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Why Does *Escherichia coli* Have Two Primary Pathways for Synthesis of Glutamate?

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***Escherichia coli* has two primary pathways for glutamate synthesis. The glutamine synthetase-glutamate synthase pathway is known to be essential for synthesis at low ammonium concentrations and for regulation of the glutamine pool, but the necessity for glutamate dehydrogenase (GDH) has been uncertain. The results of competition experiments between the wild type and a GDH-deficient mutant during nutrient-limited growth and of direct enzyme measurements suggest that GDH is used in glutamate synthesis when the cell is limited for energy (and carbon) but ammonium and phosphate are present in excess, while the glutamine synthetase-glutamate synthase pathway is used when the cell is not under energy limitation. The use of alternative routes for glutamate synthesis implies that the energy cost of biosynthesis may be less when energy is limited than when energy is unlimited.**

Most enteric bacteria and many other organisms have two primary pathways of glutamate synthesis (Fig. 1). Glutamate dehydrogenase (GDH) catalyzes the reductive amination of 2-oxoglutarate to form glutamate. In *Escherichia coli*, the enzyme is composed of six identical polypeptides encoded by the *gdhA* gene (27). The second pathway, the glutamine synthetase-glutamate synthase (GOGAT) pathway, comprises the activities of two enzymes, glutamine synthetase and glutamate synthase. In this GOGAT pathway, glutamate is amidated to form glutamine by glutamine synthetase (encoded in *glnA*) and the amide group is then transferred reductively to 2-oxoglutarate by GOGAT, resulting in the net conversion of oxoglutarate to glutamate.

Why should any organism possess two distinct pathways to the same end product? Some species in nature have only one or the other. For example, some species of the bacterial genera *Erwinia* and *Bacillus* lack GDH, while higher animals generally lack GOGAT (19, 20, 30). Furthermore, strains of *E. coli* deficient separately in GDH (*gdhA*) or GOGAT (*gltBD*) show no glutamate requirement in usual laboratory media. *gdhA gltB* double mutants require external glutamate (or aspartate, which is readily converted to glutamate) for growth, showing that the organism requires one or the other pathway for glutamate synthesis but under the usual growth conditions not both (1).

Kinetic parameters of the enzymes, enzyme levels under different growth conditions, and the properties of mutants together show that the GOGAT path plays at least two roles for which GDH cannot substitute. GOGAT can fix ammonium into organic molecules (glutamine, thence glutamate and other compounds) when the external concentration of ammonium is low, and it reduces the concentration of glutamine when that concentration becomes high (27). At an ammonium concentration of about 0.1 mM as the nitrogen source, the wild type will grow, but *gltBD* mutants fail to grow in the absence of external glutamate even if they are *gdhA*$^+$ (25). This is because the K_m for ammonium of GDH (about 3 mM) is poor relative to that of glutamine synthetase (<0.2 mM) (22), and because the uptake system for ammonium is repressed in *gltBD* mutants (28).

The necessity for GDH has seemed more problematic. Reitzer and Magasanik (27) state, "Glutamate dehydrogenase is a completely dispensable enzyme; a strain deficient in glutamate dehydrogenase has no observable growth phenotype." However, the maintenance of both GOGAT and GDH in a variety of organisms is prima facie evidence for important and distinct roles for each. If GOGAT is needed at low ammonium concentrations and for regulation of the glutamine level, what is the distinct role for GDH?

Consideration of the pathways shown in Fig. 1 suggests one such role. ATP is expended for every glutamate formed by the GOGAT pathway, but ATP is not used in the GDH reaction. Therefore, one might expect to find that GDH is favored for synthesis of glutamate during energy limitation.

A second possible role for GDH lies in adjusting to high external osmolarity (above the apparent growth optimum of 200 to 300 mosM [3]). *E. coli* and many other organisms respond to increasing osmolarity by increasing their K^+ content. Concomitantly, the glutamate pool increases (3–6, 13, 32). The increased glutamate may result in part from activation of GDH by K^+ (2, 18). The activity of GOGAT is unaffected by relevant concentrations of K^+ (18).

The *gdhA1* mutation resulted from a single-nucleotide-pair substitution. The mutant GDH is devoid of enzymatic activity as the result of replacement of an essential lysine (Lys-92) by glutamate at the active site (12). In the work reported here, I compared growth rates of a *gdhA1* mutant strain and its wild-type counterpart during competition in continuous culture under carbon and energy limitation. The mutant was at a disadvantage, presumably as the result of its restriction in glutamate synthesis.

MATERIALS AND METHODS

Strains, media, and culture conditions. All strains are derivatives of *E. coli* K-12 (Table 1). The standard 1× minimal salts medium contains 7.6 mM $(NH_4)_2SO_4$, 22 mM KH_2PO_4, 40.2 mM K_2HPO_4, and 0.8 mM $MgSO_4$ (11). The calculated osmolarity of standard medium is 189 mosM, and that of medium with 0.1× phosphate (6.22 mM total phosphate) is 41 mosM. Thiamine · HCl was present at 50 μg/liter. Glucose was

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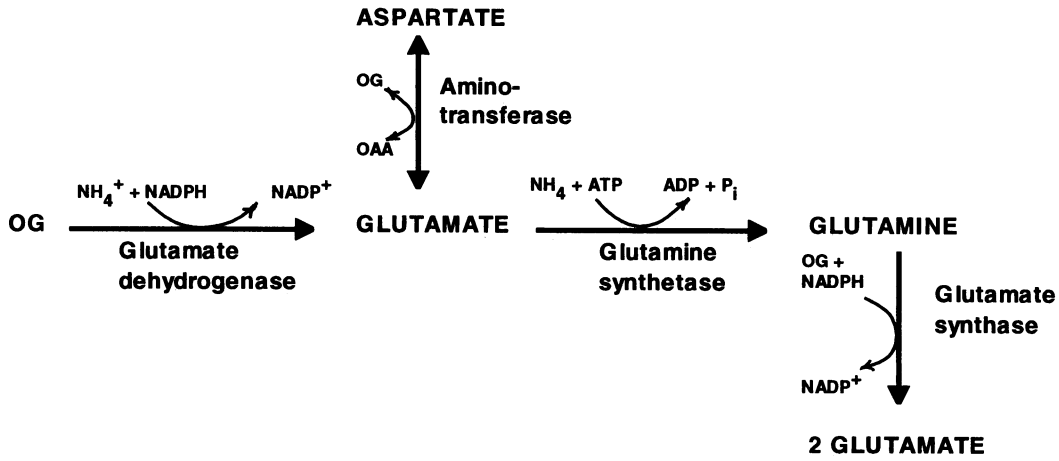

FIG. 1. Pathways for glutamate synthesis in *E. coli*. OG, 2-oxoglutarate; OAA, oxaloacetate.

added to 0.0125% (0.694 mM) for continuous culture (11) and to 0.05% for unlimited growth. All cultures were grown at 30°C, and glucose was shown to be the limiting growth factor even in media containing lower concentrations of salts. Chemostat cultures were maintained with culture volumes fixed at 190 ml and a constant dilution rate of 0.2 h^{-1} (10, 11).

For experiments conducted at reduced phosphate concentration, the two phosphate salts were reduced proportionately. The pH of a culture growing at equilibrium in the chemostat in 1× medium (62.2 mM phosphate) was 7.14, and that of a culture in 0.1× phosphate medium was 6.84. Control experiments for which the total phosphate was 62.2 mM but the pH was varied by changing the ratio of the component phosphate salts showed that over (at least) the pH range of 6.8 to 7.5, the relative fitness of the *gdh* strain did not change.

Detailed procedures for the competition experiments have been described elsewhere (10). In essence, the strains were grown separately in chemostat culture and then mixed, and during continued growth, samples were removed periodically and appropriate dilutions were plated on 1× glycerol (0.2%) agar medium. The colonies that appeared were replicated to 1× glycerol medium also containing 0.1% L-arabinose in order to determine the relative frequency of organisms resistant to arabinose. Differences in growth rates of two competing strains were calculated in the standard way by least-squares regression of the slope of the natural logarithm of the ratios of strain frequencies versus generations (see Fig. 2) (10, 31). Growth rates of the *gdhA1* mutant strains were defined as fractions of that of the *gdhA*$^+$ reference strains and are denoted relative fitnesses, as is conventional (i.e., the relative fitness of the *gdh*$^+$ reference strain was 1.0 by definition [10]). Control experiments showed that the mutation to arabinose resistance conferred a small selective disadvantage, s (relative reduction in growth rate; $s = 1 -$ relative fitness [10]) during continuous culture in 1× medium ($s = 0.021 \pm 0.005$ [see Fig. 2]), comparable to that reported earlier ($s = 0.035 \pm 0.009$ [23]). The effect of the mutation varied slightly as the medium was varied so as to be essentially neutral in medium with the phosphate concentration reduced 10-fold. The results of reciprocal competition experiments suggested that the selective effects of the mutations causing arabinose resistance were independent of the selective effects of *gdhA* mutations. Relative fitnesses were corrected for the effect of the arabinose marker. Maximum specific growth rates were determined by using chemostats operating batchwise, with glucose as the sole carbon and energy source. The frequencies of the separate strains in mixed cultures grown at maximum rates were determined by plating samples periodically and determining frequencies of the arabinose-resistant colonies as described above. All data represent averages of at least two experiments.

Enzyme measurements. Chemostat cultures were initiated with 100-ml overnight standing cultures in the same medium. About 170 ml was removed for assay after 5 to 6 generations. After centrifugation, the harvested cells were washed in 0.01 M $MgSO_4$, resuspended in 1.5 ml of Tris · HCl buffer (0.2 M, pH 7.8), and treated sonically at 75 W for 1 min. The extracts were centrifuged for 3 min in a microcentrifuge, and the supernatant solutions were assayed for GDH and glutamate synthase

TABLE 1. Strains used in this study[a]

Strain	Relevant characteristics	Source or reference
RH481	*gdhA3 gap-7*	11
RH484	*gdhA1 gltB31*	CGSC5550 (12)
RH742	Same as RH481 but *gdhA*$^+$ *zdi-276*::Tn*10*	Transduction from CAG18464 (29), score *gdh*, Tc^r
RH753	Wild-type K-12	CGSC4401
RH822	*araD139 lacY1 glnV44 mcrA gdhA2 rfbD1 thi hsdS1*	JA209 (9, 11)
RH825	Same as RH822 but *zdi-276*::Tn*10 gdh*$^+$ *gap-7*	Transduction from RH742, select Tc^r, score *gdh*, *gap*
RH828	Same as RH825 but *gdhA1 gap*$^+$, lacks Tn*10*	Transduction from RH484, select *gap*$^+$, score *gdh*, Tc^s
RH830	Same as RH825 but *gap*$^+$, lacks Tn*10*	Transduction from RH753, select *gap*$^+$, score Tc^s
RH840	Same as RH828 but arabinose resistant, Ara$^-$	Selection for growth on EMB-arabinose (11)
RH842	Same as RH830 but arabinose resistant, Ara$^-$	Same as for RH840

[a] For a map of the *gdhA* region, see reference 9.

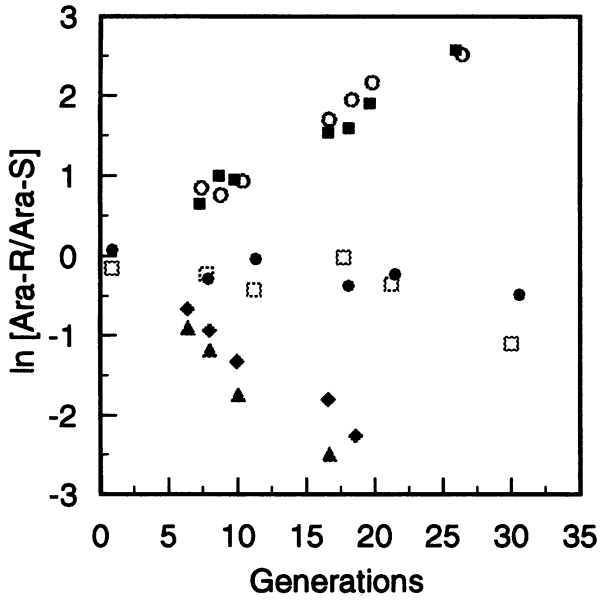

FIG. 2. Competition between *gdhA1* and *gdhA*$^+$ strains during glucose-limited growth. The results from pairs of duplicate experiments are shown. Uppermost symbols (○ and ■) show results of competition experiments between RH828 (*gdhA1* Ara^s) and RH842 (*gdhA*$^+$ Ara^r). Middle symbols (● and □) show results of competition experiments between RH830 (*gdhA*$^+$ Ara^s) and RH842 (*gdhA*$^+$ Ara^r). Lowermost symbols (▲ and ◆) show results of competition experiments between RH830 (*gdhA*$^+$ Ara^s) and RH840 (*gdhA1* Ara^r).

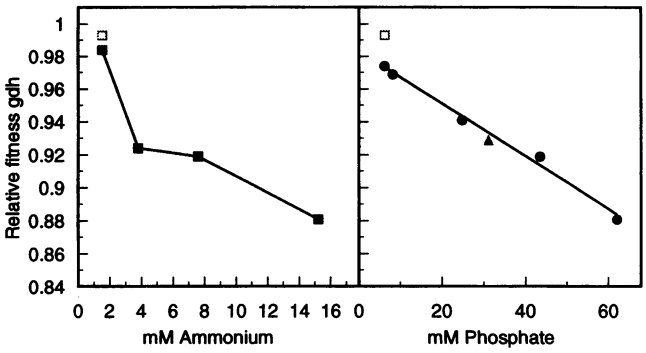

FIG. 3. Relative fitness of a *gdhA* mutant during glucose-limited growth as a function of ammonium or phosphate concentration. Relative fitness denotes the growth rate of the *gdhA1* mutant relative to that of the wild type and was calculated as described in Materials and Methods. Data are from competition experiments between RH828 (*gdhA1* Ara^s) and RH842 (*gdhA*$^+$ Ara^r) such as the ones whose results are shown in Fig. 2. The rightmost datum point in each panel shows the outcome of competition experiments using 1× medium (15.2 mM ammonium, 62.2 mM phosphate), and other similar datum points (■, ●) show the results from competition experiments using medium with decreased concentrations of ammonium or phosphate salts. □, outcome of competition experiments using medium with ammonium and phosphate concentrations both reduced to 0.1 of those in 1× medium; ▲, outcome of an experiment in which the phosphate concentration was reduced to 31.1 mM and KCl was added to bring the osmolarity to that of the 1× medium.

activities at 21°C by monitoring the oxidation of NADPH at 340 nm (20). Enzyme specific activities are expressed as nanomoles of substrate converted to product per minute per milligram of protein. All data represent averages of at least two experiments.

RESULTS

Competition experiments. When *gdhA1* and *gdhA*$^+$ strains (RH828 and RH842) were grown separately in batch culture with a saturating concentration of glucose as the sole source of carbon and energy (maximum specific growth rate), no difference in growth rates or growth yields was observed. As a more sensitive test for difference in growth rates, the strains were grown together, samples were plated periodically, and single colonies were tested to determine the frequencies of the two strains during growth (see Materials and Methods). Again, no difference in the growth rates of the two strains could be detected (data not shown).

Competition experiments with the two strains were repeated, except that this time they were performed during glucose-limited growth in continuous culture. In this case, the *gdhA*$^+$ strain had a clear growth advantage over the mutant (Fig. 2, upper portion [■ and ○]). In order to confirm that the growth advantage of RH842 was because of its *gdhA*$^+$ allele and was not a consequence of the arabinose resistance marker used to identify RH842 after it had been plated for single colonies, the experiment was repeated with the markers reversed; i.e., the *gdhA1* mutant was arabinose resistant (RH830 × RH840). This time, the frequency of arabinose-resistant cells dropped, showing that again the *gdhA*$^+$ strain had a growth advantage (Fig. 2, lower portion [▲ and ◆]). After correction for the slight effect on growth that was observed for the arabinose resistance phenotype (Fig. 2, middle portion [□ and ●]) (see Materials and Methods), the *gdhA1* strain was found to have a fitness (growth rate) of 0.88 relative to that of the wild type (Fig. 3).

In many organisms, GOGAT levels are high at low ammonium concentrations and low at high ammonium concentrations, consistent with the essential role of GOGAT at low concentration (19). (In *E. coli*, the levels do not seem to change much with ammonium concentration [27], but there is disagreement on this point, probably reflecting other differences in the experiments [e.g., see reference 14]). GDH is expected to be ineffective during growth at low ammonium concentrations. Therefore, the *gdhA1* mutant should compete better with the *gdhA*$^+$ strain at a low ammonium concentration than it did in the standard 1× medium. When the competition experiments carried out during glucose-limited growth were repeated, but at lower ammonium concentrations, the results were consistent with this expectation (Fig. 3, left panel [■]). At less than 2 mM ammonium, the growth rates of the strains were almost equivalent.

In order to see if the fitness of GDH varied with osmolarity, the results of the experiments conducted with 1× medium described above were compared with those from other competition experiments done with medium of progressively lower osmolarity. The osmolarity was reduced by decreasing the concentration of potassium phosphate (see Materials and Methods). The results show that there is, in fact, an inverse correlation between the phosphate concentration (Fig. 3, right panel [●]), hence osmolarity, and the relative fitness of the *gdhA1* mutant, suggesting that GDH may help the organism to adapt to growth as osmolarity increases. Decreasing both ammonium and phosphate at the same time increased the relative fitness more than decreasing either separately did (Fig. 3 [□]).

However, the osmolarities involved in these studies are

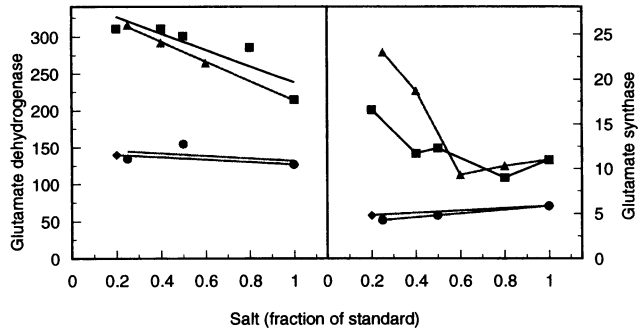

FIG. 4. Enzyme activities as a function of ammonium and phosphate concentrations. The lowermost curves (● and ◆) show results from RH842 cells grown in a chemostat with glucose limitation; the uppermost curves (■ and ▲) represent cells from unlimited-growth cultures. Ammonium (◆, ■) and phosphate (●, ▲) concentrations are represented as fractions of those in 1× medium (15.2 mM ammonium and 62.2 mM phosphate).

relatively low (3), and McLaggan et al. recently reported evidence that GDH plays no essential role in adaptation to high osmolarity (17). Thus, it seemed likely that the increase in fitness with decreasing potassium phosphate concentration might be specific to one of the ions rather than the consequence of decreasing osmolarity itself. To test this possibility, the phosphate was reduced to half the concentration in the 1× medium but the osmolarity was maintained at the standard level by adding KCl. The results show clearly that for the experiments reported here, the relative fitness is a function of the phosphate level (Fig. 3 [▲]) and is not correlated with osmolarity at the low salt concentrations involved here or with potassium.

Enzyme studies. The results described above showed that the relative fitness of a mutant lacking GDH activity increased as the ammonium or phosphate concentration decreased. This must reflect the increasing role of GOGAT (and the diminishing role of GDH) in glutamate synthesis under these conditions, which must result in turn from a change in enzyme activity (turnover number), amount, or both. In order to see if the amount of either enzyme changed as the fitness changed, I assayed the enzymes.

During glucose-limited growth in continuous culture, the specific activities of both GDH and GOGAT were low and little affected by change in either ammonium or phosphate concentration over the range in which the relative fitness of the *gdhA1* mutant varied (Fig. 4, lower curves). During unlimited growth (excess glucose), the specific activity of each enzyme was higher and increased as the ammonium or phosphate concentration was reduced (Fig. 4, upper curves). However, except for the response of GOGAT to phosphate (Fig. 4, right panel [▲]), the changes in enzyme level were slight. The amount of GOGAT during glucose-limited growth in 1× medium appeared to be unaffected by the *gdhA1* mutation (data not shown).

DISCUSSION

A broad range of literature deals with the levels of the glutamate-synthesizing enzymes under different conditions. It has not been easy to tie together previous results into a coherent picture. This is most likely because the conditions and strains varied and because the functions of the two primary pathways for glutamate synthesis are of central importance to metabolism and so are subject to multiple forms of regulation. The extraordinarily complex regulation of formation and activity of the first enzyme in the GOGAT pathway, glutamine synthetase, is well recognized (7, 8, 15, 27, 33).

The experiments reported here imply that GDH plays a role in glutamate synthesis when *E. coli* is under energy (and carbon) restriction but not under ammonium or phosphate restriction and that the GOGAT pathway is responsible for glutamate synthesis when energy is plentiful or when the ammonium or phosphate concentration becomes low. The demonstration that energy supply and ammonium level are regulators of glutamate synthesis is not unexpected, but a role for phosphate in this control may seem surprising. Phosphate starvation activates expression of the *gltBDF* operon (21) (Fig. 4), phosphorylation is thought to activate GDH itself (16), phosphorylated intermediates play important roles in the formation and activity of glutamine synthetase (7), and the importance of phosphorylated intermediates in carbon and energy metabolism is well known. Nevertheless, the phosphate concentrations employed here are high relative to the affinities of the phosphate transport systems (e.g., see reference 34), and it seems unlikely that the internal phosphate concentration varied much in the experiments reported here. Possible explanations for the marked dependence of relative fitness on phosphate concentration include activation of the GOGAT pathway or inactivation of the GDH pathway by external rather than internal phosphate (e.g., see reference 26) or by a trace contaminant present in the phosphate salts.

The details of the mechanisms by which the controls over glutamate synthesis are effected remain to be determined, but controls beyond the level of transcription and translation of *gdhA* and *gltBDF* are required. During glucose-limited growth, *gltBDF* is highly repressed and the GDH level is low, and enzyme levels do not change with ammonium or phosphate concentration over the range in which the relative fitness changes. Competition experiments provide a sensitive assay for the flux of metabolites leading to glutamate formation.

Recognition that GOGAT appears to be responsible for glutamate synthesis during growth in saturating glucose has important implications relative to estimating the energy expenditure for biosynthesis. The formation of a gram (dry weight) of cells of *E. coli* has been calculated to require the conversion of 43 mmol of ATP to ADP (24). This calculation underestimates the cost of protein synthesis (35) and ignores the cost of transport of materials other than glucose. Adjusting to include those costs, I estimate the overall cost of synthesis to be 50 to 60 mmol of ATP per g (dry weight), or (for purposes of calculation) about 55 mmol. The original calculation also assumed that glutamate is synthesized by the GDH pathway. If, instead, the GOGAT path is used, the requirement for ATP should increase by about 10 mmol (10 mg-atoms of N fixed through glutamate per g [dry weight] [27]); 1 mmol of ATP per mg-atom of N [Fig. 1]), for a total cost of 65 mmol of ATP, or an 18% increase in energy expenditure. This large a difference should be reflected in the relative growth yields of *gdh*$^+$ and *gdh* mutant strains if they differ in their modes of glutamate synthesis in batch culture; lack of difference is consistent with the conclusion that GDH plays little biosynthetic role during such growth. On the other hand, during energy-limited growth, GDH appears to be important because, through its use, the cost of biosynthesis is scaled down. The use of alternative routes for glutamate synthesis (and possibly of alternative metabolic routes for other pathways as well) implies that the energy cost of biosynthesis may be less under energy limitation than when energy is unlimited.

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