells, pgln25 directs the synthesis of one polypeptide (other than pBR322-specific peptides); this polypeptide is slightly smaller than glutamine synthetase (Fig. 2, lane 6; Fig. 3, lane 4). Because pgln22 directs the synthesis of a shorter polypeptide (Fig. 3, lane 3), we conclude that transcription of *glnG* is from right to left (as drawn in Fig. 1), and that *glnG* is located at the left end of the *E. coli* DNA carried by pgln25. Allowing about 1500 bp to code for *glnG*, we see that there is a space of approximately 1000 bp between *glnA* and *glnG*.

We constructed a plasmid, pgln35, carrying a deletion of 480 bp within the space between *glnA* and *glnG*. The behavior of this plasmid was compared with that of its parent, pgln28, when each was carried in strain YMC11, from which the *glnA–glnG* region is deleted. As shown in Table 4, pgln28 is able to confer normal regulation of both glutamine synthetase and histidase in YMC11. In contrast, pgln35 is unable to reduce expression of glutamine synthetase or histidase in media containing ammonia. This phenotype has been previously observed and called

Table 4. Expression of glutamine synthetase and histidase in plasmid-carrying strains

| Strain | Glutamine synthetase | | Histidase | |
|---|---|---|---|---|
| | Gln | Gln + $NH_3$ | Gln | Gln + $NH_3$ |
| YMC10 | 1080 | 185 | 380 | 130 |
| YMC12 | 55 | 80 | 115 | 115 |
| YMC12/pgln22 | 55 | 85 | 105 | 120 |
| YMC12/pgln25 | 1610 | 110 | 235 | 110 |
| YMC11/pgln28 | 2410 | 560 | 230 | 100 |
| YMC11/pgln35 | 5630 | 5710 | 270 | 310 |

Strains with the *glnA–glnG* region deleted (YMC11) or bearing an insertion in *glnG* (YMC12) were transformed with various plasmids and the expression of glutamine synthetase and histidase was measured in cells grown on the indicated nitrogen source. As a control, expression in untransformed YMC12 and YMC10 (wild type) was also measured. Values are given in nmol/min per mg of protein.

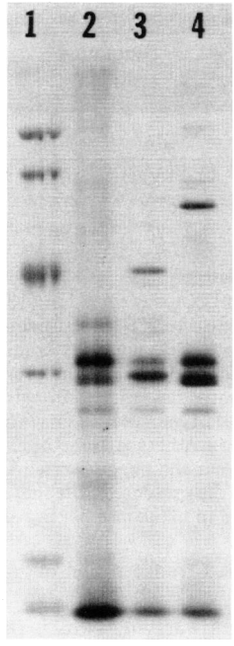


FIG. 3. Minicells harboring various plasmids were analyzed as described in the legend to Fig. 2. Lane 1, marker proteins; lane 2, pBR322; lane 3, pgln22; lane 4, pgln25.

the GlnC phenotype (10, 16). Our observation suggests that the GlnC phenotype may result from alterations wholly outside of *glnA* and *glnG*.

## DISCUSSION

The biosynthesis of glutamine synthetase is regulated in response to signals that reflect the availability of nitrogen in the cell. In order to understand the molecular nature of these signals, and the mechanisms by which they regulate the expression of *glnA* and other genes, we wish to construct a defined system that exhibits the features of *glnA* regulation. As a step toward such a system, we cloned *glnA* and adjacent DNA, and mapped *glnA* to a defined region of the DNA. We have identified the *glnG* gene and mapped it on our plasmid, and we have identified in minicells its polypeptide product, which is very similar in size to glutamine synthetase. We have also identified another gene carried on our plasmids. This gene, whose polypeptide product weighs 82,000 daltons and whose promoter is in close juxtaposition to the *glnA* promoter, neither is regulated by nor participates in the system that regulates *glnA*. There is no obvious relevance of this gene to nitrogen metabolism. For each of these genes, we have determined the direction of transcription (Fig. 1).

Certain of our results are at variance with those of Covarrubias *et al.* (17, 18) for *E. coli* and Koduri *et al.* (19) for *Salmonella typhimurium*. The 82,000-dalton polypeptide that we find encoded adjacent to *glnA* is not observed by Koduri *et al.* (19); this may be a consequence of the 2100-bp deletion adjacent to *glnA* that they describe in their plasmids. Covarrubias *et al.* (18) describe a polypeptide of 65,000 daltons from the region adjacent to *glnA*. The differences in the reported sizes may reflect differences in the gel systems or molecular weight standards used or perhaps a difference in the genes in the strains from which chromosomal DNA was originally cloned.

Both groups conclude that *glnA* is transcribed in the direction opposite to that proposed by us. To accomodate their conclusion, Covarrubias *et al.* (18) map a significant portion of *glnA* to a region immediately to the right of a *Sal* I site (position 3670 in our Fig. 1) that we have shown is not part of *glnA*. We further note that this area is the only place where our cleavage map differs significantly from theirs, which is about 600 bp shorter than ours in this region. Our assignment of the direction of transcription of *glnA* follows from our identification of a promoter located adjacent to *glnA* that transcribes toward *glnA* and is regulated in parallel with *glnA*. This conclusion is in agreement with the work of Rothstein *et al.* (14) and the note in proof in which Koduri *et al.* (19) reverse the conclusion of their manuscript.

We have located the *glnG* gene downstream from *glnA* by complementation of a *glnG* insertion mutation (Table 4). The complementation results obtained with the cloned *glnG* gene can be compared with the complementation results obtained by Pahel and Tyler (3). They found that the ability of a *glnG* mutant to utilize complex nitrogen sources was not complemented by an F factor carrying $glnG^+$ unless $glnA^+$ was also present on the F factor. In contrast, the regulation of glutamine synthetase synthesis in a *glnG* mutant was complemented by an F factor carrying $glnG^+$ whether or not $glnA^+$ was present on the F factor. Our results (Table 4) do not strictly parallel these observations: pgln25, which carries *glnG* but not *glnA*, restores normal glutamine synthetase but also restores the ability to produce histidase. One difference between our experiment and that of Pahel and Tyler (3) is the multicopy nature of our episome, suggesting that product levels of *glnG* may be important in regulation operons involved in nitrogen metabolism. In keeping with this notion is the observation of G. Pahel (personal communication) that *glnG* expression is regulated in response to the source of nitrogen in the growth medium. One model advanced to explain the unusual phenomena surrounding *glnG* postulates that *glnG* can be transcribed either from its own weak promoter or by stronger readthrough transcription from the *glnA* promoter (20). Our mapping of the positions of *glnA* and *glnG* and our determination of the direction of transcription of *glnG* and *glnA*, while not establishing the model, add to its plausibility.

If this model is valid, transcription of *glnG* from the *glnA* promoter would have to proceed across the region between *glnA* and *glnG*, whose absence leads to an inability to reduce glutamine synthetase and histidase expression in reponse to ammonia (GlnC phenotype; Table 4). Mutations conferring the GlnC phenotype have been claimed to be within *glnA* (21, 22), and postulated to be in *glnG* (3, 4). A recent study has shown that at least some GlnC mutations of *Klebsiella aerogenes* are not in *glnG* but may be in a gene belonging to the *glnA* unit of transcription (unpublished data). Our results show that an alteration wholly outside of *glnA* and *glnG* can result in a GlnC phenotype.

We imagine two likely ways in which a deletion between *glnA* and *glnG* might result in a GlnC phenotype. First, it might remove a site at which expression of *glnG* from the *glnA* promoter is ordinarily regulated. Second, it might result in the loss of a gene product. Although we have not identified in minicells a polypeptide coded between *glnA* and *glnG*, such a product might be poorly labeled by methionine or obscured by one of the other polypeptides present.

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