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cis-dominant, glutamine synthetase constitutive mutations of *Escherichia coli* independent of activation by the *glnG* and *glnF* products

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Summary. Mutants resistant to 80 μM L-methionine-DL-sulfoximine (MS) were isolated on glucose-minimal 15 mM NH_4^+ medium plates from *Escherichia coli* cells which were hypersensitive to this concentration of the analogue by virtue of their harboring *glnG* mutations. MS-resistant mutants derived from strain MX902 carried, in addition to its *glnG74*::Tn*5* allele, mutations tightly linked to *glnA*, as shown by P1-mediated transduction experiments. One particular allele, *gln-76*, which suppressed the MS-sensitivity conferred by *glnG74*::Tn*5* but not its Ntr^- phenotype (inability to transport and utilize compounds such as arginine or proline as the only nitrogen sources), was shown to allow constitutive expression of glutamine synthetase in the absence not only of a functional *glnG* product but also of a functional *glnF* product. This behavior was found to be *cis*-dominant in complementation experiments with F'14 merogenotes. In an otherwise wild-type genetic background as in MX929 (*gln-76 glnA*$^+$ *glnL*$^+$ *glnG*$^+$ *glnF*$^+$), however, normal activation, mediated by the *glnG* and *glnF* products was preferred over that mediated by *gln-76*.

Introduction

Glutamine synthetase (L-glutamate: ammonia ligase (ADP-forming), EC 6.3.1.2) is one of several proteins of enteric bacteria whose synthesis is regulated by the availability of nitrogen in the growth medium (Woolfolk et al. 1966). Under nitrogen limitation, as in the presence of glutamine, glutamate, arginine, proline or low concentrations of ammonium ions as the only nitrogen sources, glutamine synthetase is derepressed. The addition of high ammonium ion concentrations to the medium causes repression by about 10 to 20-fold. Experimental evidence obtained in different laboratories indicates that the products of several genes, *glnF*, *glnL*, *glnG*, and *glnB*, appear to mediate regulation of *glnA*, the structural gene for the glutamine synthetase monomer, presumably at the transcription level (Rothstein et al. 1980; Pahel et al. 1982; Goldie and Magasanik 1982; Guterman et al. 1982). These genes also regulate the expression of other operons or genes whose products are involved in the transport and utilization of a number of organic nitrogenous compounds such as histidine, arginine or proline, the previously named Reg phenotype (Pahel and Tyler 1979; Guterman et al. 1982; MacNeil et al. 1982), later renamed the Ntr phenotype (Magasanik 1982). *glnF* mutations first discovered in *Salmonella typhimurium* by Garcia et al. (1977), are located at min 86 on the *Escherichia coli* map (Bachmann and Low 1980). Cells carrying a mutant *glnF* are glutamine requirers, e.g., they synthesize glutamine synthetase in amounts even lower than those found under extreme repressing conditions. *glnF* mutants revert to the Gln^+ phenotype at frequencies of about 10^{-6}. Most of the revertants carry, in addition to the original *glnF* mutation, secondary mutations in a gene closely linked to *glnA* named *glnG* in *E. coli* (Pahel and Tyler 1979) and *glnR* in *S. typhimurium* (Kustu et al. 1979a). As already mentioned, *glnG* mutants are Gln^+. They synthesize low constitutive levels of glutamine synthetase and are also Ntr^- in either a *glnF*$^+$ or *glnF* background. Recent evidence (McFarland et al. 1981) indicates that *glnG* (or *glnR*) is not one but two genes. The relative orientation of these genes in the *E. coli* chromosome is *polA*...*glnG glnL glnA*...*rha*; the direction of transcription of the three genes occurs in the same counterclockwise direction (Backman et al. 1981; Pahel et al. 1982).

On the basis of their results, McFarland et al. (1981) have proposed a model in which the *glnL* and *glnG* products function as a repressor protein complex for *glnA* and other nitrogen-controlled genes. The repressor complex may be acted upon by the *glnF* product either directly or through a catalytic small molecular weight product, thus converting it to a form with positive regulatory character, i.e., an activator.

Since the frameshift mutations of McFarland et al. (1981) in either *glnL* or *glnG* affected only the expression of one protein product but not that of the other, and since we know that frameshift mutations are highly polar, it is probable that *glnL* and *glnG* belong to different transcriptional units. In accordance with these observations, Backman et al. (1981) have described a deletion within *glnL* which is nonpolar to *glnG* in a plasmid containing the whole *glnA* region. Nevertheless, *glnL* mutations polar to *glnG* have also been isolated by G. Pahel (quoted in Chen et al. 1982). According to Pahel et al. (1982) *glnA* and *glnG* are expressed under nitrogen-limiting growth conditions as a single transcriptional unit; thus the three genes, *glnA*, *glnL* and *glnG* seem to constitute an operon. Most of the evidence in support of the operon model comes from studies on β-galactosidase expression of Mud1 (*lac*) insertions in *glnA*, *glnL* or *glnG* (Rothstein et al. 1980; Guterman et al.

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1982; Pahel et al. 1982) and from suppression of the Ntr^- phenotype of *glnA* point mutants by *rho* mutations (Guterman et al. 1982).

The presence of a promoter starting transcription from *glnL* and being negatively controlled by the *glnG* product has been suggested (Pahel et al. 1982; Chen et al. 1982). Evidence is also available for the presence of a functional promoter transcribing only the *glnG* gene (Covarrubias et al., in preparation). Indeed, if expression of these genes is achieved by a series of overlapping mRNA transcripts depending upon the quality and the quantity of the nitrogen source available for growth it will be as expected. This is in agreement with observations indicating that some expression of *glnG* takes place in strains in which *glnA* (Pahel and Tyler 1979; Pahel et al. 1982; Goldie and Magasanik 1982) or *glnL* (Urbina and Bastarrachea, unpublished data) have been mutated by insertions.

We have characterized a number of *glnG* mutants and have found, in addition to their low level constitutivity for glutamine synthetase and their Ntr^- phenotypes, that they are also hypersensitive to the glutamine analogue L-methionine-DL-sulfoximine (MS). We have used MS-resistance as a selection marker for wild-type recombinants in crosses involving *glnG* mutations, as well as for complementation experiments with F'14 merogenotes. Selection for MS-resistant revertants of the *glnG74*::Tn*5* allele led to the isolation of *cis*-dominant mutations tightly linked to *glnA*, which allowed constitutive synthesis of glutamine synthetase independent of the *glnG* and *glnF* products under otherwise ammonium repressing conditions. Our results indicate that *gln-76* may lie in the control region of the *glnA* transcriptional unit making it independent of activation and poorly repressible, if at all, by the normal regulatory effectors within the general framework of the McFarland et al. (1981) model.

Materials and Methods

Bacterial Strains. All strains were derivatives of *E. coli* K12 and are listed in Table 1.

Culture Media. The NN minimal and the complex Luria media used have been described (Covarrubias et al. 1980). Additions to minimal medium, in final concentrations, were as follows: G, 0.2% glucose, LA and HA, 0.5 mM and 15 mM ammonium chloride, respectively; gln, L-glutamine (1 mg/ml). The utilization of 0.2% L-arginine as the only nitrogen source (Aut^+) was used as an indicator of the Ntr phenotype. Other supplements of minimal medium to satisfy nutritional requirements were added at predetermined optimal concentrations ranging from 5×10^{-4} M to 2×10^{-3} M. Glutamine auxotrophs were maintained in slants of Luria medium supplemented with L-glutamine (1 mg/ml). Merodiploid strains were maintained on slants of minimal medium without isoleucine and valine.

Isolation of Mutants. Tn*5* insertions were isolated following infection of MX615 (Table 1) with λKan2 (Berg et al. 1975). P1 lysates were then prepared from pooled samples of a large number of kanamycin resistant (Km^r) individual colonies. Following transduction of MX727 (*glnA71*::Tn*5*), selection was made for Gln^+ [Km^r Aut^-] colonies which turned out to carry Tn*5* insertions into either *glnG* or *glnL*.

Genetic Procedures. Preparation of P1 lysates and the protocol for transductions were according to Miller (1972). Merodiploid strains were constructed by transfer of F'14 merogenotes from AB1206 or its derivatives to appropriate recipients and selection for Ilv^+ complementation. Since F'14 merodiploids constructed in a *recA56* background were highly unstable, all our recipients were $recA^+$. Their merodiploid state was verified by curing with acridine orange (Bastarrachea and Willetts 1969). The *polA* genotype was recognized by its sensitivity to methylmethanesulfonate (De Lucia and Cairns 1969) and the Tn*5* transposon by its resistance to kanamycin at 30 μg/ml. Curing of MX927 (*glnG74*::Tn*5*) from its transposon was achieved by selection of Aut^+ [Km^s] spontaneous revertants.

Glutamine Synthetase Activity. The γ-glutamyl transferase assay used, the preparation of cell-free extracts and the determination of the average state of adenylylation, were as previously reported (Covarrubias et al. 1980). Specific activities are given as nanomoles γ-glutamyl hydroxomate formed per min per mg protein at 37° C. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. The screening of individual colonies for high constitutive level synthesis was carried out using the qualitative assay described by Pahel et al. (1978).

Southern Hybridizations. Southern hybridizations were carried out using purified DNA's (Betlach et al. 1976) from plasmid probes pACR2 and pACR10. pACR2 carries an *E. coli* DNA chromosomal fragment of approximately 10.5 kb that contains the *glnA*, *glnL* and *glnG* genes (Fig. 2B). pACR10 is pBR322 carrying a Tn*5* insertion of unknown location. Chromosomal DNA from strains MX615 and MX902 was purified (Marmur 1961) and digested to completion with endonucleases *Bam*HI and *Pst*I. The digested mixtures were subjected to agarose gel electrophoresis and the DNA's transferred to nitrocellulose paper (Schleicher and Schüel BA85) for hybridization against nick-translated pACR2 or pACR10 DNA's as described by Southern (1975).

Reagents. All amino acids and vitamins as well as the L-methionine-DL-sulfoximine were from Sigma Chem. Co., St Louis, Mo, USA. The restriction endonucleases were purified by the procedure of Greene et al. (1978). The plasmid DNA's used as probes were labeled using a nick-translation kit (code PBS100) from Amersham International, Bucks, England. All other reagents and media used were of analytical grade.

Genetic Nomenclature. In as much as the gene designations *glnF*, *glnL* and *glnG* have been used extensively in *E. coli* (see for instance Chen et al. 1982; MacNeil et al. 1982) they are preferred here over *ntrA*, *ntrB* and *ntrC*, their counterparts in *S. typhimurium* (McFarland et al. 1981). All other genotypic symbols are those recommended by Bachmann and Low (1980). The genotypic designation *msr*, for the mutation associated with the MS resistance of strain MX981 (Table 1) is tentative. Alleles *glnA70* and *glnA71*::Tn*5* are those previously known as *glnA20* and *glnA21*::Tn*5* (Covarrubias et al. 1980; Bastarrachea et al. 1980; MacNeil 1981; Pahel et al. 1982), respectively.

Table 1. Bacterial strains

Strain	Genotype	Source or derivation
MX614	*Δ(pro-lac) galE ilv-680 thi-1*	Bastarrachea et al. (1980)
MX615	*Δ(pro-lac) Δ(gal attλ bio) ilv-680 thi-1*	Bastarrachea et al. (1980)
MX705	*leu-6 proA2 lacY1 galK2 rpsL20 glnA70 thi-1 hsdSr*$^-{}_B m^-{}_B$	Covarrubias et al. (1980)
MX727	*Δ(pro-lac) Δ(gal attλ bio) ilv-680 glnA71*::Tn*5 thi-1*	Bastarrachea et al. (1980)
MX734	*Δ(pro-lac) galE ilv-680 glnA71*::Tn*5 thi-1*	P1 (MX727) × MX614; Km^r [Gln$^-$] selection
MX752	*Δ(pro-lac) Δ(gal attλ bio) supF ilv-680 zih*::Tn*5*λTn*5 thi-1 lamB*	Bastarrachea et al. (1980)
AB1206	*proA2 tfr-3 lacY1 supE44 galK2 his-4 rpsL8 Δ*DE2(*ilvE-argH*) *thi-1*/F′14	B. Bachmann
MX818	*proA2 tfr-3 lacY1 supE44 galK2 his-4 rpsL8 Δ*DE2(*ilvE-argH*) *thi-1*/F′14 *rha*	Ethyl metanesulphonate mutagenesis of AB1206
MX821	*proA2 tfr-3 lacY1 supE44 galK2 his-4 rpsL8 Δ*DE2(*ilvE-argH*) *thi-1*/F′14 *glnA71*::Tn*5*	P1 (MX727) × MX818, Rha$^+$ [Km^r Gln$^-$] selection
MX822	*proA2 tfr-3 lacY1 supE44 galK2 his-4 rpsL8 Δ*DE2(*ilvE-argH*) *thi-1*/F′14 *glnA70*	P1 (MX705) × MX818; Rha$^+$ [Gln$^-$] selection
MX848	*Δ(pro-lac) galE glnF73*::Tn*5 ilv-680 thi-1*	P1 (MX615, λKan2 infected) × MX614; Km^r, 44% cotransductible with *argG*
MX850	*Δ(pro-lac)Δ*(gal attλ *bio) supF ilv-680 Δ(glnGLA-rha) zih*::Tn*5 thi-1 lamB*	Heat induction of MX752
MX852	*Δ(pro-lac) Δ(gal attλ bio) supF ilv-680 Δ(glnA-rha) zih*::Tn*5 thi-1 lamB*	Heat induction of MX752
MX900	*proA2 tfr3 lacY1 supE44 galK2 his-4 rpsL8 Δ*E2(*ilv-argH*) *thi-1*/F′14 *glnG74*::Tn*5*	P1 (MX902) × AB1206; Km^r [Aut$^-$] selection
MX902	*Δ(pro-lac) galE ilv-680 glnG74*::Tn*5 thi-1*	This paper
MX914	*Δ(pro-lac) (gal attλ bio) supF ilv-680 Δ(glnGLA-rha) thi-1 lamB*	Precise excision of Tn*5* in MX850
MX915	*Δ(pro-lac) Δ(gal attλ bio) supF ilv-680 glnF73*::Tn*5 Δ(glnGLA-rha) thi-1 lamB*	P1 (MX848) × MX914; Km^r selection
MX927	*Δ(pro-lac) galE ilv-680 glnG74*::Tn*5 gln-76 thi-1*	MS^r spontaneous mutant of MX902
MX929	*Δ(pro-lac) galE ilv-680 gln-76 thi-1*	Aut$^+$ [Km^s] spontaneous derivative of MX927
MX931	*Δ(pro-lac) galE glnF73*::Tn*5 ilv-680 gln-76 thi-1*	P1 (MX848) × MX929; Km^r [Aut$^-$] selection
MX932	*Δ(pro-lac) galE ilv-680 glnG74*::Tn*5 thi-1*/F′14	Mating AB1206 × MX902; Ilv$^+$ [His$^+$] selection
MX933	*Δ(pro-lac) galE ilv-680 glnG74*::Tn*5 thi-1*/F′14 *glnA71*::Tn*5*	Mating MX821 × MX902; Ilv$^+$ [His$^+$] selection
MX936	*Δ(pro-lac) galE ilv-680 glnG74*::Tn*5 gln-76 thi-1*/F′14	Mating AB1206 × MX927; Ilv$^+$ [His$^+$] selection
MX937	*Δ(pro-lac) galE ilv-680 glnG74*::Tn*5 gln-76 thi-1*/F′14 *glnA71*::Tn*5*	Mating MX821 × MX927; Ilv$^+$ [His$^+$] selection
MX981	*Δ(pro-lac) galE glnF73*::Tn*5 ilv-680 gln-76 thi-1 msr-1*	MS^r spontaneous mutant of MX931
MX982	*Δ(pro-lac) galE glnF73*::Tn*5 ilv-680 glnG77 gln-76 thi-1*	MS^r spontaneous mutant of MX931
W3110 *polA1*	*nalA thyA polA1*	MC. Gómez-Eichelmann

Results

Hypersensitivity to L-*Methionine*-DL-Sulfoximine of *glnG Mutants*

The GlnR phenotype of *glnG* mutants in enteric and other bacteria is characterized by an inability to transport and utilize compounds such as arginine, proline or histidine as the only nitrogen sources (i.e., the Ntr phenotype) and by a low level constitutive expression of glutamine synthetase (Kustu et al. 1979a; Pahel and Tyler 1979). Based on the known irreversible inhibition of glutamine synthetase by the glutamine analogue, L-methionine-S-sulfoximine (MS) in the presence of ATP (Ronzio and Meister 1968; Ronzio et al. 1969; Rowe et al. 1969; Manning et al. 1969), we reasoned that cells synthesizing small amounts of this enzyme should be hypersensitive to MS. Indeed, we have found that all the *glnG* mutants of *E. coli* we have tested are hypersensitive to MS. At 80 μM MS in GHA medium, *glnG* cells fail to grow while *glnG*$^+$ cells do grow (Fig. 1). In the presence of glutamine as the only nitrogen source, MS inhibition of growth of either *glnG*$^+$ or *glnG* cells was not observed; methionine also prevented the growth inhibitory effects of MS on MX902 cells after a prolonged lag (Fig. 1; Ayling and Bridgeland 1972; Betteridge and Ayling 1975).

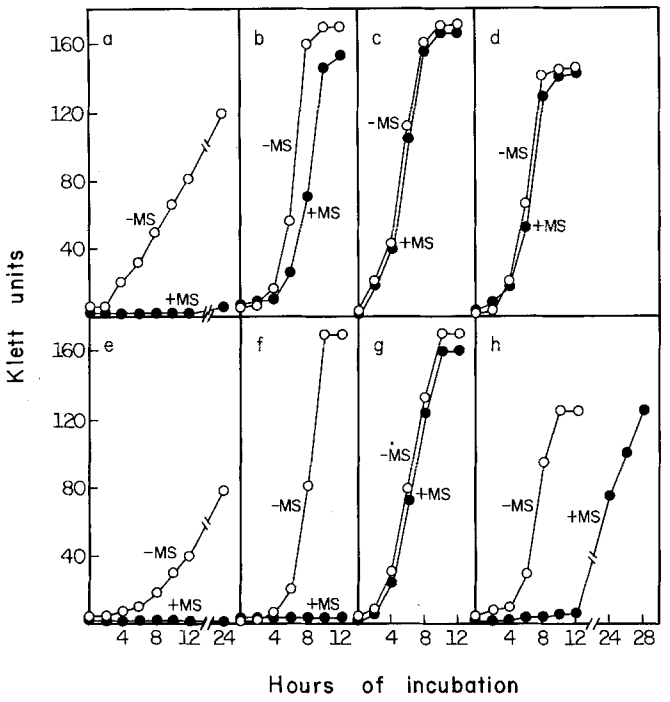

Fig. 1 a–h. Growth responses of strains MX614 (wild-type) and MX902 (*glnG74*::Tn*5*) in minimal medium at 37° C with (•) or without (o) 80 μM MS, as a function of the nitrogen source. **a–d**, MX614; **e–h**, MX902. **a** and **e**, GLA medium; **b** and **f**, GHA medium; **c** and **g**, Ggln medium; **d** and **h**, GHA medium plus 80 μM L-methionine

Genetic and Physical Characterization of the glnG74::Tn5 Mutation

glnG mutations can be isolated either as supressors of *glnF* mutations or as Ntr$^-$ mutations tightly linked to *glnA*. The particular mutation *glnG74*::Tn*5* was isolated by the latter procedure but yet, was able to suppress the *glnF73*::Tn*5* mutation. In a conjugal cross made between the AB1206 derivative, MX900, carrying *glnG74*::Tn*5* in the F-merogenote, as donor and MX915, a *glnF73*::Tn*5* derivative of the MX850 deletion strain (Table 1), as recipient, we obtained 87% Gln$^+$ (Ntr$^-$) progeny among the Ilv$^+$ sexductants.

All the phenotypic properties of the *glnG74*::Tn*5* allele, i.e., its low level constitutivity for glutamine synthetase, and its Ntr$^-$ and MS^s phenotypes, were shown to be recessive to the wild-type allele as shown by complementation of MX902 cells by the F′14 merogenote carrying the entire wild-type *glnA* region (strain MX932, Table 3). Cells of strain MX933 carrying *glnG74*::Tn*5* *glnA*$^+$ on the chromosome and *glnG*$^+$ *glnA71*::Tn*5* on the F-merogenote (Table 3) synthesized normal, regulated levels of glutamine synthetase and were MS^r. In agreement with the observation of Pahel and Tyler (1979), no complementation for Aut$^+$ was observed in these cells. Pahel et al. (1982) have explained these results in terms of the polarity exerted by the *glnA71*::Tn*5* mutation on expression of *glnG cis* to it and to the limited transcription of this gene from a *glnA* distal promoter insufficient to activate Ntr expression but sufficient to activate *glnA* expression in *trans*.

glnG74::Tn*5* was found to be 93% linked to *glnA* and 70% linked to *polA* in P1 transduction experiments (Table 2). These results were consistent with those obtained

Table 2. Mapping of *glnG74*::Tn*5* by transduction

P1 donor	Recipient	Marker		% Cotransduction
		Selected	Unselected	
W3110 (*polA1*)	MX902 (*glnG74*::Tn*5*)	Aut$^+$	MMS^s	70 (284/406)
MX902	MX727	Gln$^+$	Aut$^-$ MS^s	93 (340/365)

MMS, Methyl methanesulphonate; MS, L-methionine-DL-sulfoximine. The superscripts indicate sensitivity to 0.04% MMS or to 80 μM MS

in crosses with mutants carrying well-defined deletions entering the *glnA* region. In transductional crosses using MX902 (*glnG74*::Tn*5*) as a recipient and P1 propagated on either MX850 *Δ(glnG glnL glnA...rha)* or MX852 *Δ(glnA...rha)*, we obtained GlnG$^+$ recombinants only with MX852 but not with MX850 as the donor.

To locate the *glnG74*::Tn*5* mutation more precisely. Southern type hybridization experiments were carried out using the two plasmids, pACR2, which carries the *glnA – glnL – glnG* region (Fig. 2B) and pACR10, a derivative of pBR322 carrying a Tn*5* insertion as probes. These plasmids were nick-translated and hybridized against chromosomal DNA from strains MX615 (wild-type) and MX902 (*glnG74*::Tn*5*) previously digested with endonucleases *Bam*HI and *Pst*I. As shown in Fig. 2A, five *Bam*HI-*Pst*I DNA fragments from MX615 were found to hybridize with pACR2 DNA. Three of them seemingly corresponded to the internal pACR2 DNA region, since similar bands were observed in the pACR2 vs pACR2 hybridization (Fig. 2A). The remaining two fragments represented the junctions between each of the ends of the chromosomal DNA fragment carried by pACR2. In the case of the hybridization of MX902 against pACR2, we noticed the disappearance of the 3.9 kb *Bam*HI-*Pst*I fragment. Instead, two fragments of 2.77 kb and 2.55 kb were visualized. These results indicate that the *glnG74*::Tn*5* insertion occurred inside the 3.9 kb *Bam*HI-*Pst*I fragment, approximately 2.77 kb from the *Bam*HI site. The data were confirmed by results obtained using pACR10 DNA as probe (Fig. 2A). Our hybridization results, therefore, are consistent with the conclusion that the *glnG74*::Tn*5* insertion readily interrupts the *glnG* gene sequence (Fig. 2C).

Isolation of Constitutive Mutations for Glutamine Synthetase as Suppresors of glnG74::Tn5

From MX614 cells, we isolated and characterized 17 independent mutants carrying Tn*5* insertions tightly linked to *glnA* in P1 transduction experiments. Most of them were shown to be insertions in *glnG* but a few were insertions in *glnL* that were polar to *glnG*. Yet all were MS^s and Aut$^-$. None of the *glnG*::Tn*5* insertions reverted to Aut$^+$ in the presence of kanamycin, but they did revert, to the MS^r phenotype whether or not kanamycin was present in the selection medium. Strain MX902, for instance, reverted to MS^r at a frequency of 1 in 10^7. Ten independent MS^r revertants from strain MX902 were screened for glutamine synthetase activity after growth on GHA medium. Specific

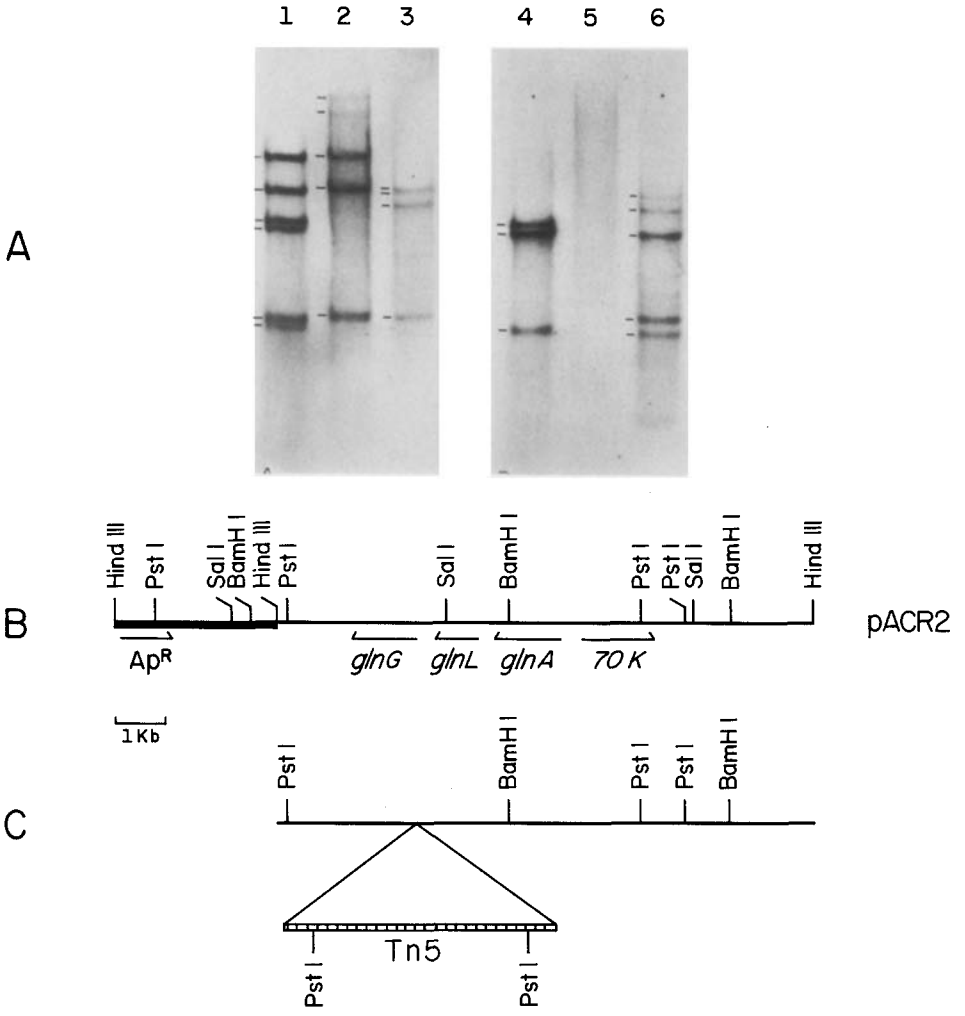

Fig. 2A–C. Blotting hybridization of *Bam*HI + *Pst*I digests of MX615 and MX902 *E. coli* chromosomal DNA's with ^{32}P-pACR2 (lanes 1–3) or ^{32}P-pACR10 (lanes 4–6) DNA's as probes for *glnG* and *Tn5*, respectively. **A** lane 1, pACR2 vs pACR2; lane 2, MX615 vs pACR2; lane 3, MX902 vs pACR2; lane 4, pACR2 vs pACR10; lane 5, MX615 vs pACR10, lane 6, MX902 vs pACR10. **B** Diagram of plasmid pACR2. **C** Diagram of the Tn*5* insertion in MX902; the *Bam*HI sites of Tn*5* are not shown since its orientation in MX902 is not known; very likely, in lane 6 bands 3–6 (from top to bottom) correspond to *Bam*HI-Tn*5* internal fragments while bands 1 and 2 (of 2.77 kb and 2.55 kb, respectively) are fusions between Tn*5* and the chromosome. The black bars correspond to pBR327 DNA, the thin lines to chromosomal DNA and the cross-hatched bars to Tn*5*. The arrows indicate the direction of gene transcription

activities ranged between 370 and 1900. The MS^r strain with the highest glutamine synthetase specific activity under otherwise repressing conditions was selected for detailed study.

This MS^r revertant strain, designated MX927, synthesized high constitutive levels of glutamine synthetase but failed to utilize arginine as a nitrogen source (Table 3). Thus, in addition to the original *glnG74*::Tn*5* mutation, MX927 carried a secondary mutation that suppressed only the *glnG* functions related to *glnA* expression and not those related to expression of the Aut$^+$ phenotype.

Mapping of gln-76

P1 lysates made on MX927 (*glnG74*::Tn*5 gln-76*) were used to transduce two independent glutamine auxotrophs, MX727 and MX822, to prototrophy. *glnG74*::Tn*5* was found to cotransduce 82% and 96% with the wild-type chromosomal regions corresponding to the *glnA71*::Tn*5* and *glnA70* mutations, respectively (Table 4). This is consistent with an ordering of the mutations as *glnG74*::Tn*5-glnA70-glnA71*::Tn*5*. The fact that all the *glnA*$^+$ *glnG74*::Tn*5* transductants from either cross also coinherited *gln-76* was indicative of the tight linkage of this mutation with *glnA*. A third cross was performed to locate *gln-76* unambigously. Phage P1 was propagated on MX852, a strain carrying *Δ(glnA rha)* with one deletion end cutting the *glnA* structural gene near the middle while leaving the *glnG*$^+$ and *glnL*$^+$ genes intact. This lysate was used to transduce MX927 recipient cells. All Aut$^+$ recombinants tested were Km^s (Table 4). Recombinants were expected to be wild-type for all *gln* genes and to have regulated levels of glutamine synthetase unless they coinherited the *gln-76* mutation. Since the only difference between cells carrying *gln-76* (i.e., MX929, see below) and wild-type cells (i.e., MX614) is a two-fold derepression of glutamine synthetase by MX929, after growth on high ammonium ion concentrations (Table 3), 14 individual transductants from the above cross were purified and tested for glutamine synthetase activity after growth on GHA medium. All of the isolates showed two-fold derepressed levels of the enzyme, thus affording the conclusion that the chromosomal region corresponding

Table 3. Phenotypic characterization of *gln-76* in different genetic backgrounds

Strain	Relevant genotype	Gln	Aut	MS	Glutamine synthetase		
					GLA	GHA	Ggln
MX614	Wild-type	+	+	Resistant	1,990 (4)	260 (9)	1,760 (6)
AB1206	*Δ(glnA-glnG)*/F′14	+	+	Resistant	1,220	190	1,470
MX848	*glnF73*::Tn*5*	−	−		−	−	10
MX902	*glnG74*::Tn*5*	+	−	Sensitive	20 (8)	50 (6)	50 (9)
MX927	*glnG74*::Tn*5 gln-76*	+	−	Resistant	4,130 (5)	1,870 (11)	2,860 (6)
MX929	*gln-76*	+	+	Resistant	1,970 (5)	530 (10)	1,400 (5)
MX931	*glnF73*::Tn*5 gln-76*	+	−	Sensitive	150 (4)	600 (9)	370 (7)
MX932	*glnG74*::Tn*5*/F′14	+	+/−	Resistant	n.d.	120	1,960
MX933	*glnG74*::Tn*5*/F′14 *glnA71*::Tn*5*	+	−	Resistant	n.d.	140	1,470
MX936	*glnG74*::Tn*5 gln-76*/F′14	+	+/−	Resistant	n.d.	870	2,360
MX937	*glnG74*::Tn*5 gln-76*/F′14 *glnA71*::Tn*5*	+	−	Resistant	n.d.	1,550	2,140
MX981	*glnF73*::Tn*5 gln-76 msr-1*	+	−	Resistant	150 (4)	540 (8)	270 (7)
MX982	*glnF73*::Tn*5 gln-76 glnG77*	+	−	Resistant	4,180 (5)	1,390 (11)	2,670 (6)

In the cases where the γ glutamyl transferase assays were run with and without 60 mM Mg^{2+}, the average state of adenylylation, $\bar{n}$, is indicated in parenthesis; n.d., not determined

Table 4. Mapping of the *gln-76* mutation by transduction

P1 donor	Recipient	Marker		% Cotransduction	GS activity[a]
		Selected	Unselected		
MX927 (*glnG74*::Tn*5 gln-76*)	MX727 (*glnA71*::Tn*5*)	Gln^+	Km^r	82	Constitutive (40/40)[b]
MX927	MX822	Gln^+	Km^r	96	Constitutive (40/40)[b]
MX852 *Δ(rha-glnA)*	MX927	Aut^+	Km^s	100	Constitutive (14/14)[c]

[a] After growth of individual transductants in GHA medium
[b] High level constitutivity (qualitative assay)
[c] 2-fold derepressed as compared to wild-type (quantitative assay)

to the *gln-76* mutation was deleted in MX852. Due to its tight linkage with *glnA*, *gln-76*, therefore must lie very close to the NH_2-terminal end of *glnA*.

Regulation of Glutamine Synthetase in Strains Carrying the gln-76 Allele

The most striking feature of the *gln-76* allele was that it mediated synthesis of glutamine synthetase at high levels in the absence of a functional *glnG* product. In fact, cells carrying the *gln-76* mutation made considerably more enzyme when the cells lacked a normal activating system than in its presence. When compared with the wild-type, strain MX614, the *gln-76* strain had double the activity when both strains were grown on media containing low ammonium ion concentrations; it had about 1.6-fold higher activity when glutamine was the nitrogen source and 7-fold higher activity when both strains were grown on high ammonium ion concentrations (Table 3).

Using the curing procedure outlined in Materials and Methods, we obtained $glnG^+$ derivatives from MX927 that were Km^s due to precise excision of the Tn*5* transposon. One of these Gln^+ Km^s derivatives, termed MX929, was shown to be Aut^+ MS^r and synthesized normal levels of glutamine synthetase, except when grown on GHA medium where the activity was two-fold derepressed as compared to wild-type MX614 cells grown under similar conditions. This implied that *gln-76*, the mutation conferring the MS^r phenotype to MX927, was still present in the Tn*5*-cured strain, MX929. These results suggest that the putative *glnG-glnF* activator complex still bound and initiated transcription at the *glnA* operon control region of MX929 cells despite the fact that it is not needed.

The next logical step was to introduce a mutant *glnF* allele into the *gln-76* background in order to study the effects of the *gln-76* mutation on glutamine synthetase in more detail. Accordingly we transduced the *glnF73*::Tn*5* mutation from MX848 into MX929 with bacteriophage P1, selecting for Km^r transductants. One Km^r transductant was purfied and designated MX931. The *glnF73*::Tn*5 gln-76* genotype of this isolate was verified by cotransduction with *argG* in the case of *glnF73*::Tn*5* and with *glnA* in the case of *gln-76*. MX931 was glutamine independent due to its ability to synthesize sufficient amounts of glutamine synthetase for growth (Table 3). The glutamine synthetase specific activities of strain MX931 varied between 150 and 600 (Table 3). Surprisingly, they showed the inverse relationship of having higher levels of activity in cells grown on high ammonium and lower in those grown on low ammonium ion concentrations. MX931 was also Aut^- and MS^s; the Aut^- phenotype being a clear indication of an absolute requirement of a functional *glnF* product for Aut^+ (Ntr^+) expression.

MS^r Derivatives of MX931

Plating of MX931 (*glnF73*::Tn*5 gln-76*) cells on minimal-glucose-15 mM NH_4^+-80 μM MS medium in the presence

of kanamycin, led to the isolation of MS^r spontaneous mutants at a frequency of 3×10^{-5}. Two classes of MS^r mutants were easily distinguised after the colonies were screened with the qualitative assay for glutamine transferase activity. One class (35%) synthesized high constitutive levels of the enzyme, similar to those made by MX927 (*glnG74*::Tn*5 gln-76*) and another class (66%) which synthesized intermediate constitutive levels practically identical to those made by the parental strains, MX931. As expected, representatives of the high level constitutive class, exemplified by MX982 (Table 3), apparently carried, in addition to *glnF73*::Tn*5* and *gln-76*, *glnG* mutations which conferred on them the MS^r phenotype. This was suggested by results of transduction tests in which P1 lysates made on three isolates of the high constitutive level class, including MX982, were used to transduce MX734 (*glnA71*::Tn*5*) to glutamine independence. Approximately 85% of the Gln$^+$ [Km^s] progeny resulting from each of the three crosses were Aut$^-$ MS^r and high level constitutive for glutamine synthetase. These results suggest that MX931 cells spontaneously revert to *glnG*$^-$ at a frequency of 1 in 10^5 which is characteristic of the *glnF73*::Tn*5* mutation. Thus, inability of the cells to synthesize glutamine does not seem to be a condition under which the suppressors of *glnF* mutations will arise, as they take place in MX931 capable of synthesizing sufficient amounts of the enzyme to support cellular growth.

The other class of MS^r mutants derived from MX931 was shown not to affect *glnG*. P1 propagated in three of the mutants including MX981, the prototype strain of this class, was used to transduce the MX734 recipient to Gln$^+$ [Km^s]. None of the 200 transductants tested from each cross was Aut$^-$. The nature and location of the mutations rendering these mutants MS^r has not been investigated further.

Finally, the pattern of adenylylation of the glutamine synthetase made by wild-type MX614 cells, which is a function of the quality and abundance of the nitrogen source in the medium, was similar to those shown by the different strains carrying the *gln-76* allele (Table 3). This was true, even if the amount of enzyme made by a particular strain was unusually high. Thus, the *gln-76* mutation does not seem to affect the normal adenylylation-deadenylylation system of glutamine synthetase in these cells.

cis-Dominance of gln-76 in Heterozygous Merodiploids

We reasoned that if *gln-76* lay in the *glnA* control region, it should behave as *cis*-dominant in +/− heterozygotes. To test this assumption we constructed two strains by conjugal transfer of F′14 merogenotes into MX927 (*glnG74*::Tn*5 gln-76*) recipients. One strain, MX936, received an F′14 merogenote carrying the entire wild-type *glnA* region, and the other, MX937, received a similar F-merogenote but carrying the *glnA-71*::Tn*5* insertion (Table 3).

MX936 was weakly Aut$^+$ similar to MX932. In both strains the F′14 merogenote carrying the wild-type *glnA* region complemented the *glnG74*::Tn*5* chromosomal mutation, albeit weakly. Apparently, some *trans* effects exerted by a chromosomal product had negative effects on *glnG* expression from the merogenote, since the wild-type F′14 merogenote expressed Aut$^+$ in the haploid state (i.e., in AB1206, Table 3). Strain MX937 was Aut$^-$, as expected, due to the *glnG74*::Tn*5* mutation of its chromosome and the *glnA71*::Tn*5* mutation of its merogenote which probably exerted polar effects on expression of the *glnG*$^+$ gene positioned *cis* to it.

Table 5. Inhibition of Glutamine Synthetase activity by 80 μM MS in cell-free extracts of different *E. coli* strains

Strain	Without Mg^{2+}			With 60 mM Mg^{2+}		
	−MS	+MS	% Inhibition	−MS	+MS	% Inhibition
MX614	1,320	170	87	1,000	63	97
MX902	35	6	84	35	5	84
MX927	1,900	160	91	1,870	180	90
MX931	130	16	87	150	17	89
MX981	150	36	76	91	12	87
MX982	2,160	108	95	2,000	360	82

Glutamine synthetase activities were determined in cell-free extracts prepared from strains grown on GLA medium. MS (final concentration of 80 μM) was added 10 min before the reactions were started by the addition of L-glutamine

In the haploid state, as in AB1206, the wild-type F′14 merogenote directed synthesis of normally repressible glutamine synthetase (Table 3). Therefore, the high level constitutivity of glutamine synthetase in both MX936 and MX937 was ascribed to *cis*-dominance of *gln-76* on expression of the chromosomal *glnA* gene; the higher maximum levels of constitutivity observed in MX937 over those of MX936 being an additional indication of the negative effects of the limiting amounts of *glnG*$^+$ product made from the F′14 merogenote of MX936, and the inability of the *glnG*$^+$ gene in the F′14 *glnA71*::Tn*5* merogenote to be expressed.

An independent MS^r mutation from MX902 was also isolated and termed *gln-75*. The behavior of *gln-75* was similar to that of *gln-76* in many respects. It was tightly linked to *glnA* in P1 transduction experiments. It also mediated *cis*-dominant constitutive synthesis of glutamine synthetase in the absence of the *glnG-glnF* activator system (data not shown).

Inhibition of Glutamine Synthetase Activity by MS

From evidence presented here we propose that the control region of the *glnA* operon is the site wherein the *gln-76* mutation must lie. The possibility that *gln-76* could lie within the *glnA* structural gene, thus rendering the glutamine synthetase enzyme resistant to MS inhibition, as occurred for the *Salmonella* mutants of Miller and Brenchley (1981) was considered unlikely. Nonetheless, experiments to rule out this possibility were undertaken. As expected, cell-free extracts prepared from strains carrying *gln-76* showed glutamine synthetase activities equally as sensitive to MS as those prepared from wild-type MX614 or MX902 (*glnG74*::Tn*5*) cells (Table 5).

Discussion

E. coli strain MX902 which is hypersensitive to MS by virtue of harboring the *glnG74*::Tn*5* mutation, was used to isolate and characterize spontaneous MS^r variants. One of these, MX927, carried in addition to *glnG74*::Tn*5*, a secondary mutation, *gln-76*, which allowed the constitutive expression of glutamine synthetase.

This constitutive behavior of *gln-76* was shown to be *cis*-dominant when complemented with F'14-merogenotes carrying the entire wild-type *glnA* region (Table 3). Mapping experiments using transduction with bacteriophage P1 demonstrated that the *gln-76* allele was nearly 100% linked to the *glnA* structural gene. Moreover, all Aut^+ transductants that were tested from a cross between MX852 $glnG^+$ $glnL^+$ *Δ(glnA-rha)* as donor and MX927 (*glnG74*::Tn*5* *gln-76*) as recipient still had the *gln-76* mutation (Table 4); therefore, *gln-76* must lie on the *glnA* side opposite to *glnL* and *glnG*. By several additional criteria we have been able to show that the *gln-76* allele does not affect the *glnL* gene. This was considered necessary since mutations in *glnL* are also known to lead to constitutivity for glutamine synthetase, i.e., the GlnC phenotype (Backman et al. 1981; Chen et al. 1982). Contrary to the *cis*-dominant constitutive behavior for glutamine synthetase expression mediated by *gln-76*, constitutivity of all the *glnL* mutations we have tested, as those reported by Chen et al. (1982), was *trans* recessive to the wild-type allele in complementation experiments; in addition, the *gln-76* allele did not suppress the Ntr^- phenotype conferred on *E. coli* cells by the *gltB31* mutation (data not shown) as has been reported for certain *glnL* mutations (Pahel et al. 1978; Chen et al. 1982).

One of the most striking features of the *gln-76* mutation is that it allows *glnA* expression in the absence of a functional *glnG* product, e.g., in strain MX927. The later construction of strain MX982 (Table 3), persumably devoid of both the *glnG* and *glnF* products, further suggests that *gln-76* promotes a highly efficient expression of *glnA* in the absence of both the *glnG* and *glnF* products. Bacterial mutants synthesizing high constitutive levels of glutamine synthetase in the absence of the *glnG-glnF* activator system had not been reported previously. The only precedent was a mutation known as *nifT* isolated in *Klebsiella pneumoniae* by Ausubel et al. (1976, 1977); one such mutation was shown to be *cis*-dominant. These mutations presumably affected the regulatory region of the *nifLA* operon since they allowed *nif* expression in the apparent absence of activation by the *glnG* product, i.e., in a GlnR background.

A *cis*-dominant mutation, *gln-153*, that affects the *glnA* control region of *Klebsiella aerogenes*, has been reported previously (Rothstein and Magasanik 1980). It was selected as a glutamine-independent derivative of a strain carrying a *glnF* mutation and merodiploid for the $glnA^+$ region. Some properties inherent to our *gln-76* mutation are shared by the *gln-153* mutation. However, others such as the high constitutive level of expression of glutamine synthetase mediated by *gln-76* in the absence of a *glnG* product have not been documented for cells carrying *gln-153*. Thus, whether or not *gln-76* and *gln-153* are alike remains an open question.

The molecular basis for *glnA* activation by the *glnG* and *glnF* products and for repression by the *glnG* and *glnL* products, remains veiled. Whatever the mechanisms turn out to be, it is clear that the binding site for the activator remains largely unaltered in cells carrying *gln-76* according to the following reasoning. It was noticed that the maximal level of glutamine synthetase expression in MX927, which occurred when these cells were grown under nitrogen-limiting conditions, was increased by a factor of two when compared to that of wild-type MX614 cells grown under similar conditions (Table 3). However, in strain MX929 which carries *gln-76* and all the *gln* regulatory genes in the wild state, the maximal level of glutamine synthetase expression dropped back to that of wild-type cells (Table 3). Thus, in MX929 cells activation of *glnA* expression by the *glnG* and *glnF* products is apparently preferred over that mediated by *gln-76*. It seems, therefore, as if the *gln-76* mediated expression of glutamine synthetase takes place only in the absence of a functional *glnG* product, regardless of whether or not a *glnF* product is present (compare MX927 with MX981, Table 3).

Another important feature of the constitutive expression of glutamine synthetase in MX927 and in MX982 was its incompleteness; in other words, when these strains were grown on GHA, normally a repressing medium, the enzyme activity was derepressed but was still half of that found under derepressing conditions, as when the cells were grown with glutamine as the only nitrogen source. Since neither normal activation nor repression of the *glnA* operon can take place in MX927 or MX982 cells due to their lack of a functional *glnG* product, this effect may represent a hitherto undefined type of negative control created by exposure of cells carrying *gln-76* to an excess of ammonium ions. A similar inability to attain full derepression of glutamine synthetase in the presence of an excess of ammonium ions, but not necessarily of the same origin, has been observed in *E. coli* strains of quite different genetic constitution, as those carrying *glnL* mutations nonpolar to *glnG* displaying the GlnC phenotype (Chen et al. 1982; Bastarrachea et al., unpublished data).

Introduction of the *glnF73*::Tn*5* allele into MX929 led to strain MX931 (*glnF73*::Tn*5* *gln-76*), the behavior of which was peculiar in several aspects. In *glnF* cells carrying the site corresponding to the *gln-76* mutation in the wild state, i.e. MX848 (Table 3), the *glnF* mutation causes glutamine auxotrophy. In these cells only repression takes place since no activator is present. In strain MX931, however, the *gln-76* mutation prevented *glnF73*::Tn*5* from causing glutamine auxotrophy by allowing synthesis of glutamine synthetase in amounts sufficient to support growth under ammonium assimilating conditions. Curiously enough, the glutamine synthetase activity made by MX931 cells was consistently higher, by a factor of two, when the cells were grown in ammonium excess than when they were grown at low ammonium ion concentrations. The weak expression and the variations of glutamine synthetase activity in MX931 cells could be due to repression exerted by the *glnG* product. This possibility is in agreement with the finding that the intracellular concentration of *glnG*, as measured by β-galactosidase activity in *glnG*::Mud1 fusion strains, is high in cells grown under nitrogen limitation and low in those grown in an excess of nitrogen (Rothstein et al., 1980).

Our method of isolating mutations such as *gln-76* precluded selection of cells overproducing glutamine synthetase in the absence of the *glnG* product. It could be anticipated that at least some of the mutations that arose were affected in the *glnA* promoter. The high constitutive level of *glnA* expression mediated by *gln-76* readily suggests that a high efficiency promoter was probably created by this mutation. Even though *gln-76* made *glnA* expression independent of its normal activator system, it did not affect either the binding to its specific recognition site(s) or its functionality when such an activator was present. Other results described above indicate that *gln-76* altered or des-

troyed a site near *glnA* where the repressor binds. We may tentatively conclude, therefore, that the binding sites for the activator and the repressor are not the same, which is tantamount to saying that they are independent of each other. The results also indicate that if a high efficiency promoter was created by the *gln-76* mutation, it should overlap, at least in part, with the binding domains of the repressor. Our efforts to this end suggest that the *glnA* regulatory region contains more than one DNA sequence which could function as promoters (Covarrubias and Bastarrachea 1983). These findings raise another possibility, that the *glnA* operator region may overlap one of the promoters, thus making both coding sequences susceptible to a single mutational event, which could be the case for *gln-76*. The location of the *gln-76* mutation by base sequence analysis and identification of the start of the mRNA transcripts made from wild-type and *glnG74*::Tn*5 gln-76* DNA's, which are now in progress in our laboratory, may shed some light on the mechanisms that are operative.

From the results presented in Table 3 it is clear that neither the normal pattern of adenylylation of glutamine synthetase nor the inability of the cells to express the Ntr^+ (Aut^+) phenotype in the absence of either one or both the *glnG* and *glnF* functional products were altered by *gln-76*. The phenotypic response of the strains to the glutamine analogue, MS, however, deserves further comments.

Based on the irreversible inhibition of glutamine synthetase activity by MS in the presente of ATP (Ronzio and Meister 1968; Ronzio et al. 1969; Rowe et al. 1969; Manning et al. 1969) we postulated that cells making abnormally small amounts of glutamine synthetase were more sensitive than wild-type cells to growth inhibition by MS. In fact all the *glnG* mutants we have tested were hypersensitive to MS. Previous evidence from Betteridge and Ayling (1975) suggested that MS penetrates the *S. typhimurium* cell via the high affinity glutamine transport system. It is also known that one of the manifestations of the Ntr^- phenotype of *S. typhimurium glnG* mutants is a reduced ability to transport a number of amino acids inside the cells, including glutamine (Kustu et al. 1979b; Wei and Kustu 1981). If the internal MS concentration attained by *glnG* cells is indeed diminished, it should be sufficient to titrate most of the reduced number of glutamine synthetase molecules made by these cells, in order to leave too few active enzyme molecules incompatible with cellular growth.

In our laboratory, accumulation of enzymatically inactive glutamine synthetase-MS molecular complexes has been shown to occur during growth of *E. coli* wild-type cells on GHA-80 μM MS medium (data not shown). Although this result can be taken in support of the titration hypothesis, we still find many examples of strains synthesizing high glutamine synthetase activities which are MS^s. MX931 (*glnF73*::Tn*5 gln-76*), for instance, synthesized twice the glutamine synthetase activity made by wild-type MX614 cells when both were grown on high ammonium ion concentrations. Yet, under these growth conditions, MX931 is sensitive and MX614 is resistant to 80 μM MS. Further experimentation along these lines should lead to new insights into this complex problem of the regulation of glutamine biosynthesis.

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