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Differential contribution of the proline and glutamine pathways to glutamate biosynthesis and nitrogen assimilation in yeast lacking glutamate dehydrogenase

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Abstract

In *Saccharomyces cerevisiae*, the glutamate dehydrogenase (GDH) enzymes play a pivotal role in glutamate biosynthesis and nitrogen assimilation. It has been proposed that, in *GDH*-deficient yeast, either the proline utilization (PUT) or the glutamine synthetase-glutamate synthase (GS/GOGAT) pathway serves as the alternative pathway for glutamate production and nitrogen assimilation to the exclusion of the other. Using a *gdh*-null mutant (*gdh1*Δ*2*Δ*3*Δ), this ambiguity was addressed using a combination of growth studies and pathway-specific enzyme assays on a variety of nitrogen sources (ammonia, glutamine, proline and urea). The *GDH*-null mutant was viable on all nitrogen sources tested, confirming that alternate pathways for nitrogen assimilation exist in the *gdh*-null strain. Enzyme assays point to GS/GOGAT as the primary alternative pathway on the preferred nitrogen sources ammonia and glutamine, whereas growth on proline required both the PUT and GS/GOGAT pathways. In contrast, growth on glucose-urea media elicited a decrease in GOGAT activity along with an increase in activity of the PUT pathway specific enzyme Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH). Together, these results suggest the alternative pathway for nitrogen assimilation in strains lacking the preferred *GDH*-dependent route is nitrogen source dependent and that neither GS/GOGAT nor PUT serves as the sole compensatory pathway.

Keywords

Yeast; *Saccharomyces cerevisiae*; glutamate dehydrogenase; nitrogen assimilation

1. Introduction

Like many other microorganisms, *Saccharomyces cerevisiae* is able to utilize a wide array of nitrogen-containing compounds as its sole source of nitrogen (Magasanik and Kaiser,

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2002). In order to utilize diverse nitrogen sources, *S. cerevisiae* uses two strategies, both of which converge on the amino acid glutamate. This is achieved either through the assimilation of the metabolic by-product ammonia into α-ketoglutarate or by catabolism of precursor molecules to yield glutamate directly. Glutamate produced by these routes can then react with another molecule of α-ketoglutarate to form glutamine. The two amino acids that are formed—glutamate and glutamine—are responsible for 85% and 15% of total cellular nitrogen, respectively (Cooper, 1982). The primary enzyme responsible for generating glutamate *in vivo* is glutamate dehydrogenase (GDH) (Avendano *et al*., 1997; De Luna *et al*., 2001; Miller and Magasanik, 1990). The *S. cerevisiae* genome contains three *GDH* genes encoding for enzymes with different functional roles. Two of the genes, *GDH* and *GDH3*, encode NADPH-dependent enzymes that catalyze the reductive amination of α-ketoglutarate to form glutamate, whereas the other, *GDH2*, encodes an NAD^+-dependent enzyme that catalyzes the reverse reaction (DeLuna *et al*., 2001). Although both Gdh1p and Gdh3p catalyze the formation of glutamate, Gdh1p is the preferred biosynthetic enzyme under all conditions (DeLuna *et al*., 2001). The third GDH gene, *GDH2*, plays an equally important role in nitrogen metabolism, where it has been shown to be the primary source of ammonia generation in *S. cerevisiae* (Miller and Magasanik, 1990).

Although previous research posits a central role for GDH in glutamate metabolism and nitrogen assimilation, it does not constitute the sole pathway for these processes (Figure 1) (Avendano *et al*., 1997; De Luna *et al*., 2001Miller and Magasanik, 1990). In fact, at least two other pathways have been shown to exist in *S. cerevisiae* for the synthesis of glutamate and assimilation of nitrogen—viz. the proline utilization (PUT) and glutamine synthetase-glutamate synthase pathways (GS/GOGAT). Proline gains entry into the cells via either the general amino acid permease, Gap1p, or the proline-specific permease, Put4p (Magasanik and Kaiser, 2002). The enzymes that constitute the PUT pathway are proline oxidase (Put1p) and Δ^1-pyrroline-5-carboxylate dehydrogenase (Put2p), which are encoded by the *PUT* and *PUT* genes, respectively (Brandriss and Magasanik, 1979; Wanduragala *et al*., 2010). In laboratory strains of *S. cerevisiae*, both the PUT pathway and permease genes (Gap1p and Put4p) are tightly controlled by nitrogen catabolite repression (NCR) due to the status of proline as a non-preferred nitrogen source (ter Schure *et al*., 2000). The second alternative pathway for glutamate production, GS/GOGAT, consists of two enzymes acting consecutively to catalyze: (1) the ATP-dependent amidation of glutamate using free ammonia (GS) and (2) the reductive amination of α-ketoglutarate using the amide nitrogen of glutamine (GOGAT) (Figure 1) (Magasanik and Kaiser, 2002; ter Schure *et al*., 2000). Ultimately, this sequence of reactions yields two molecules of glutamate for every one that enters the pathway.

Using mutagenesis on a glutamate auxotroph stalled in the citric acid cycle at aconitase (in their nomenclature, $glt_{1\text{-}1}$), Lundgren *et al*. showed that the PUT pathway could serve as a source of glutamate, as the *put2*-$glt_{1\text{-}1}$mutant they isolated was no longer able to utilize proline as a nitrogen source (Lundgren *et al*., 1972). In contrast with the results from Lundgren *et al*., Avendano and colleagues asserted that the GS/GOGAT pathway, in combination with the GDHs, represents the sole pathway for glutamate biosynthesis in *S. cerevisiae* (Avendano *et al*., 1997). In order to address this apparent discrepancy, a *gdh*-null

mutant was created and subjected to experiments exploring the relative contribution of the PUT and GS/GOGAT pathways to glutamate biosynthesis and nitrogen assimilation following adaptation to growth on various nitrogen sources. This approach allowed for an unprecedented exploration of nitrogen assimilation in *S. cerevisiae* and enabled both the clarification previous findings and the discovery of previously unappreciated mechanisms for nitrogen assimilation in this organism

2. Materials and Methods

2.1. Construction of GDH mutants

The yeast strains used in these studies and their genotypes are listed in Table 1. The strains were obtained using a combination of traditional and molecular genetic techniques. Briefly, the single mutants (i.e. *gdh1*Δ, *gdh2*Δ and *gdh3*Δ) were crossed with a wild-type strain (SEY6211) and mutants with novel genetic markers were isolated following sporulation, tetrad dissection and genotyping (Sherman, 2002).

The disrupted genes, *gdh1*Δ*::TRP1*and *gdh2*Δ*::URA3*, were introduced into *gdh* single mutants via linearization and homologous recombination from the YEp352-*gdh1*Δ*::TRP1* and pUC18-*gdh2*Δ::*URA3*plasmids, respectively. *GDH1*and *GDH2* were amplified from genomic DNA using restriction site (underlined)-flagged primer pairs *GDH1*-BamF (5'-CAGGATCCACGATTGGCTG-GATAAGAGGA-3') / *GDH1*-PstR (5'-TGTCTGCAGTGCCAAATTGGACGAGTAAG-3') and *GDH2*-XbaF (5'-GCTCTAGATCGACATCAACACTGACAAGC-3') / *GDH2*-KpnR (5'-GGGGTACCAAC-CTGTTTCAATGCTGCCT-3'), respectively (Integrated DNA Technologies). Genes amplified from genomic DNA were restriction digested and ligated into the YEp352 (Hill *et al*., 1986) and pUC18 (Stratagene; now Agilent Technologies) plasmids using restriction enzyme pairs BamHI-PstI and XbaI-KpnI, respectively. The *TRP1* gene was excised from the PJJ281 (Jones and Prakash, 1990) plasmid using restriction enzymes SalI and BglII and ligated into the YEp352-*GDH* plasmid to create YEp352-*gdh1*Δ*::TRP1*. *URA3* was amplified from genomic DNA using primers *URA3* F-AvaI (5'-CCCTCGAGCGGCATCAGAGCAGATTGTA-3') and *URA3* R-ClaI (5'-CCATCGATCGT-TGGAGTCCACGTTCTTT-3') (Integrated DNA Technologies) and inserted between the AvaI and ClaI sites in the pUC18-*GDH* plasmid. Linear fragments were isolated using restriction enzyme pairs PacI-KpnI (*gdh1*Δ*::TRP1*) and XbaI-KpnI (*gdh2*Δ*::URA3*), gel purified and introduced into the appropriate single and double mutants via homologous recombination using the YEASTMAKER Yeast Transformation System 2 (ClonTech). Following recombination, the mutants were selected for on the appropriate drop out media. Genotypes were confirmed by assessing the growth phenotypes for nutritional markers and through PCR analysis of *GDH* alleles (data not shown). All restriction enzymes were purchased from New England Biolabs.

2.2. Media and Growth

Minimal Media (Y-Min) contained 0.67% yeast nitrogen base without amino acids or ammonium sulfate, 2% glucose and the appropriate amino acid supplements (Table 1) (Sherman, 2002). The nitrogen sources were added at either 0.2% for ammonium sulfate or

0.1% for glutamine, proline or urea (Miller and Magasanik, 1991). All media reagents were obtained from either Difco or Sigma chemical.

Doubling times were determined for cells during logarithmic growth. Cells pre-grown overnight in Y-Min supplemented with ammonia and the appropriate amino acids were diluted in Y-Min plus the nitrogen source being tested to an optical density of 0.05–0.2 at 600 nm (OD_{600}). Following a lag-phase of 7–8 hours, OD_{600} readings were recorded every hour for 6–8 hours. At each step, cells were at 30°C with shaking (200 rpm).

2.3. Enzyme Assays

For the determination of enzyme activities, culture conditions were identical to those used in the experiments. Crude lysates were prepared from cultures in late-log phase as described previously (Trotter *et al*., 2005). Aliquots were stored at −80° C and thawed on the day of the assay. Protein in the crude lysates were determined prior to enzyme assays using the BCA (bicinchoninic acid) method (Sigma Chemical). All chemicals were purchased from either Sigma Chemical or Dow Chemical.

GDH activities were measured by monitoring the oxidation of NADPH (Gdh1/3p) or NADH (Gdh2p) spectrophotometrically according the method of Doherty (Doherty, 1970). Citrate synthase were measured by following the reaction of coenzyme A and 5,5'-dithiobis-(2-nitro-benzoate) (DTNB) at 412 nm in a reaction mixture containing 1 mM DTNB, 10 mM acetyl-CoA, 10 mM that was initiated by adding ~0.06 mg of protein from crude lysate (Srere, 1969).

Glutamate synthase (GOGAT) and Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH) activities measured by monitoring either the oxidation (GOGAT) or reduction (P5CDH) of NAD(H) at 340. GOGAT assays were performed in 1-mL reaction volumes (100 mM potassium phosphate, pH 7.8; 0.2 mM NADH; 10 mM glutamine; ~0.2–0.4 mg protein from crude lysates) and initiated by the addition of α-ketoglutarate (10 mM) (Roon *et al*., 1974). DL-Δ^1-Pyrroline-5-carboxylate (P5C) was synthesized to the method of Williams and Frank and was adjusted to a pH of 7.0 prior to use in assays (Williams and Frank, 1975). P5CDH assays were carried out in 1-mL reaction volumes (50 mM tris(hydroxylmethyl)methyl-3-aminopropanesulfonic acid (TAPS), pH 9.0; 0.8 μM P5C, pH 7.0; 0.2 mM NAD^+) and initiated by the addition of between 0.2–0.4 mg of protein from crude lysate (Brandriss and Magasanik, 1979; Williams and Frank, 1975).

3. Results

3.1. Strain Creation and Confirmation

Although the isolation and characterization of several *GDH* mutants have been reported elsewhere, the *gdh2*Δ*3*Δ and *gdh*1Δ2Δ*3*Δ strains have yet to be described (Avendano *et al*., 1997; De Luna *et al*., 2001; Miller and Magasanik, 1990). For this study, the full complement of double and triple mutants was isolated using standard genetic techniques (see Methods). Confirmation of mutant strains was accomplished via PCR amplification of *GDH* alleles and growth phenotype for auxotrophic markers (data not shown). To further test the integrity of the isolated strains, NADP-GDH (Gdh1p and Gdh3p) and NAD-GDH

(Gdh2p) activities were determined (Table 2). The lack of detectable NADP-GDH activity in the *gdh1*-deficient strains corroborates well with both our PCR findings and other studies showing the primacy of Gdh1p over Gdh3p in strains grown on glucose (Avendano *et al*., 1997; De Luna *et al*., 2001). While significant levels of NAD-GDH activity were not observed for the *gdh2*Δ, *gdh1*Δ*2*Δ or *gdh1*Δ*2*Δ*3*Δ strains, the *gdh2*Δ*3*Δ mutant exhibited consistently elevated activity when grown on glucose-ammonia media. It has been previously reported that the *GDH2* allele is subject to glucose repression, which can be relieved when strains are grown on either glucose-limiting media or non-fermentable carbon sources (Coschigano *et al*., 1991). To further test for NAD-GDH activity in putative *gdh* mutants, wild-type and mutant strains were grown on the non-fermentable carbon source raffinose, which has previously been used to maximize Gdh2p activity (Tang *et al*., 2011). As expected, when raffinose was substituted for glucose, the putative *gdh2* mutants showed no detectable NAD-GDH activity as compared to the wild-type strain, which exhibited a four-fold increase in activity (see Table 2, c). Taken together, these results confirm the isolation of the full complement of *GDH* mutants.

3.2. Experimental Conditions and Growth Data

Four nitrogen sources were selected to explore the differential contributions of the PUT and GS/GOGAT pathways to glutamate biosynthesis and nitrogen assimilation in adapted *gdh*-deficient yeast. As a result of nitrogen catabolite repression (NCR) in *S. cerevisiae*, pathways for utilization of non-preferred sources of nitrogen—e.g. proline—are down-regulated in the presence of the preferred nitrogen sources ammonia, glutamine and asparagine (ter Schure *et al*., 2000). In addition to NCR status, nitrogen sources in *S. cerevisiae* differ in their mode of utilization proceeding either through an ammonium intermediate (Ai) or via direct conversion to glutamate (Glu) (Magasanik and Kaiser, 2002; ter Schure *et al*., 2000). Four nitrogen sources were chosen corresponding to the four possible combinations of NCR-status and pathways for utilization: (i) ammonia – Ai/repressing; (ii) glutamine – Glu/repressing; (iii) urea – Ai/de-repressing; (iv) proline – Glu/de-repressing (Magasanik and Kaiser, 2002; ter Schure *et al*., 2000). Figure 1details the relevant pathways for the utilization of these nitrogen sources including the enzymes whose activities were assayed in this study.

Upon transfer and adaptation to fresh medium containing any of the four nitrogen sources, all strains exhibited a lag-phase between 7–8 hours (data not shown). The *gdh1*-deficient strains had doubling times 56–67% as compared to wild-type when grown on glucose-ammonia media (Table 2). When the *gdh1*-deficient strains were grown on glutamine as the sole nitrogen source, low growth rates were observed (39–51% of wild-type) with the exception of *gdh1*Δ and *gdh1*Δ*2*Δ*3*Δ, which exhibited slightly higher (79% and 83% of wild-type) growth rates (Table 2). Growth of the *gdh2*Δ*3*Δ strain on glucose-glutamine media was approximately 130% of wild-type (Table 2). Rates of growth for the GDH mutants when urea was utilized as the sole nitrogen source yielded a pattern analogous to that seen on glucose-glutamine media—i.e. a reduction of growth rate in *gdh1*-deficient strains and an elevation in the *gdh2*Δ*3*Δ mutant (Table 2). In contrast, growth on proline yielded a reduction in growth rate to approximately 80% for all mutants with the exception of *gdh3*Δ. For strains exhibiting severely attenuated growth rates (i.e. those below 50% of

WT), growth phenotypes were confirmed using spot assays of serial dilutions (data not shown). Growth phenotypes for the remaining mutants were undiscerable due to the assay's lack of resolution. Because the elevated growth rate of the *gdh2*Δ*3*Δ mutant in glucose-glutamine and glucose-urea media was unexpected, this strain was chosen for inclusion in subsequent experiments.

3.3. Enzyme Assays

Because glutamate metabolism and nitrogen assimilation are intricately linked with the citric acid cycle (CAC; or tricarboxylic acid, TCA, cycle) through the intermediate α-ketoglutarate, the activity of the enzyme citrate synthase (encoded by the gene *CIT1*) was measured as a proxy for CAC function in *GDH*-deficient mutants. In the wild-type strain, citrate synthase activities were similar for nitrogen sources that either converge on glutamate (30.9 and 48.7 μmol/min/mg-protein for glutamine and proline, respectively) or proceed through an ammonium intermediate (58.0 and 75.7 μmol/min/mg-protein for ammonia and urea, respectively). On nitrogen sources where the *gdh*2Δ3Δ strain exhibited enhanced growth rates—i.e. glutamine and urea—citrate synthase activities followed a similar pattern in which the *gdh*2Δ3Δ exhibited slightly up-regulated and the triple-mutant exhibited either no change or slightly down-regulated activity compared to wild-type ($p < 0.05$ for both the up-regulations in *gdh*2Δ3Δ and the down-regulation of the triple-mutant on urea; see Figure 2). While citrate synthase activities showed an analogous pattern on the aforementioned nitrogen sources, the pattern for proline grown cells differed greatly. Although an increase in citrate synthase activity for the *gdh*2Δ3Δ strain was also observed on proline, the up-regulation was far more pronounced (2.5-fold increase as compared to wild-type, $p < 0.01$; Figure 2). Additionally, an approximately 2-fold elevation in citrate synthase activity was observed for the *gdh1*Δ*2*Δ*3*Δ strain grown on glucose-proline media (relative to WT, $p < 0.01$; Figure 2).

Previous work in *S. cerevisiae* has suggested that either the GS/GOGAT or the PUT pathway is responsible for glutamate production in strains deficient in *gdh* activity (Avendano *et al*., 1997; Valenzuela *et al*., 1998). In order to assess the relative contributions of these pathways, activities of the Put2p (P5CDH) and Glt1p (GOGAT) enzymes were determined for the *gdh*2Δ3Δ and *gdh1*Δ *2*Δ*3*Δ strains following adaptation to various nitrogen sources. P5CDH activities in the *gdh*2Δ3Δ mutant were slightly decreased when either proline or urea were supplied as the sole nitrogen source (Figures 5 & 6; ~0.8-fold compared to wild-type, $p < 0.08$ and $p < 0.05$ for proline and urea, respectively). When the *gdh1*Δ*2*Δ*3*Δ strain was grown in the presence of either ammonia or urea, an increase in P5CDH activity was observed (Figures 3 & 6; ~1.6-fold and ~1.2-fold increase compared to WT for ammonia and urea, respectively, $p < 0.05$). P5CDH activities for both mutants on the remaining nitrogen sources remained indistinguishable from wild-type. Analysis of GOGAT activity in the *gdh1*Δ*2*Δ*3*Δ strain showed a significant up-regulation when ammonia, proline or glutamine were utilized as the sole nitrogen source (between 1.4–1.8-fold relative to wild-type, $p < 0.08$, 0.05 and 0.01 for ammonia, proline and glutamine, respectively; Figures 3–Figure 5). Up-regulation of GOGAT activity was also observed for the *gdh*2Δ3Δ mutant grown on glucose-ammonia media (~1.6-fold compared to wild-type, $p < 0.08$; Figure 3B).

On the remaining nitrogen source, urea, a decrease in GOGAT activity of 40–60% relative to wild-type was observed for the *gdh*2Δ3Δ and *gdh1*Δ*2*Δ*3*Δ strains ($p < 0.05$; Figure 6).

4. Discussion

In *S. cerevisiae*, the amino acid glutamate plays an integral role in nitrogen metabolism, where it is responsible for 85% of total cellular nitrogen (Cooper, 1982; ter Schure *et al*., 2000). The ability of glutamate to serve as progenitor of 85% of total cellular nitrogen is due, in large part, to the action of glutamate dehydrogenase (GDH), which catalyzes the direct assimilation of free ammonia via reductive amination of α-ketoglutarate (ter Schure *et al*., 2000). The *S. cerevisiae* genome contains three *GDH* genes encoding for enzymes that favor either the assimilation—Gdh1p & Gdh3p—or release—Gdh2p—of ammonia (see Figure 1) (Cooper, 1982; ter Schure *et al*., 2000). Although previous studies report that Gdh1p serves as the main conduit for nitrogen assimilation and glutamate biosynthesis *in vivo*, *gdh1*Δ strains are viable and do not exhibit glutamate auxotrophy suggesting that alternative pathways exist (DeLuna *et al*., 2001). While the proline utilization (PUT) and glutamine synthetase-glutamate synthase (GS/GOGAT) pathways have been previously identified for their ability to facilitate glutamate biosynthesis in *gdh*-deficient yeast, questions remain as to the relative contribution of these pathways in mutant strains grown on various nitrogen sources (Avendano *et al*., 1997; Valenzuela *et al*., 1998). A *gdh*-null mutant was created. The strain was able to adapt and grow on various nitrogen sources which allowed the examination of the relative contributions of the PUT and GS/GOGAT pathways to glutamate biosynthesis and nitrogen assimilation in the absence of the favored GDH pathway.

Although previous studies examining the growth characteristics of *GDH* mutants have primarily used ammonia as the sole nitrogen source, data for wild-type growth on the remaining nitrogen sources have been documented (Miller and Magasanik, 1991). Doubling times for the wild-type strain used in these studies, BY4742, correspond well with these previous results (Table 2, a). When ammonia was supplied as the sole nitrogen source, *gdh1*-deficient strains exhibited decreased growth rates in the range of 47–63% compared to wild-type, whereas loss of either *GDH2* or *GDH3* resulted in little reduction in growth rate; these rates largely paralleled those reported previously (DeLuna *et al*., 2001; Miller and Magasanik, 1990). In a previous study, the growth rate of the *gdh1*Δ mutant on glucose-glutamine media was found to be 56% of the wild-type rate (Miller and Magasanik, 1991), and our results are generally similar. The elevated growth rate of the *gdh2*Δ*3*Δ mutant grown on glucose-glutamine or glucose-urea media, however, was unexpected. While this strain was expected to grow at a near wild-type rate—as seen in the *gdh2*Δ and *gdh3*Δ single mutants—a persistent elevation in growth rate was not predicted. In glucose-limiting conditions, the concentration of α-ketoglutarate decreases leading to a switch in preference from GDH to the GS/GOGAT pathway for nitrogen assimilation (Magasanik, 2003; Roon *et al*., 1974). The preference for the GS/GOGAT pathway in response to low intracellular α-ketoglutarate levels has been proposed to result from a more efficient use of glucose by this pathway as well as the intrinsic properties of the GOGAT enzyme—viz. a three-fold greater affinity for α-ketoglutarate and the irreversibility of the reaction it catalyzes (Magasanik, 2003). Our data suggest that this pathway may be operative in the *gdh2*Δ*3*Δ mutant, as the

inability of this strain to regenerate α-ketoglutarate through the Gdh2p catalyzed reaction may have led to an analogous drop in intracellular α-ketoglutarate, thus ensuring the use of the more efficient GS/GOGAT pathway. While the observed increase in citrate synthase activity on glucose-glutamine and glucose-urea provides indirect evidence of decreased α-ketoglutarate concentrations in the *gdh2*Δ*3*Δ mutant, this explanation is weakened by the absence of a growth-rate enhancement in the *gdh2*Δ mutant. A possible explanation for this discrepancy is that the Gdh3p isoform may catalyze the oxidative deamination of glutamate to α-ketoglutarate in strains lacking Gdh2p activity. While there is no literature precedent for this alternative activity of the Gdh3p isoform, our growth data for the *gdh2*Δ*3*Δ mutant grown on glucose-glutamine or glucose-urea media suggest Gdh3p may compensate for loss of Gdh2p activity under certain physiological conditions.

Previously, Avendano *et al*. have shown that yeast lacking both NADP-GDH and GOGAT enzymatic activity (*gdh1*Δ*gdh3*Δ*glt1*Δ) are strict glutamate auxotrophs (Avendano *et al*., 1997) arguing for the importance of the GS/GOGAT pathway in *gdh*-deficient yeast. As expected, our data show an increase in GOGAT activity for the *gdh*-null mutant grown on glucose-ammonia media (Figure 3A) (Avendano *et al*., 1997; Valenzuela *et al*., 1998). Interestingly, despite the repressing activity of ammonia on the PUT pathway (ter Schure *et al*., 2000; Magasanik and Kaiser, 2002), a 1.6-fold increase in P5CDH activity was also observed, which suggests that the GS/GOGAT pathway is not solely responsible for glutamate production under these conditions (Figure 3B). When the *gdh*-null mutant was grown on glucose-glutamine media, glutamate production was derived primarily from the GS/GOGAT pathway as evidenced by a nearly 2-fold increase in GOGAT activity relative to the wild-type strain grown on this nitrogen source (Figure 4B). Together, these results suggest that the GS/GOGAT pathway serves as the primary, but not sole, route for glutamate production in *gdh*-null yeast adapted to the preferred nitrogen sources ammonia and glutamine (Figures 3C and 4C).

While an up-regulation in P5CDH activity compared to the wild-type strain was not observed for the *gdh*-null mutant grown on glucose-proline media, the absolute level of P5CDH activity in the null mutant was significantly elevated on this nitrogen source (83.9 vs. 23.5 nmol/min-mg for glucose-proline and glucose-ammonia grown cells, respectively). Elevated activity of the P5CDH enzyme argues for the importance of the PUT pathway in *gdh*-null yeast grown on glucose-proline media. Yet, the observed increases in both citrate synthase and GOGAT activities suggest that alternative pathways may become important when levels of glutamate are significantly depleted. In a previous report, Valenzuela and co-workers observed a slight up-regulation in GOGAT activity when the *gdh1*Δ mutant was grown on glucose-proline media (~1.3-fold) (Valenzuela *et al*., 1998). Our results indicate that elimination of the GDH pathway yields an approximately 1.6-fold up-regulation in GOGAT activity on glucose-proline media (Figure 5B). Because citrate synthase catalyzes the rate-limiting step in the CAC, the nearly 2-fold increase in its activity observed for the *gdh*-null mutant grown on glucose-proline media is evidence of enhanced CAC function (Figure 2C) (Kim *et al*., 1986). Enhanced CAC activity would be expected to result in elevated levels of α-ketoglutarate, which could then be utilized by the GS/GOGAT pathway to synthesize an additional molecule of glutamate from the one already derived from

breakdown of proline. Taken together, these results demonstrate that, in yeast lacking the preferred GDH pathway, the GS/GOGAT and PUT pathways may act in a coordinated manner to ensure that glutamate concentrations do not reach prohibitively low levels (Figure 5C).

In contrast to cells grown in the presence of ammonia, glutamine or proline as sole nitrogen sources, growth of the *gdh*-null mutant on glucose-urea media elicited a 40% decrease in GOGAT activity (Figure 6B). Utilization of urea as a nitrogen source requires the action of the bi-functional enzyme Dur1,2p, whose catalytic cycle involves the carboxylation of urea followed by hydrolysis of the resulting urea-1-carboxylate intermediate to yield two equivalents of carbon dioxide and ammonia (Cooper *et al*., 1980; Cooper, 1982; ter Schure *et al*., 2000). In the absence of NADP-GDH activity, assimilation of urea-derived ammonia was expected to proceed via the action of glutamine synthetase (GS) (see Figure 1) (Minehart and Magasanik, 1992). However, assimilation of urea-derived ammonia via this route would require glutamate as a substrate for the GS catalyzed reaction. This requirement would necessitate the acquisition of glutamate from alternative sources in *gdh*-null yeast. Our results indicate that the PUT pathway, which exhibits a small up-regulation in the *gdh*-null mutant (Figure 6A), may serve as an important source of the glutamate required for GS-dependent assimilation of urea-derived ammonia in *gdh*-null yeast. While the PUT pathway represents a plausible source of glutamate in this strain, we cannot rule out the possibility of other sources. In particular, transaminase enzymes involved in the degradation of various amino acids could represent an unappreciated source of glutamate (see Figure 6C). Further experimentation will be needed to confirm these hypotheses.

In this report, we have described the characterization of a newly isolated *gdh1*Δ*2*Δ*3*Δ triple mutant and its application towards assessing the relative contribution of the PUT and GS/GOGAT pathways to glutamate production and nitrogen assimilation following adaptation on various nitrogen sources—an issue where previous research had yielded discrepant results (Avendano *et al*., 1997; Valenzuela *et al*., 1998). Analysis of pathway-specific enzyme activities in the *gdh*-null strain adapted to various nitrogen sources suggests that the GS/GOGAT pathway is primarily responsible for glutamate production when yeast strains are grown on the preferred nitrogen sources ammonia or glutamine. In contrast, utilization of the non-preferred nitrogen source proline appears to require a combination of both the PUT and GS/GOGAT pathways. Finally, utilization of urea as sole nitrogen source resulted in a down-regulation in GOGAT activity with a concomitant increase in P5CDH activity. This result suggests that the PUT pathway serves as an important source of glutamate needed for GS/GOGAT-dependent assimilation of nitrogen on this nitrogen source. Taken together, these results establish that both the PUT and GS/GOGAT pathways play important—and often complementary—roles in glutamate and nitrogen metabolism in *S. cerevisiae*.

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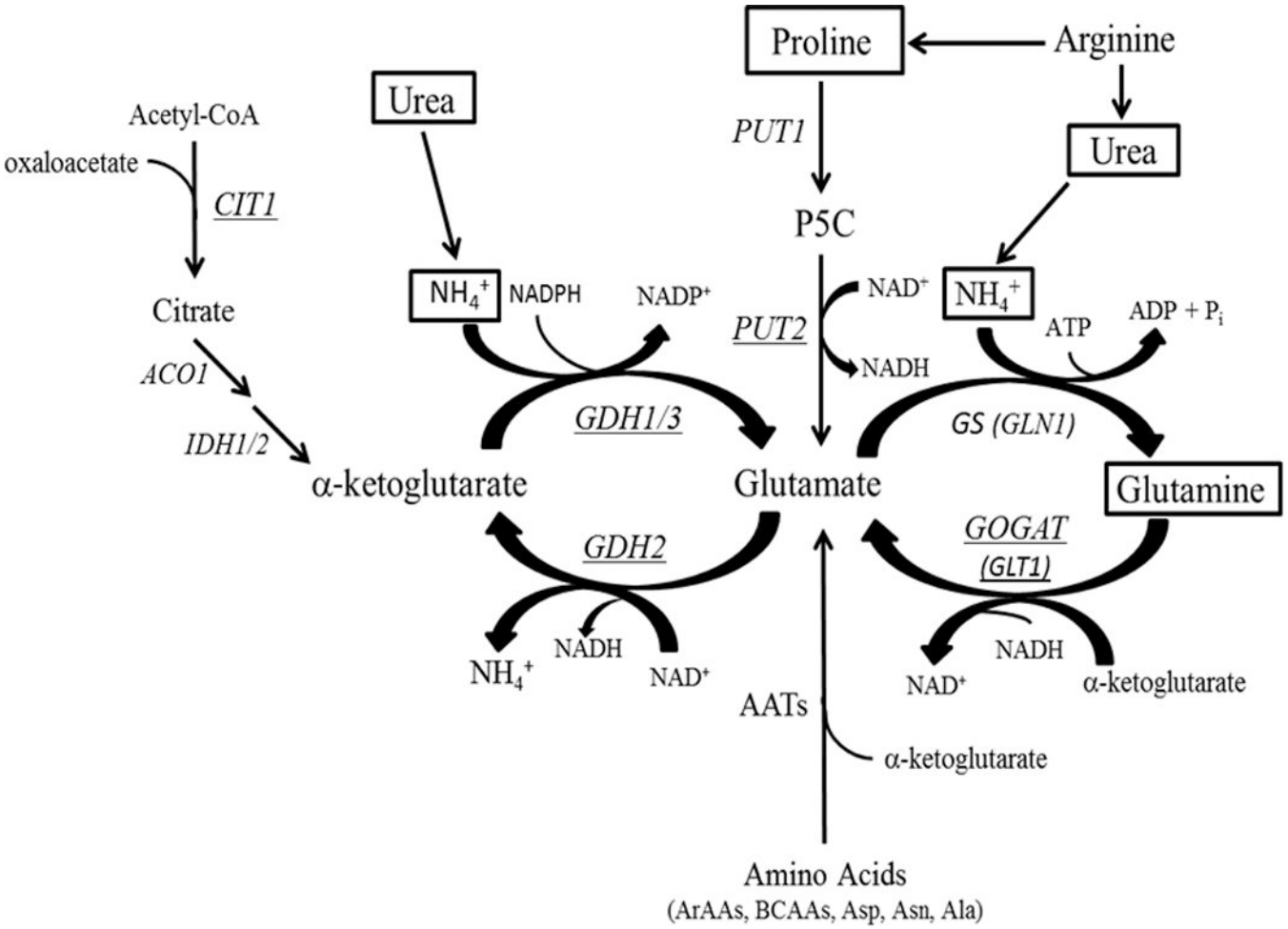

Figure 1.

Relevant pathways for glutamate metabolism and nitrogen assimilation in *S. cerevisiae*. The three primary pathways for glutamate synthesis and nitrogen assimilation are detailed including the two pathways (GS/GOGAT and PUT) tested in this study for up-regulation in response to abrogation of *GDH* activity. Molecules surrounded by a box denote that it was used as a nitrogen source in this study and genes encoding for enzymes that were assayed are underlined. Co-factors utilized in enzyme assays are also listed. Abbreviations: P5C = Δ^1-pyrroline-5-carboxylate; AATs = amino acid transaminases; ArAAs = aromatic amino acids; BCAAs = branched-chain amino acids; BCKA = branched chain keto acids. Figure was adapted from Magasanik, 2002.

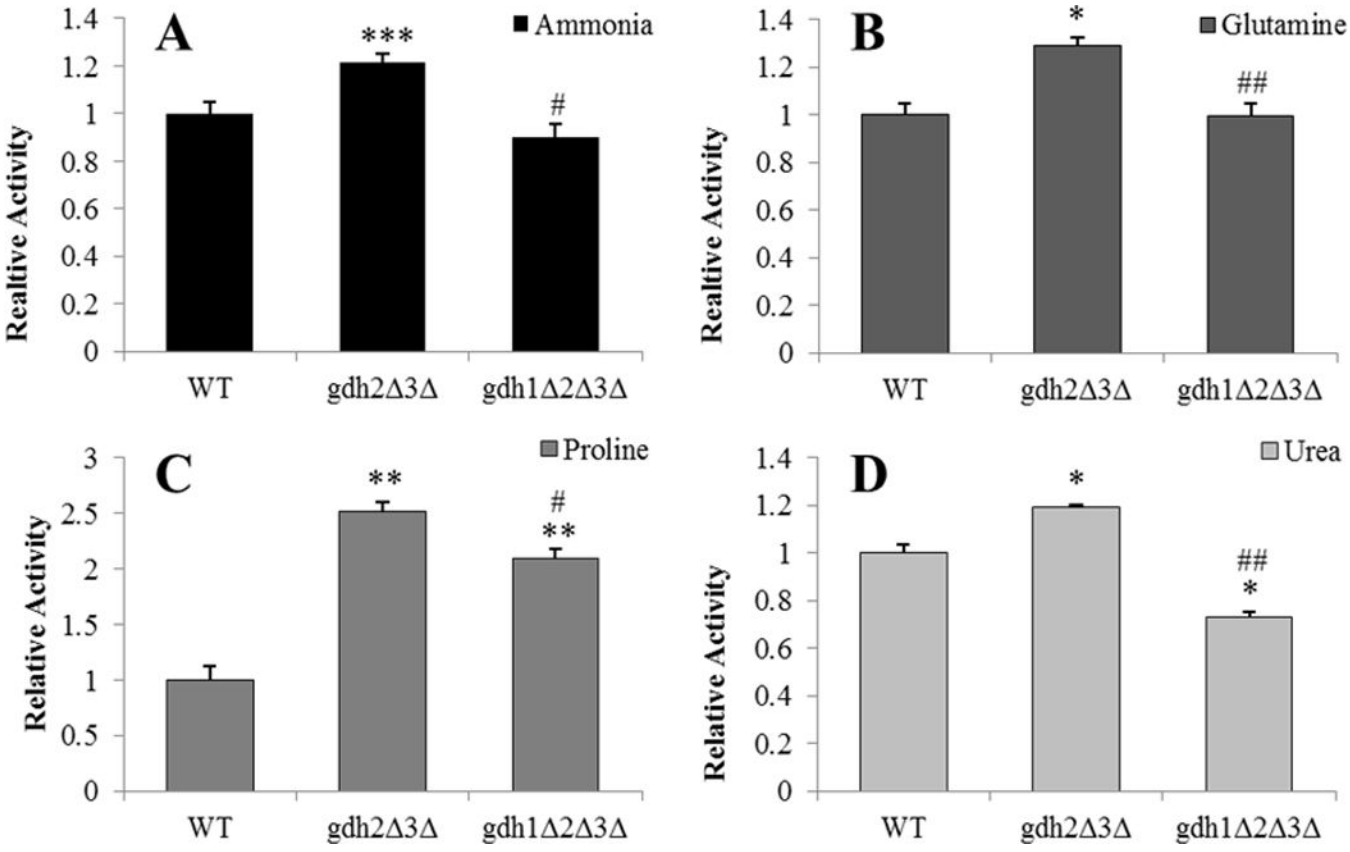

Figure 2.

Citrate synthase activities in *gdh2*Δ3Δ and *gdh1*Δ2Δ3Δ mutants. Enzyme activities were determined following growth on various nitrogen sources (A–D) and results expressed as the ratio of mutant to wild-type (BY4742) activity ± SEM from three separate experiments. Citrate synthase activities in the wild-type strain were 58.0, 30.9, 48.7 and 75.6 μmol/min-mg for ammonia, glutamine, proline and urea grown cells, respectively. * $p < 0.05$, ** $p <$ 0.and *** $p < 0.0$between wild-type and strain indicated by paired t-test. # $P < 0.05$ and ## $P < 0.01$ between double and triple-mutant strains by paired t-test.

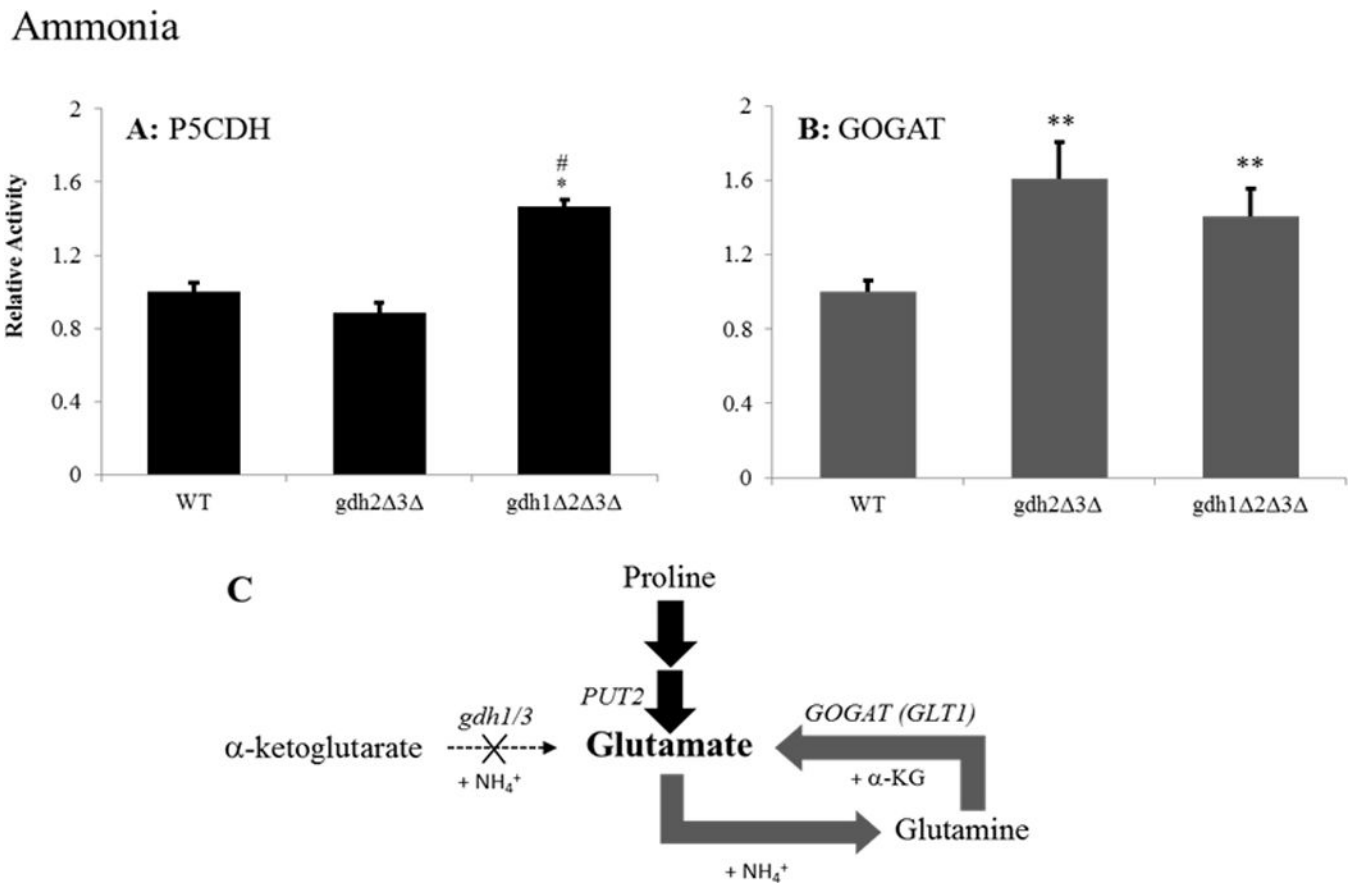

Figure 3.

Both the PUT and GS/GOGAT pathways contribute to glutamate biosynthesis and nitrogen assimilation in *gdh*-null grown on glucose-ammonia media. Enzyme activities were determined for (A) Δ^1-pyrroline-5-carboxylate dehydrogenase (Put2p) or (B) glutamate synthase (GOGAT/Glt1p) following adaptation to growth in glucose-ammonia media. Results are expressed as the ratio of mutant to wild-type (BY4742) activity ± SEM from three separate experiments. Activities in the wild-type strain were 17.and 7.nmol/min-mg for Put2p and GOGAT, respectively. (C) Graphical summary of findings for the triple mutant. Gene designations are indicated in italics for each reaction along with the appropriate co-factors. Bolded arrows indicate pathway up-regulated relative to the wild-type strain. * $P < 0.05$ and **$P < 0.08$ between wild-type and strain indicated by paired t-test. # $P < 0.01$ between double and triple-mutants by paired t-test.

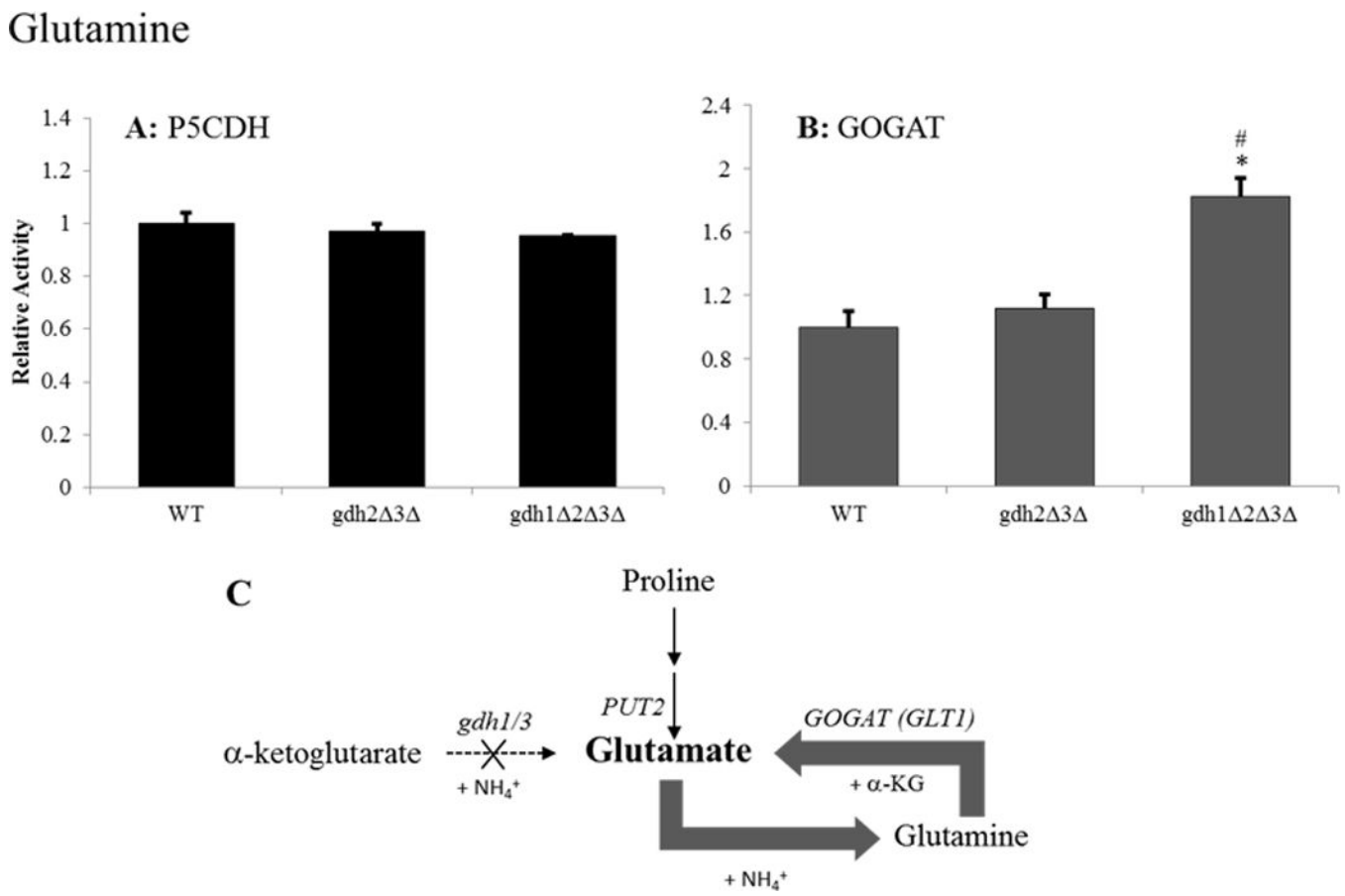

Figure 4.

GS/GOGAT is the primary pathway for glutamate biosynthesis and nitrogen assimilation in *gdh*-null grown on glucose-glutamine media. Enzyme activities were determined for (A) Δ^1-pyrroline-5-carboxylate dehydrogenase (Put2p) or (B) glutamate synthase (GOGAT/Glt1p) following adaptation to growth in glucose-glutamine media. Results are expressed as the ratio of mutant to wild-type (BY4742) activity ± SEM from three separate experiments. Activities in the wild-type strain were 21.and 5.2 nmol/min-mg for Put2p and GOGAT, respectively. (C) Graphical summary of findings for the triple mutant. Gene designations are indicated in italics for each reaction along with the appropriate co-factors. Bolded arrows indicate pathway up-regulation whereas regular weighted arrows indicate no observed up-regulation relative to the wild-type strain. * $P < 0.01$ between wild-type and strain indicated by paired t-test. # $P < 0.05$ between double and triple-mutants by paired t-test.

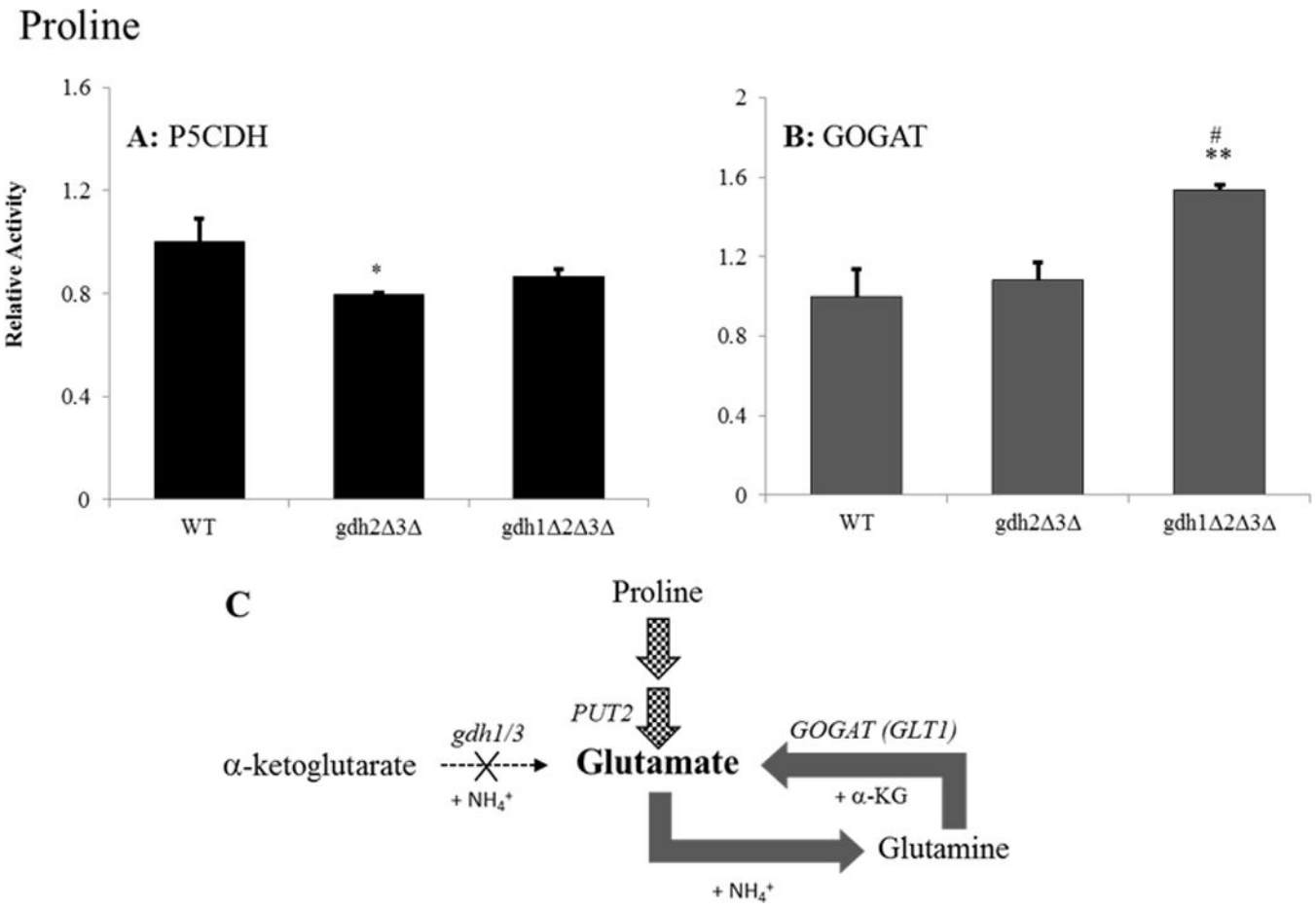

Figure 5.

Both the PUT and GS/GOGAT pathways contribute to glutamate biosynthesis and nitrogen assimilation in *gdh*-null grown on glucose-proline media. Enzyme activities were determined for (A) Δ^1-pyrroline-5-carboxylate dehydrogenase (Put2p) or (B) glutamate synthase (GOGAT/Glt1p) following adaptation to growth in glucose-proline media. Results are expressed as the ratio of mutant to wild-type (BY4742) activity ± SEM from three separate experiments. Activities in the wild-type strain were 97.1 and 11.8 nmol/min-mg for Put2p and GOGAT, respectively. (C) Graphical summary of findings for the triple mutant. Gene designations are indicated in italics for each reaction along with the appropriate co-factors. Bolded arrows indicate pathway up-regulation relative to the wild-type strain. Checkered arrows indicated an absolute increase in Put2p activity compared to the same strain grown on glucose-ammonia (83.9 vs. 23.5 nmol/min-mg) * $P < 0.05$ and ** $P < 0.008$ between wild-type and strain indicated by paired t-test. # $P < 0.05$ between double and triple-mutants by paired t-test.

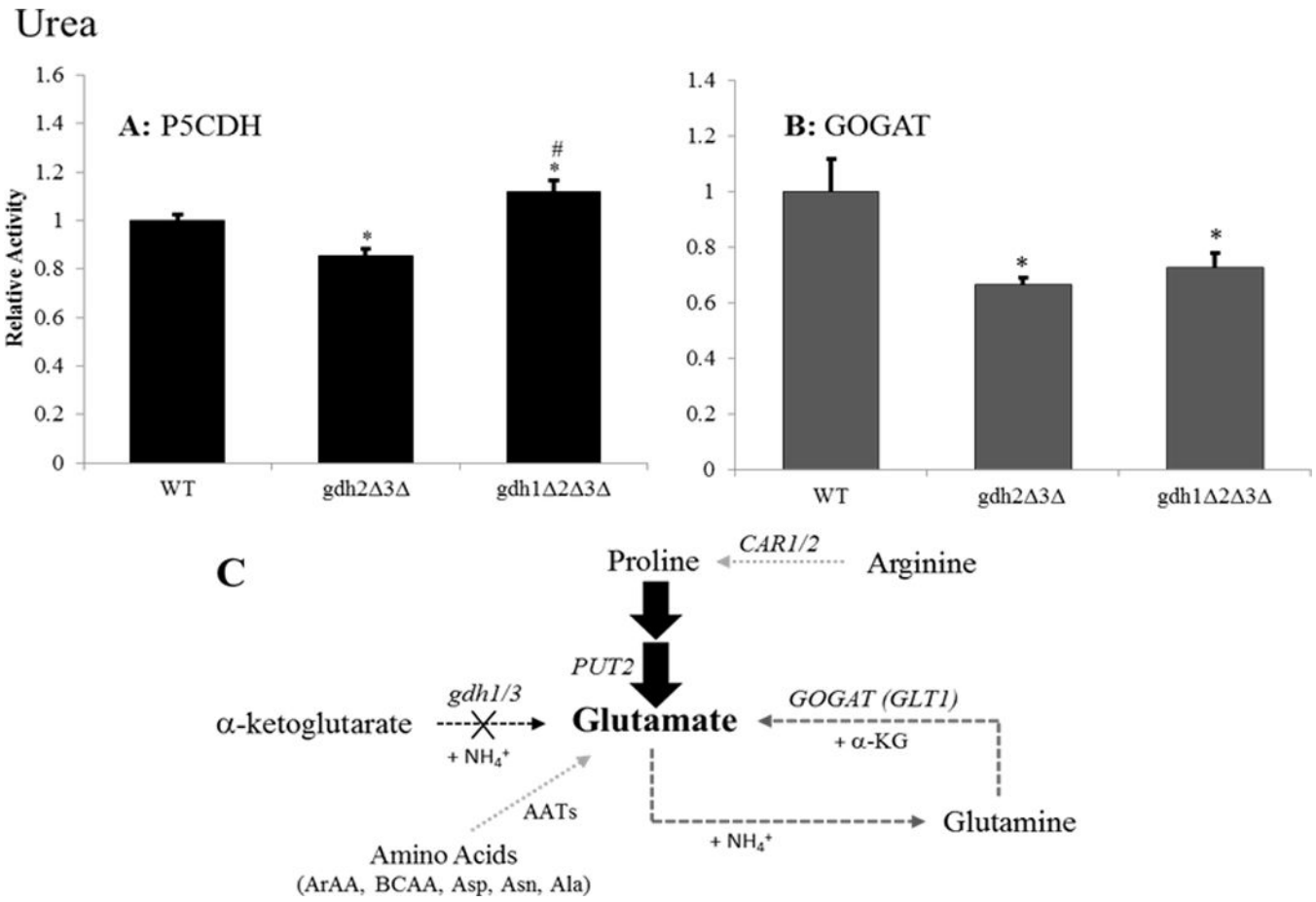

Figure 6.

The PUT pathway may represent an important source of glutamate in the *gdh*-null mutant grown on glucose-urea. Enzyme activities were determined for (A) Δ^1-pyrroline-5-carboxylate dehydrogenase (Put2p) or (B) glutamate synthase (GOGAT/Glt1p) following adaptation to growth in glucose-urea media. Results are expressed as the ratio of mutant to wild-type (BY4742) activity ± SEM from three separate experiments. Activities in the wild-type strain were 24.1 and 21.9 nmol/min-mg for Put2p and GOGAT, respectively. (C) Graphical summary of findings for the triple mutant. Gene designations are indicated in italics for each reaction along with the appropriate co-factors. Bolded arrows indicate pathway up-regulation whereas dashed arrows indicated pathway down-regulation relative to the wild-type strain. Potential alternative sources of glutamate are indicated by light gray arrows (Brandriss and Magasanik, 1980; Eden *et al*., 1996; García-Campusano *et al*., 2009; Iraqui *et al*., 1998; Kispal *et al*., 1996; Morin *et al*., 1992; Urrestarazu *et al*., 1998). Abbreviations: AATs = amino acid transaminases; ArAAs = aromatic amino acids; BCAAs = branched-chain amino acids; BCKA = branched chain keto acids. * $P < 0.05$ between wild-type and strain indicated by paired t-test. # $P < 0.05$ between double and triple-mutants by paired t-test.

Table 1

Strains used in this study.

Strain	Genotype	Source
SEY6211	*ade2 trp1 ura3 leu2 his3 suc2*	M. Yaffe
BY4742 (*YSC1049*)	*his3 leu2 lys2 ura3*	Research Genetics
*gdh1*Δ (*YOR375c*)	*his3 leu2 lys2 ura3 gdh1*Δ*::kanMX4*	Research Genetics[a], This study[b]
*gdh2*Δ (*YDL215c*)	*his3 leu2 lys2 ura3 gdh2*Δ*::kanMX4*	Research Genetics[a], This study[b]
*gdh3*Δ (*YAL062w*)	*his3 leu2 lys2 ura3 gdh3*Δ*::kanMX4*	Research Genetics[a], This study[b]
*gdh1*Δ*2*Δ (PTY84)	*his3 leu2 lys2 trp1 ura3 gdh1*Δ*::TRP1 gdh2*Δ*::URA3*	This study
*gdh1*Δ*3*Δ (ASY18A)	*his3 leu2 trp1 ura3 gdh1*Δ*::TRP1 gdh3*Δ*::kanMX4*	This study
*gdh2*Δ*3*Δ (ASY19A)	*his3 leu2 trp1 ura3 gdh2*Δ*::URA3 gdh3*Δ*::kanMX4*	This study
*gdh1*Δ*2*Δ*3*Δ (ASY20A)	*his3 leu2 trp1 ura3 gdh1*Δ*::TRP1 gdh2*Δ*::URA3 gdh3*Δ*::kanMX4*	This study

[a] Now available from Open Biosystems (Thermo Scientific).

[b] Variations of these strains with novel markers were generated as explained in methods.

Table 2

Strains and doubling times on various nitrogen sources

Strain	Relative Growth[a]				NADP-GDH Sp. Activity[b] (Gdh1p & Gdh3p)	NAD-GDH Sp. Activity[b] (Gdh2p)
	NH_3	Glutamine	Proline	Urea		
BY4742	1.00 ± 0.01	1.00 ± 0.01	1.00 ± 0.03	1.01 ± 0.07	88.4 ± 4.1	7.7 ± 1.7
*gdh1*Δ	0.64 ± 0.05[e]	0.79 ± 0.03[f]	0.74 ± 0.01[e]	0.73 ± 0.04[d]	ND	19.8 ± 3.9
*gdh2*Δ	0.93 ± 0.05	1.02 ± 0.03	0.79 ± 0.03[e]	0.99 ± 0.07	87.9 ± 12.0	ND
*gdh3*Δ	0.95 ± 0.01	0.95 ± 0.03	0.95 ± 0.05	1.00 ± 0.05	95.0 ± 11.9	9.0 ± 0.9
*gdh1*Δ*2*Δ	0.56 ± 0.03[f]	0.51 ± 0.07[f]	0.80 ± 0.03[f]	0.79 ± 0.04	ND	2.1 ± 1.7[c]
*gdh1*Δ*3*Δ	0.58 ± 0.01[f]	0.39 ± 0.03[f]	0.84 ± 0.01[e]	0.77 ± 0.03[d]	ND	9.9 ± 4.3
*gdh2*Δ*3*Δ	1.06 ± 0.05	1.27 ± 0.04[e]	0.86 ± 0.01[e]	1.21 ± 0.02[d]	83.5 ± 8.1	4.4 ± 0.7[c]
*gdh1*Δ*2*Δ*3*Δ	0.67 ± 0.02[e]	0.83 ± 0.01[f]	0.87 ± 0.03[e]	0.83 ± 0.02[d]	ND	1.8 ± 1.7[c]

[a] Doubling times calculated for cultures during logarithmic growth on various nitrogen sources as described in methods. Values are shown relative to the doubling-time of BY4742 (i.e. 2.3, 2.1, 4.5 and 3.6 hours with NH_3, Glutamine, Proline and Urea as nitrogen sources, respectively). Average of 4 separate experiments ± SEM.

[b] NAD(P)-GDH activities were determined on Y-Min + NH_3 media as described in methods. Values are expressed in nmol/min/mg of protein. Average of three separate experiments ± SEM. ND, not detectable.

[c] No detectable NAD-GDH activity as compared to wild-type (~33 μmol/min/mg of protein) when grown on raffinose as a carbon source (n=1, data not shown).

[d] $P < 0.05$ by t-test as compared to BY4742 for the same nitrogen source.

[e] $P < 0.01$ by t-test as compared to BY4742 for the same nitrogen source.

[f] $P < 0.001$ by t-test as compared to BY4742 for the same nitrogen source.