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gltBDF Operon of *Escherichia coli*

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A 2.0-kilobase DNA fragment carrying antibiotic resistance markers was inserted into the *gltB* gene of *Escherichia coli* previously cloned in a multicopy plasmid. Replacement of the chromosomal *gltB*$^+$ gene by the *gltB225*::Ω mutation led to cells unable to synthesize glutamate synthase, utilize growth rate-limiting nitrogen sources, or derepress their glutamine synthetase. The existence of a *gltBDF* operon encoding the large (*gltB*) and small (*gltD*) subunits of glutamate synthase and a regulatory peptide (*gltF*) at 69 min of the *E. coli* linkage map was deduced from complementation analysis. A plasmid carrying the entire *gltB*$^+$*D*$^+$*F*$^+$ operon complemented cells for all three of the mutant phenotypes associated with the polar *gltB225*::Ω mutation in the chromosome. By contrast, plasmids carrying *gltB*$^+$ only complemented cells for glutamate synthase activity. A major tricistronic mRNA molecule was detected from Northern (RNA blot) DNA-RNA hybridization experiments with DNA probes containing single genes of the operon. A 30,200-dalton polypeptide was identified as the *gltF* product, the lack of which was responsible for the inability of cells to use nitrogen-limiting sources associated with *gltB225*::Ω.

In many microorganisms undergoing nitrogen limitation, the concerted action of two enzymes, glutamine synthetase (EC 6.3.1.2) and glutamate synthase (EC 2.6.1.53), leads to net synthesis of L-glutamate from α-ketoglutarate and ammonia. In enteric bacteria, another enzyme, glutamate dehydrogenase (EC 1.1.1.1) also functions in the biosynthesis of L-glutamate by catalyzing the reductive amination of α-ketoglutarate with ammonia. Loss of both glutamate dehydrogenase and glutamate synthase, but not either enzyme alone, renders the cells auxotrophic for glutamate (4).

Glutamate synthase of *Escherichia coli* catalyzes the formation of two glutamate molecules from glutamine and α-ketoglutarate. The *E. coli* enzyme is composed of four dimers, each dimer consisting of two different subunits with molecular masses of 135 and 53 kilodaltons (20a, 24). In *Salmonella typhimurium*, the structural genes for the large and small glutamate synthase subunits have been termed *gltB* and *gltD*, respectively; both genes are contiguous and probably form an operon at 69 min of the linkage map (18). In *E. coli*, two genes apparently analogous to and having the same genetic organization as *gltB* and *gltD* of *S. typhimurium* have been cloned (12, 17). *gltB* mutations affecting biosynthesis of glutamate synthase in *E. coli* simultaneously impose the Ntr$^-$ phenotype (26) on the cells, i.e., the inability to utilize substrates such as arginine or proline, whose degradation yields either NH_4^+ or glutamate. *gltB* cells also display the Gsd$^-$ phenotype, which is the inability of the cells to derepress glutamine synthetase when they are cultured on glutamine, an N-limiting amino acid that supports sustained growth (8, 19). A relationship between glutamate synthase biosynthesis and the Ntr network is further suggested by the finding that the Ntr$^-$ phenotype of *gltB* mutants can be suppressed by mutations in *glnL* (*ntrB*) (19, 26) which result in a high concentration of glutamine synthetase regardless of the nitrogen source of the medium (GlnC phenotype).

In this work, we established the existence in *E. coli* of a *gltBDF* operon comprising genes coding for the large (*gltB*) and small (*gltD*) glutamate synthase subunits and a third downstream gene, *gltF*, the product of which appears to be involved in Ntr regulation.

MATERIALS AND METHODS

Bacteria and bacteriophage strains. All of the bacterial strains used were derivatives of *E. coli* K-12, except RR1, which is an *E. coli* K-12–B hybrid (Table 1). Strain PA340 carries a Δ*gltBDF* mutation in addition to an unmapped mutation that affects the level of expression of the wild-type *gltBDF* operon, as in plasmid pRSP21, when introduced into PA340 cells by transformation. A strain carrying only the deletion was obtained by transducing the *argG31* derivative MX971 with P1 propagated on PA340. Among *argG*$^+$ transductants unable to make glutamate synthase activity, one was isolated and termed MX988. Inheritance of the Δ(*gltBDF*) mutation by this strain was confirmed by colony hybridization with plasmid pGOP3 or pRSP21 (which contain *gltB* and *gltBDF* genes, respectively, see Fig. 1) as a probe. *rpoN73*::Tn*5* was formerly known as *glnF73*::Tn*5* (9).

Culture media. Complex LB broth and NN minimal medium have been previously described (11). The additions to NN (final concentrations) were 0.2% glucose as the carbon source and 15 mM NH_4Cl for N excess medium or 1 mg of L-glutamine per ml for N-limiting medium. Other nutritional requirements were satisfied by adding the filter-sterilized compounds at predetermined concentrations (1). The final concentrations of antibiotics were as follows: kanamycin, 30 μg/ml; spectinomycin, 20 μg/ml; streptomycin, 100 μg/ml; tetracycline, 10 μg/ml; ampicillin, 100 μg/ml.

Plasmids. All of the plasmids used are shown in Table 1. Plasmid DNA was purified as described by Betlach et al. (5). The recombinant plasmids constructed were derived from pBR322 (6), pBR327, or pBR328 (31).

Enzyme activity determinations. Glutamine synthetase activity was determined by the γ-glutamyltransferase assay (11); specific activities are expressed as nanomoles of γ-glutamyl hydroxamate formed per minute per milligram of protein at 37°C. Glutamate synthase and glutamate dehydro-

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TABLE 1. Bacterial strains and plasmids

E. coli strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
RR1	*hsd-20* (*hsdR hsdM*)$_B$	6
P678-54	F *thr leu thi-1 supE lacY tonA gal mal xyl ara mtl min*	21
PolATs	*polA214*(Ts)	15
PA340	*gltB31 gdh-1*	B. Bachmann
MX614	Δ(*pro-lac*) *galE ilv-680 thi-1*	3
MX848	*rpoN73*::Tn*5*	9
MX971	Same as MX614 but *argG31*	9
MX1172	Same as MX614 but *gdh-1*	This work
MX1176	Same as MX614 but *gdh-1 gltB225*::Ω	This work
MX1178	Same as MX614 but *gltB225*::Ω	This work
Plasmids		
pRSP20	*gltB*$^+$*D*$^+$*F*$^+$ derivative of ColE1	12
pRSP21	*gltB*$^+$*D*$^+$*F*$^+$ Cm^r derivative of pRSP20	This work
pGOP1	*gltB*$^+$ *gltD*::pBR328 *gltF*$^+$ Ap^r Tc^r derivative of pRSP20	12
pGOP3	*gltB*$^+$ Tc^r derivative of pBR327	12
pGOL2	*gltD*$^+$ Ap^r derivative of pBR327	12
pGOP3::Ω	*gltB225*::Ω Tc^r Spc^r Spm^r derivative of pGOP3	This work
pGOG1	*gltF*$^+$ Ap^r derivative of pGOP1	This work
pGOG2	Same as pGOG1 but *gltF1*::Ω Ap^r Spc^r Sm^r	This work
pGOG3	*gltF*$^+$ Tc^r derivative of pBR322	This work

genase activities were determined by the methods of Meers et al. (22); specific activities are expressed as nanomoles of NADPH oxidized per minute per milligram of protein at room temperature. Protein was measured by the method of Lowry et al. (16), with bovine serum albumin as the standard.

Reagents. Enzymes were obtained from P-L Biochemicals, Inc. (St. Goar, Federal Republic of Germany). Amino acids, vitamins, and antibiotics were from Sigma Chemical Co. (St. Louis, Mo.). Radiochemicals were from Amersham Corp. (Arlington Heights, Ill.). LB medium ingredients were from Difco Laboratories (Detroit, Mich.), and other reagents were of analytical grade.

Phenotypic characterization. The pleiotropic Ntr phenotype was scored as cell growth on 0.2% L-arginine as the only nitrogen source (Aut). The ability of cells to derepress their glutamine synthetase under nitrogen-limiting conditions of growth (Gsd) is another Ntr-associated phenotype (13, 14, 29). Thus, the specific activity of glutamine synthetase of cells grown with L-glutamine as the sole nitrogen source is a quantitative measure of the Ntr function. The enzyme made under this condition is fully active biosynthetically, i.e., unadenylylated. In fact, glutamine synthetase derepression is more sensitive to low levels of the general Ntr activator NR_I than are other Ntr-associated phenotypes, such as Aut (27). Certain *E. coli* mutants, for instance, may weakly derepress their glutamine synthetase, clearly indicating Ntr functionality, yet insufficient to make them Aut$^+$ (see Table 2).

Throughout this paper, a distinction between the Gsd and Ntr phenotypes is kept only for the eventuality that an additional regulatory factor (i.e., the *gltF* product [see Results and Discussion]) could directly derepress glutamine synthetase without activating the entire Ntr system.

Genetic and recombinant DNA procedures. Preparation of P1 *virA* lysates and transduction protocols were as described by Miller (23). *E. coli* cells were prepared for transformation as described by Cohen et al. (10).

For construction of the *gltB225*::Ω insertion, plasmid pGOP3 (12), which contains the complete *gltB* gene for the large glutamate synthase subunit (Fig. 1), was cut at the *Bgl*II site, which interrupts this gene at 1.3 kilobases from its 5′ end. On the other hand, DNA of plasmid pHP45Ω (28) was digested with enzyme *Bam*HI, which generates two fragments of 2.3 and 2.0 kilobases. The 2.0-kilobase DNA fragment has been shown to contain the gene which confers resistance to streptomycin and spectinomycin (Sm^r Spc^r), in addition to transcription and translation termination sequences (28). This so-called Ω fragment was ligated to pGOP3 previously linearized with *Bgl*II, and the ligation mixture was used to transform RR1 competent cells (Table 1). Selection was performed on LB plates supplemented with streptomycin. The Sm^r colonies that appeared upon incubation were tested for the accompanying Spc^r and Tc^r phenotypes. Restriction enzyme mapping of one of the resulting plasmids confirmed the insert at the *Bgl*II site of pGOP3, thus interrupting *gltB* within the 5′ half of the gene.

To subclone the *gltF* gene, plasmid pGOP1 (13; Fig. 1) was digested with *Sma*I, and *Bam*HI linkers were added. After *Bam*HI digestion, the mixture was ligated and used to transform *E. coli* RR1 cells; selection was done for Ap^r (Tc^s) to allow for recircularization from the *Bam*HI site in pBR328 to the *Sma*I site (to which *Bam*HI linkers had been added) in pGOP1 without interruption of *gltF*. Ap^r Tc^s transformants were screened for the presence of the desired plasmid by restriction enzyme analysis. The resultant plasmid, pGOG1 (Fig. 1), contained a 1.8-kilobase chromosomal fragment immediately downstream of *gltD*.

To construct the *gltF1*::Ω insertion, plasmid pGOG1 was cut at the *Hin*dIII site that interrupts *gltF* (Fig. 1) and ligated with the Ω DNA fragment carrying *Hin*dIII ends. The ligation mixture was used to transform RR1 competent cells with selection for Ap^r Sm^r Spc^r transformants. Restriction enzyme mapping of the resultant plasmid, pGOG2, confirmed that the Ω fragment was inserted at the unique *Hin*dIII site.

With the purpose of constructing a plasmid in which *gltF* was transcribed from the anti-*tet* promoter of pBR322, we subcloned the chromosomal fragment of pRSP20 (12; Fig. 1) from its second *Eco*RI site immediately downstream of *gltD*

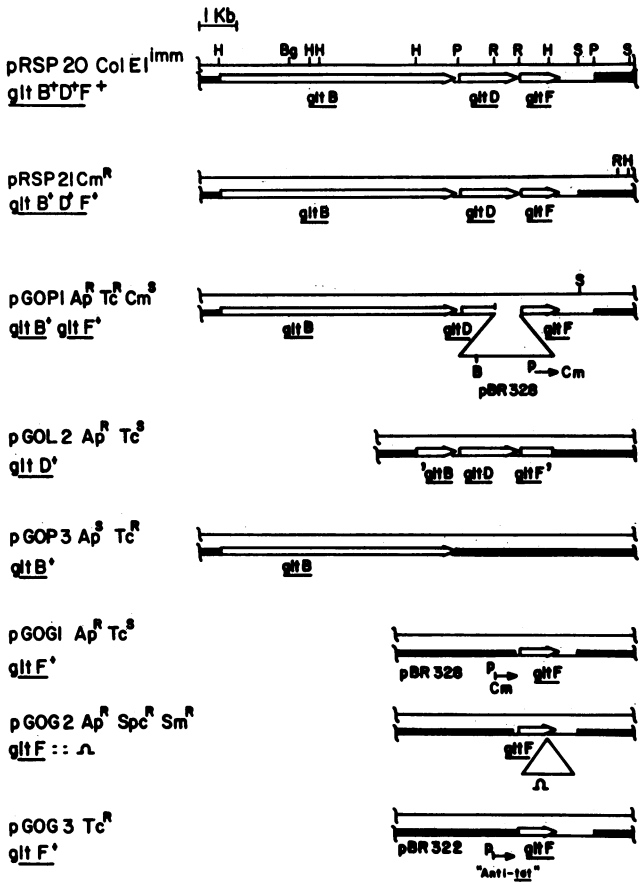

FIG. 1. Schematic representation of the plasmids used. The arrowheads indicate direction of transcription. Vector DNA is not drawn to scale. Only relevant restriction enzyme sites are shown in pRSP20 (12), pRSP21, and pGOP1. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; S, *Sma*I. In plasmid pGOP1, the small *Eco*RI fragment that makes up the *gltD* 3′ end was substituted by *Eco*RI-linearized pBR328 (12). In pGOG2, the Ω fragment (Spc^r Sm^r) was inserted at the *Hin*dIII site. Relevant vehicle promoters (P Cm and P anti-*tet*) and their direction of transcription are indicated (32). Superscripts R and S indicate resistance and sensitivity, respectively, to the antibiotic. Prime symbols, as in '*gltB* and *gltF*', indicate discontinuity at the 5′ and 3′ ends, respectively. In pGOL2, only *gltD* is complete. The carboxy terminus of *gltF* was deleted during the construction of this plasmid. Symbols: —, chromosomal DNA; ▭, coding regions; ▬, vector DNA.

up to the *Pst*I site of the vector. This fragment of approximately 2.8 kilobases was cloned into plasmid pBR322 previously digested with *Eco*RI and *Pst*I. Transformation of RR1 competent cells led to Tc^r (Ap^s) clones carrying plasmid pGOG3 in which *gltF* is transcribed from the anti-*tet* promoter.

Northern (RNA) blot RNA-DNA hybridizations. Total cellular RNA was isolated as described by Aiba et al. (2). Agarose gels containing 2.2 M formaldehyde were prepared, and the RNA preparations were electrophoresed as described before (20), except that 1 mM Na_2HPO_4 and 1 mM KH_2PO_4 were added to all of the buffers (pH 6.0). Gel transfers and nitrocellulose filter hybridizations were carried out as described before (33).

Polypeptide synthesis by minicells. Minicells were purified, by three sequential sucrose gradient centrifugations (21), from strain P678-54 (Table 1) previously transformed with the different plasmids. The minicells were suspended in methionine assay medium (Difco) and incubated with ^{35}S-labeled L-methionine (50 μCi/ml) for 30 min at 37°C with shaking. Radioactively labeled minicells were centrifuged and suspended in 50 μl of sample buffer (10% glycerol, 2.3% sodium dodecyl sulfate, 0.625 M Tris [pH 6.8], 5% β-mercaptoethanol) and boiled from 2 to 4 min before 25-μl samples were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels.

RESULTS AND DISCUSSION

Characterization of the *gltB225*::Ω mutation. The *glt B225*::Ω insertion was constructed as described in Materials and Methods. We induced homologous recombination of the *gltB225*::Ω allele into the chromosome by transforming a *polA*(Ts) strain with plasmid pGOP3 (*gltB225*::Ω) at 30°C. Since this strain bears a temperature-sensitive DNA polymerase I, it is unable to replicate ColE1-type plasmids at 42°C (15). After 5 h at 42°C, the cell suspension was spread on LB-streptomycin-spectinomycin plates. Independent colonies that appeared upon incubation at 37°C were screened for tetracycline sensitivity. Of 800 colonies tested, 2 had the desired Tc^s Sm^r Spc^r phenotype. P1 *virA* was propagated on one of these strains, and the resulting lysate was used to transduce strain MX1172 (*gdh-1*) to Sm^r and Spc^r. P1 *virA* grown on one of the transductants, MX1176, was then used to transduce strains MX614 (wild type), MX848 (*rpoN73*::Tn*5*), MX971 *argG31*, and MX1172 (*gdh-1*) to Sm^r and Spc^r (Table 1). Counterselection was done for Aut$^-$, Km^r Gln$^-$, Arg$^+$ Aut$^-$, and Glt$^-$, respectively. The results showed *gltB225*::Ω cotransduction with *argG31* (40%) and *rpoN73*::Tn*5* (95%) but not with *gdh-1*.

To check whether the recombined chromosomal mutation was the same as that in plasmid pGOP3, DNA-DNA hybridization was carried out with pGOP3 DNA as the probe. The results confirmed that homologous recombination had occurred in the transductants (data not shown).

Transcription of *gltBDF* as an operon. Bulk RNAs from four different strains were purified from cultures grown on either 15 mM NH_4Cl or 0.2% glutamate as the sole nitrogen source. The search for *gltBDF*-specific mRNA transcripts was carried out with three different hybridization probes: a 1.8-kilobase *Hin*dIII-*gltB* internal fragment, a 0.6-kilobase *Eco*RI DNA fragment corresponding to the 3′ half of *gltD*, and a 0.45-kilobase *Hin*dIII-*Bam*HI fragment downstream of *gltD* which includes part of *gltF* but is devoid of *gltB* and *gltD* sequences (Fig. 2).

A major large transcript of approximately 7.5 kilobases was detected from MX614 or MX614(pRSP21) cells with each of the three probes tested. The transcript is 1.5 kilobases longer than the size expected for a *gltB-gltD* mRNA, and so it carries enough RNA to encode synthesis of a small *gltF* polypeptide. No such transcript was detected with any of the probes in the total RNA purified from either MX1176 (*gltB225*::Ω *gdh-1*) or PA340 (Δ*gltBDF*).

Whether other mRNA bands in the gels correspond to transcripts of either one or two genes of the operon or to degradation products of the three gene mRNAs requires more careful examination.

Our results also indicate that synthesis of the 7.5-kilobase RNA is repressed by glutamate when this is used as the sole nitrogen source in the growth medium (compare lanes G and N of Fig. 2), in agreement with the previously observed

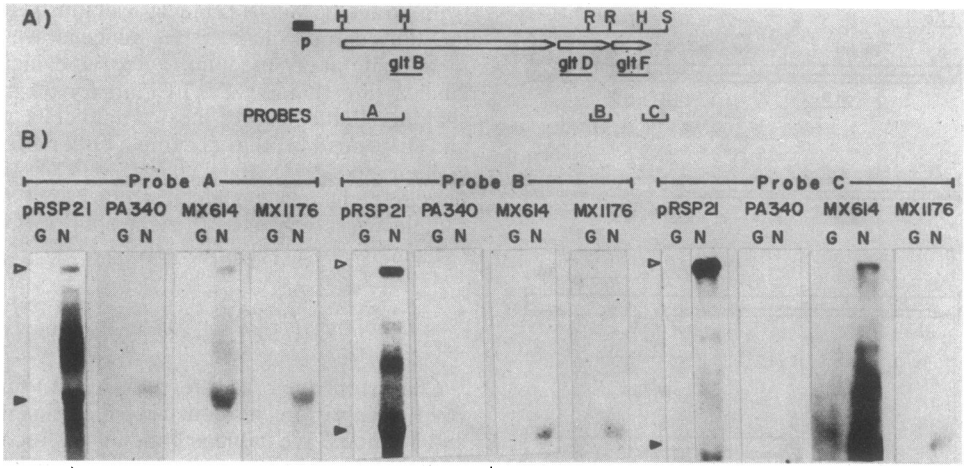

FIG. 2. Northern blot RNA-DNA experiments. Cultures from which total RNAs were purified are indicated as having N (minimal medium containing 15 mM NH_4Cl) or G (0.2% L-glutamate) as the sole nitrogen source. N cultures of strain PA340 also carried 0.02% L-glutamate to satisfy the requirement of the strain. (A) Schematic representation of the *glt* region from which probes A, B, and C were purified. (B) Total RNAs (10 μg) from the strains indicated were blotted and hybridized with probes A, B, and C. Lanes labeled pRSP21 contain 10 μg of total RNA purified from strain MX614(pRSP21). When total RNA purified from strain MX614(pRSP21) was used, the lane was heavily labeled when hybridized with probe A; since the background radioactivity was progressively diminished as the more downstream probes were used, this implies specific 3′ to 5′ degradation. Closed arrowheads indicate the migration of 16S rRNA (3,000 nucleotides). Open arrowheads show a large transcript of 7,500 nucleotides or more detected with all probes.

repression of glutamate synthase by glutamate (7, 30). The mechanism underlying this effect of glutamate is unknown but appears to be unrelated to its action as a nitrogen-limiting growth source. Glutamine, which also acts as a nitrogen-limiting source for *E. coli*, does not repress glutamate synthase synthesis (Table 2).

Identification of the *gltF* polypeptide product. The translation products of the *glt* region were characterized in the minicell system. In addition to the large and small subunits of glutamate synthase, the polypeptide products of the *gltB* and *gltD* genes (12, 24), respectively, we identified the *gltF* product as a 30,200-dalton polypeptide (Fig. 3) not present in minicells carrying either the control plasmid pBR329 (Fig. 3, lane 2) or pGOG2 (*gltF1*::Ω; Fig. 3, lane 7). Synthesis of the

TABLE 2. Enzyme activities and growth phenotypes of *E. coli* strains[a]

Strain (plasmid) and relevant genotype	Growth phenotype		Specific activity of:					
			Glutamate synthase		Glutamate dehydrogenase		Glutamine synthetase	
	Glt	Ntr	N limiting	N excess	N limiting	N excess	N limiting	N excess
MX614 wild type	+	+	71	67	205	206	1,040	128
PA340 *gdh-1 ΔgltBDF*	−	−	0	0	0	0	307	280
MX988 *ΔgltBDF*	+	−	0	0	261	185	200	128
MX1172 *gdh-1*	+	+	79	72	0	0	1,100	120
MX1176 *gdh-1 gltB225*::Ω	−	−	0	0	0	0	390	330
MX1178 *gltB225*::Ω	+	−	0	0	282	214	190	100
MX614(pRSP21) *gltB⁺D⁺F⁺*	+	+	781	751	ND	229	745	150
MX988(pRSP21) *gltB⁺D⁺F⁺*	+	+	784	ND	121	ND	1,117	160
MX1176(pRSP21) *gltB⁺D⁺F⁺*	+	+	575	607	0	0	731	210
MX1176(pGOP1) *gltB⁺ gltF⁺*	+	+	18	16	ND	ND	ND	ND
MX1178(pGOP1) *gltB⁺ gltF⁺*	+	+	15	18	188	229	270	180
MX988(pGOP3) *gltB⁺*	+	−	0	0	141	ND	260	130
MX1176(pGOP3) *gltB⁺*	+	−	37	54	0	0	320	200
MX1178(pGOP3) *gltB⁺*	+	−	31	34	332	174	690	258
MX988(pGOL2) *gltD⁺*	+	−	0	0	112	ND	170	260
MX1176(pGOL2) *gltD⁺*	−	−	0	0	0	0	450	332
MX1178(pGOG1) *gltF⁺*	+	+	0	0	278	219	410	110
MX1178(pGOG2) *gltF1*::Ω	+	−	0	0	253	234	440	150
MX1178(pGOG3) *gltF⁺*	+	+	0	0	234	245	305	140

[a] Enzyme activities are expressed as nanomoles of NADPH oxidized per minute per milligram of protein at 21°C for glutamate synthase and glutamate dehydrogenase and as nanomoles of γ-glutamyl hydroxamate formed per minute per milligram of protein at 37°C for glutamine synthetase. Zero values correspond to ≤2.0 U above the controls without glutamine for glutamate synthase or without ammonia for glutamate dehydrogenase. N limiting and N excess indicate growth on NN medium containing 6.8 mM L-glutamine on 15 mM NH_4Cl as the sole nitrogen source, respectively. Enzyme specific activities are the means of two determinations, with standard deviations of less than 15% of the mean, except for glutamate synthase, for which the values are the means of five determinations and the standard deviations are as high as 40% of the mean. Glt denotes prototrophy (+) or auxotrophy (−) for glutamate. Ntr was scored by the ability of cells to utilize 0.2% L-arginine as the sole nitrogen source. ND, Not determined.

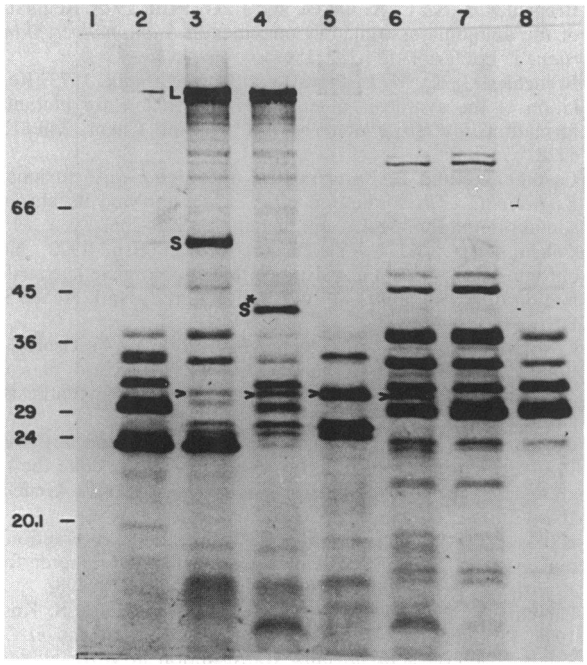

FIG. 3. Autoradiograph of a 10 to 15% polyacrylamide-sodium dodecyl sulfate gradient gel, showing [^{35}S]methionine-labeled proteins encoded by various plasmids. Lanes: 2, P678-54(pBR329); 3, P678-54(pRSP21); 4, P678-54(pGOP1); 5, P678-54(pGOG3); 6, P678-54(pGOG1); 7, P678-54(pGOG2); 8, P678-54(pHP45Ω) (28). The numbers at the left margin of the figure indicate molecular mass markers in kilodaltons. The *gltF* protein product of 30,200 daltons is indicated by arrowheads. Large and small glutamate synthase subunits made by minicells containing plasmid pRSP21 (lane 3) are indicated by L and S, respectively. The truncated small subunit synthesized by minicells with pGOP1 is indicated as S*.

30,200-dalton polypeptide was more pronounced when the *gltF* gene was transcribed from the cloramphenicol promoter of pGOP1 or pGOG1 than when it originated from the wild-type promoter as in pRSP21 (compare lanes 3, 4, and 6 of Fig. 3). As expected, the *gltF* polypeptide was overproduced from the anti-*tet* promoter of plasmid pGOG3 (32; Fig. 3, lane 5).

The aforementioned biochemical evidence that genes *gltB*, *gltD*, and *gltF* are transcribed in a single mRNA species and the fact that the *gltB225*::Ω mutation prevents its synthesis, and thus, translation of the corresponding polypeptide products, support the model of a *gltBDF* operon in *E. coli*.

Complementation tests. In assessing complementation of the three phenotypes defective in strains carrying the *gltB225*::Ω insertion with plasmids carrying all or individual genes of the *gltBDF* operon, we took into account the following considerations. Strain MX1176 (*gdh-1 gltB225*::Ω) cannot make either glutamate synthase or glutamate dehydrogenase activity and, consequently, is a glutamate auxotroph (4). This strain was adequate for testing plasmid complementation for glutamate synthase activity simultaneously with glutamate prototrophy. The glutamate auxotrophy of MX1176, however, did not allow testing of complementation for the Ntr and Gsd phenotypes unless glutamate was added to the medium. Plasmid complementation for Ntr$^+$ and Gsd$^+$ was preferentially carried out in strain MX1178 (*gltB225*::Ω), which does not require glutamate for growth, since this is provided by glutamate dehydrogenase. The Ntr and Gsd phenotypes could then be scored with 15 mM NH_4Cl (nitrogen excess) or 1 mg of glutamine per ml (nitrogen limitation) as the sole nitrogen source in the medium. It is noteworthy that neither glutamate synthase nor glutamate dehydrogenase appeared to be regulated by either nitrogen source (Table 2).

The three phenotypic defects associated with the chromosomal *gltB225*::Ω allele in MX1176 or MX1178 cells, i.e., lack of glutamate synthase activity, Ntr$^-$ and Gsd$^-$, and the glutamate auxotrophy of MX1176, were complemented by plasmid pRSP20 or pRSP21 (Table 2), in which the entire *glt* region was cloned in an 8.55-kilobase DNA segment (Fig. 1). None of the plasmids carrying individual operon genes, however, could complement all of the phenotypic defects. From these results, we conclude that in MX1176 and MX1178, the *gltB225*::Ω insertion is polar over the downstream genes of the operon, in agreement with previous evidence on the direction of transcription from *gltB* to *gltD* (12) and apparently to *gltF*.

Complementation for glutamate synthase activity was also achieved in MX1176 by pGOP3 (*gltB*$^+$) or pGOP1 (*gltB*$^+$ *gltF*$^+$). pRSP21 directed 8- to 9-fold increased levels of glutamate synthase activity over that of wild-type cells, whereas pGOP1 and pGOP3 directed 5.5- and 1.6-fold reduced levels, respectively (Table 2). It is possible that MX1176 (pGOP1) cells synthesize hybrid glutamate synthase molecules that contain the normal large enzyme subunit associated with either the truncated 40-kilodalton small subunit encoded by pGOP1 (Fig. 1) or the wild-type small subunit encoded by the chromosomal *gltD* gene. If so, the relative amounts of these two molecules may account for the lower glutamate synthase activity of MX1176(pGOP1) than MX1176(pGOP3).

The nearly wild-type level of glutamate synthase made by this last strain suggests some expression of the chromosomal *gltD* gene despite the *gltB225*:Ω insertion. This is in agreement with Garcíarrubio et al. (12), who suggested *gltD* transcription from its own promoter.

A previous suggestion of glutamate synthase activity exclusively due to the large enzyme subunit (18) as an alternative explanation for this enzyme activity in MX1176(pGOP3) or MX1178(pGOP3) probably does not apply, since pGOP3 was unable to express this enzyme in MX988 (Δ*gltBDF*) cells (Table 2).

Involvement of the *gltF* product in the activation of the Ntr system is suggested by several lines of evidence. *gltF* was cloned in two different plasmids, pGOG1 and pGOG3. Even though *gltF* is transcribed from the chloramphenicol promoter of pBR328 in pGOG1 and from the anti-*tet* promoter of pBR322 in pGOG3, both plasmids complemented MX1178 (*gltB225*::Ω) cells to Ntr$^+$ but not Gsd$^+$ (Table 2). In these cells, Ntr$^+$ complementation took place in the absence of glutamate synthase, a result that speaks in favor of activation of Ntr either directly or indirectly by the *gltF* product but not via its possible activation of the *gltBDF* operon. For reasons not known, neither pGOP1 nor pGOG1 was able to complement MX988 (Δ*gltBDF*)to Ntr$^+$. The inability of pGOP3 (*gltB*$^+$) to complement the Ntr$^-$ phenotype of MX1176 (*gltB225*::Ω) cells (Table 2) suggests that, in these cells, *gltF* cannot be efficiently transcribed as part of a bicistronic message from a putative *gltD* promoter or from a promoter of its own.

The results of noncomplementation of the Gsd$^-$ phenotype of MX1178 by *gltF*$^+$ from either pGOG1 or pGOG3 (Table 2) are taken as an indication that the glutamate synthase molecule or certain threshold levels of intracellular glutamate, not achieved solely by the biosynthetic action of

glutamate dehydrogenase, in place of or in addition to the *gltF* product, are responsible for Gsd^+ expression.

Glutamate has two opposite effects on expression of the operons coding for glutamate synthase and glutamine synthetase. As previously suggested (7, 30), and confirmed by us (Fig. 2), glutamate represses glutamate synthase in wild-type cells. It also completely derepresses glutamine synthetase (30), but this effect is not exerted solely by action as a nitrogen-limiting source as in other cases, i.e., low NH_4^+, arginine, or proline, for it does so in the *gltBDF* deletion strain MX988 (A. V. Osorio and F. Bastarrachea, unpublished data). Apparently, glutamate overcomes the requirement for the *gltF* product in the activation of the Ntr system and satisfies the requirement of a functional *gltBDF* operon for Gsd^+. Consequently, the *gltF* product, the level of intracellular glutamate, or both appear to regulate *glnALG* expression by an unknown mechanism.

It is known that the Ntr system is turned on in *gltB* mutants undergoing nitrogen limitation by suppressor mutations in *glnL*, leading to the GlnC phenotype (26). Since such suppression does not act by restoring glutamate synthase activity, it may act by bypassing a requirement for the *gltF* product. Further experimentation on the possible roles of the *gltF* product and glutamate in expression of *glnL* and *glnG* or on the functionality of their products seems warranted, particularly in light of recent evidence associating a specific *glnL* mutation conferring the GlnC phenotype with loss of the ability of its *glnL* product, NR_{II} (in the presence of the *glnB* product, P_{II}), to inactivate (dephosphorylate) the activated *glnG* product NR_I-P (25).

Experiments in progress are intended to define more precisely the gene product-phenotype relationships of the complex *gltBDF* operon.

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