Protection of the glutamate pool concentration in enteric bacteria

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The central nitrogen metabolic circuit in enteric bacteria consists of three enzymes: glutamine synthetase, glutamate synthase (GOGAT), and glutamate dehydrogenase (GDH). With the carbon skeleton provided by 2-oxoglutarate, ammonia/ammonium (NH_4^+) is assimilated into two central nitrogen intermediates, glutamate and glutamine. Although both serve as nitrogen donors for all biosynthetic needs, glutamate and glutamine play different roles. Internal glutamine serves as a sensor of external nitrogen availability, and its pool concentration decreases upon nitrogen limitation. A high glutamate pool concentration is required to maintain the internal K^+ pool. The configuration of high glutamate and low glutamine pools was disrupted in GOGAT⁻ mutants under low NH_4^+ conditions: the glutamate pool was low, the difference between glutamate and glutamine was diminished, and growth was defective. When a GOGAT⁻ mutant was cultured in an NH_4^+-limited chemostat, two sequential spontaneous mutations occurred. Each resulted in a suppressor mutant that outgrew its predecessor in the chemostat. The first suppressor overexpressed GDH, and the second also had a partially impaired glutamine synthetase. The result was a triple mutant in which NH_4^+ was assimilated by two enzymes instead of the normal three and yet glutamate and glutamine pools and growth were essentially normal. The results indicate preference for the usual ratio of glutamate and glutamine and the resilient and compensatory nature of the circuit on pool control. Analysis of other suppressor mutants selected on solid medium suggests that increased GDH expression is the key for rescue of the growth defect of GOGAT⁻ mutants under low NH_4^+ conditions.

ammonium assimilation | glutamine | metabolic circuit | suppressor

To understand a living organism in a comprehensive way, systems biology has to integrate transcriptomics, proteomics, and metabolomics. Although global metabolite profiling has recently been made possible by advances in analytical technologies, metabolomics and physiology remain to be quantitatively bridged. To fill this deficit, quantitative studies of smaller systems are warranted. The outcome of such studies may reveal novel principles and provide strategic guidance for genome-wide research.

The intensively studied central nitrogen metabolic circuit in enteric bacteria is a subsystem well suited for this purpose (1, 2). The circuit is compact, consisting of only three enzymes and three metabolites (Fig. 1). By assimilating the preferred N-source ammonia/ammonium (NH_4^+) using 2-oxoglutarate (a metabolite derived from the tricarboxylic acid cycle), the circuit synthesizes the other two metabolites, glutamine and glutamate. Glutamine synthetase (GS, encoded by *glnA*) catalyzes the only pathway for glutamine biosynthesis. Glutamate can be synthesized by two pathways: through the combined actions of GS and glutamate synthase (GOGAT, encoded by *gltBD*) forming the so called GS/GOGAT cycle, or by the action of glutamate dehydrogenase (GDH, encoded by *gdhA*). The GS/GOGAT cycle has a high affinity for NH_4^+ [$K_m < 0.2$ mM for GS (3)], whereas GDH has a low affinity [$K_m > 1$ mM (3, 4)]. The two central nitrogen intermediates, glutamine and glutamate, provide N for the synthesis of all other N-containing components: ≈88% of
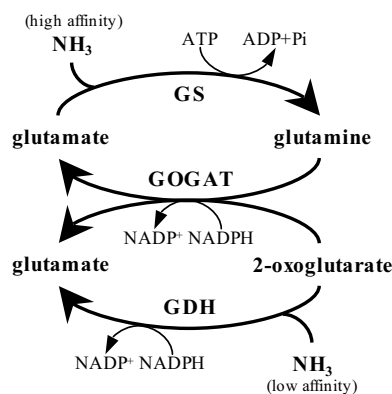

Fig. 1. The central nitrogen metabolic circuit in enteric bacteria. The three-enzyme circuit assimilates NH_4^+ and produces two central intermediates, glutamine and glutamate. GS catalyzes glutamine synthesis. Glutamate can be synthesized by the action of either GS/GOGAT or GDH, respectively, with high or low affinity for NH_4^+. The two glutamate molecules shown have no known functional difference.

cellular N comes from glutamate, and the rest comes from glutamine (5).

Beyond their necessity for biosynthesis, glutamine and glutamate play different physiological roles. It has been demonstrated that internal glutamine serves as a sensor of external N availability and may be responsible for modulating growth rate (6). The glutamine pool concentration is lower than that of glutamate under all conditions of N availability and drops as external N becomes scarce. The setting and response fit well with the multicascade regulatory systems governing GS synthesis and activity, for which extensive knowledge has been acquired over decades of research by many groups (2). On the other hand, the internal glutamate pool concentration is relatively high and does not respond to N status (6). Instead, it responds to another environmental stimulus: external osmolarity (7, 8). Internal glutamate accumulates as external osmolarity increases (9–11), and glutamate is the largest anion pool to partially counterbalance the internal pool of K^+, the most prevalent osmolyte inside a cell (7). K^+ uptake and glutamate accumulation are among the primary cellular responses upon a hyperosmotic shock (9, 10). Moreover, glutamate is also required to maintain the internal K^+ pool (12). Manipulated glutamate deficit (at levels apparently still above biosynthetic needs) results in a suboptimal K^+ pool concentration and a growth defect. The molecular mechanism for coordinating the pool levels of glutamate and K^+ has yet to be understood.

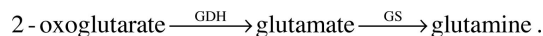MICROBIOLOGY

$$2\text{-}oxoglutarate \xrightarrow{\quad GDH \quad} glutamate \xrightarrow{\quad GS \quad} glutamine \,.$$

Scheme 1.

The setting of glutamine and glutamate in the intertwined circuitry needs to be further explored to understand the different roles of these two metabolites. Mutants missing either glutamate biosynthetic enzyme (GOGAT$^-$ or GDH$^-$) provide simplified versions to address the system configuration. GOGAT$^-$ is particularly useful. It turns the intertwined circuitry (Fig. 1) into a linear pathway (Scheme 1). With only the low-affinity NH$_4^+$ assimilatory pathway for glutamate biosynthesis, the metabolic flux through GDH can be limited experimentally under low NH$_4^+$ conditions. This results in a growth defect (e.g., the doubling time of a GOGAT$^-$ strain was $\approx$2 h in comparison to $\approx$1 h of its congenic wild type in a medium of intermediate osmolarity with 1 mM NH$_4^+$), a decreased glutamate pool accompanied by a suboptimal K$^+$ pool, and an abnormally increased glutamine pool (12, 13). The ratio of glutamate to glutamine pools of a GOGAT$^-$ strain growing in low NH$_4^+$ is $\approx$0.5–1.5, much lower than the $\approx$5–12 in wild-type strains (13). The relative pool values of glutamate and glutamine are drastically disturbed in this two-enzyme linear pathway.

I report here suppressor mutant selections of *Salmonella enterica* serovar Typhimurium and *Escherichia coli* GOGAT$^-$ strains under low NH$_4^+$ conditions by two independent methods. All suppressor mutants showed elevated GDH activities to different degrees that correlated with the extent of growth rescue. Most interesting was the restoration of a high glutamate pool and a low glutamine pool with the two-enzyme linear pathway in these suppressor mutants. I conclude that the central nitrogen metabolic circuit is a resilient and compensatory system, within which the two central nitrogen intermediates, glutamate and glutamine, coordinate with each other to fulfill their diverse roles: K$^+$ pool maintenance for glutamate and N limitation sensor for glutamine.

Results

Isolation of Suppressors of an *S. typhimurium* GOGAT$^-$ Deletion Mutant in an NH$_4^+$-Limited Chemostat. In GOGAT$^-$ strains, glutamate biosynthesis depends solely on the GDH pathway (Fig. 1), which has a low affinity for NH$_4^+$. The metabolic flux through GDH is titratable with NH$_4^+$ concentrations close to or below the K_m of GDH (13). I attempted to futher explore this by growing an *S. typhimurium* GOGAT$^-$ deletion strain in an NH$_4^+$-limited chemostat (Fig. 2). SK3062 ($\Delta gltB824$) was used to prevent GOGAT$^+$ revertants. A low-osmolarity, diluted minimal medium was used; in this medium, the growth defect of SK3062 is mild. High osmolarity would cause a more severe growth problem (12, 13).

After an initial equilibration of the chemostat, SK3062 appeared to reach a steady-state growth on day 1 (from $\approx$18 to 30 h in Fig. 2). The culture showed the signature of GOGAT$^-$ growth in an NH$_4^+$-limited chemostat. At a slow growth rate (0.25 h^{-1}; doubling time of 168 min), SK3062 failed to exhaust NH$_4^+$; $\approx$0.5 mM NH$_4^+$ (of the initial 2 mM) remained in the medium. This was different from wild type or a GDH$^-$ strain under similar situations, where free NH$_4^+$ dropped below 0.02 mM (6, 13, 14). After the dilution rate was adjusted to approximately maximum, the cell density on day 2 declined. This "washed-out" phase likely resulted from setting the dilution rate beyond the ability of cell growth. Two subsequent decreases of the dilution rate (days 3 and 4) failed to stabilize the culture: the cell density continually increased. The instability was puzzling because equilibration should have been established overnight. During the same period, the unused NH$_4^+$ dropped to $\approx$0.1 mM. One possible explanation was that the cells were evolving to use more of the NH$_4^+$.
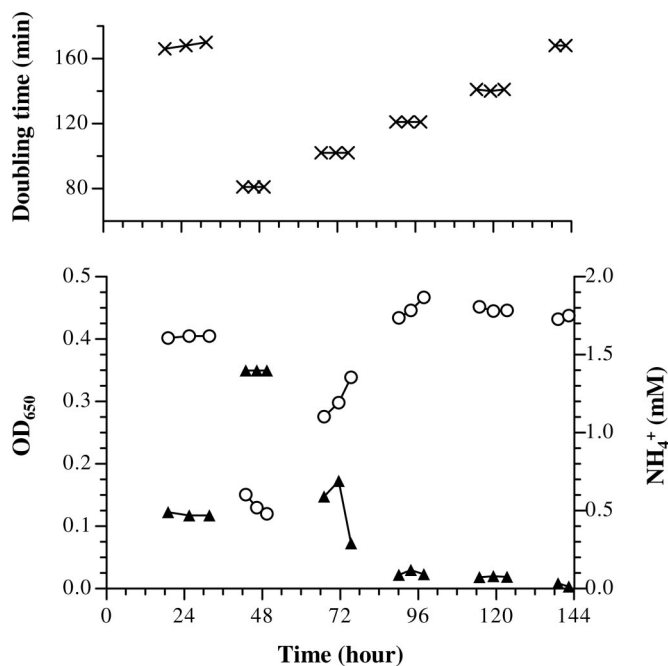
Fig. 2. GOGAT$^-$ SK3062 ($\Delta gltB824$) in an NH$_4^+$-limited chemostat. The diluted minimal medium (0.2 $\times$ N$^-$C$^-$DB) contained 2 mM NH$_4^+$ as the limiting N-source, and 0.2% glycerol as the C-source. Five dilution rates were chosen from 0.52 h^{-1} (close to maximal growth rate, doubling time of 81 min, day 2) to 0.25 h^{-1} (about half of the maximal growth rate, doubling time of 168 min, days 1 and 6), with each interval corresponding to a change in doubling time of $\approx$20 min. After each overnight equilibration, two to three samples were collected several hours apart. (*Upper*) Cell doubling time calculated from dilution rate measurement after each sample collection. (*Lower*) Cell density ($\bigcirc$) and free NH$_4^+$ concentration in the medium ($\blacktriangle$).

The population stabilized during day 5 with residual NH$_4^+$ at $\approx$0.08 mM. The experiment was stopped after day 6, when the dilution rate was set the same as on day 1. Comparing cultures between day 6 and day 1, the cell density increased while free NH$_4^+$ decreased (to <0.02 mM in the last sample). The cells apparently changed into a state that closely resembled wild-type cells in similar chemostat cultures (6, 13).

To investigate whether the original SK3062 was mutated, 10 single colonies were isolated from the first sample of each day. Their abilities to use arginine as the sole N-source were examined (Aut phenotype). Aut$^-$ is one of the pleiotropic phenotypes of GOGAT$^-$ (15). The isolates were checked by using a four-grade system: from Aut^{++} of wild-type SK2633 to Aut$^-$ of SK3062. The results were as follows: Aut$^-$ for all isolates from days 1 to 3; Aut$^{+/-}$ for 19 of 20 isolates from days 4 and 5; and seven Aut$^{+/-}$ and three Aut$^+$ for isolates from day 6. These phenotypes fit well with the chemostat growth (Fig. 2). Aut$^{+/-}$ was replacing Aut$^-$ between days 3 and 4, which coincided with increasing cell density and decreasing free NH$_4^+$ (from $\approx$0.5 to $\approx$0.1 mM). And Aut$^+$ was in the process of outgrowing Aut$^{+/-}$ during the last day, exhausting remaining NH$_4^+$ to <0.02 mM. These results suggested that two sequential spontaneous mutational events had occurred during the experiment. One Aut$^{+/-}$ isolate was designated as SK3074 (early suppressor), and one Aut$^+$ was designated as SK3075 (late suppressor).

Growth Characterization and Pools Measurements for the Suppressor Mutants in Batch Cultures. The growth of suppressor strains SK3074 and SK3075 was analyzed in low NH$_4^+$ (2 mM) batch cultures, together with that of parental strain SK3062 ($\Delta gltB824$) and wild-type SK2633 (Table 1). Growth was tested in media of

Table 1. Batch culture doubling times and pool sizes

Media osmolarity	Strain	Genotype	Doubling time, min	Pools, nmol/ml·OD$_{650}$		Ratio, glutamate:glutamine
				Glutamate	Glutamine	
Low	SK2633	WT	63	20	4.0	5.1
	SK3062	GOGAT$^-$	81, 140	16, 12	5.8, 8.5	2.7, 1.4
	SK3074	Chemostat early suppressor	62	18	3.4	5.3
	SK3075	Chemostat late suppressor	65	22	1.5	14.7
Medium	SK2633	WT	67	62	7.3	8.5
	SK3062	GOGAT$^-$	105, 165	26, 16	18.7, 22.8	1.4, 0.7
	SK3074	Chemostat early suppressor	66	52	10.4	5.0
	SK3075	Chemostat late suppressor	70	55	4.5	12.2
High	SK2633	WT	77	93	11.7	8.0
	SK3062	GOGAT$^-$	143, 206	33, 20	32.6, 34.7	1.0, 0.6
	SK3074	Chemostat early suppressor	85	73	26.3	2.8
	SK3075	Chemostat late suppressor	90	85	8.2	10.4

Media were 0.2× N$^-$C$^-$DB (low osmolarity), N$^-$C$^-$ (medium osmolarity), or 0.2× N$^-$C$^-$DB plus 0.3 M NaCl (high osmolarity), with 2 mM NH$_4^+$ as N-source, and 0.2% glycerol as the C-source. Cultures were inoculated with overnight cultures grown in the indicated media with 5 mM NH$_4^+$ and started with an initial OD$_{650}$ of 0.05–0.06. Two single samples were taken from each culture at OD$_{650}$ ≈0.2 and ≈0.4. Pool values of SK2633, SK3074, and SK3075 were expressed as averages (mean coefficient of variation = 8%). Values of SK3062 were given separately (mean coefficient of variation = 21%). The doubling time for each SK3062 sample was calculated based on two OD$_{650}$ readings sandwiching the sampling point.

three different osmolarities. During the exponential phase of growth, wild-type cells had stable high glutamate and low glutamine pools, a normal combination. Different external osmolarities caused only small changes in their ratio (5.1–8.5). SK3062 ($\Delta gltB824$) did not grow exponentially in low NH$_4^+$ media (growth curves not shown). It had a progressive growth defect, with increasing doubling times as NH$_4^+$ was consumed by cell growth (the reason for showing individual rather than average values in Table 1). This was expected because of the decreasing availability of NH$_4^+$ to GDH. The glutamate pool of SK3062 ($\Delta gltB824$) was decreasing and lower than that of wild type, and the glutamine pool was increasing and higher. As a result, the ratio of glutamate to glutamine dropped to 0.6–2.7. In contrast to their parent, both suppressors exhibited normal exponential phase of growth. Their growth was almost indistinguishable from that of wild type in low and medium osmolarity difference media. Their recovered growth corresponded well with the pool values. The pool ratios in the early suppressor SK3074 were 2.8–5.3 and further increased in the late suppressor SK3075 to ≈10–15. Taken together, there was a good correlation between the growth rate and the glutamate pool.

Characterization of Suppressor Mutations. The presence of the $\Delta gltB824$ allele in the suppressors was verified first. A GDH$^-$ strain, carrying a null $gdh-51$ allele and a linked $zch-1436$::Tn10 marker (90% linkage by P22-mediated transduction), was used as the donor to transduce into SK3074 or SK3075. In both crosses, ≈80% of the Tet^r transductants were glutamate auxotrophs (Glu$^-$), confirming that the $\Delta gltB824$ allele was retained.

Reasoning that the growth rescue could be due to enhanced GDH activity either as a result of an altered enzyme with a higher affinity for NH$_4^+$ or overexpression, I next looked for linkage of suppressor mutations to the $gdhA$ locus. By using the Glu$^-$ strain SK3064 ($\Delta gltB824$ $gdh-51$ $zch-1436$::Tn10) as the recipient, glutamate prototrophic colonies (Glu$^+$) were obtained by transducing P22 phage grown on SK3074 or SK3075. In both crosses, all Glu$^+$ transductants were identical to SK3074 but not SK3075. The percentage of Tet^s among Glu$^+$ transductants resembled the linkage between $gdhA$ and $zch-1436$::Tn10. This suggested the two suppressors shared one mutation (later designated as $gdh-515$), either at or closely linked to $gdhA$, that was solely responsible for the phenotypes of the early suppressor SK3074. With the linked $zch-1436$::Tn10, $gdh-515$ was moved into wild-type SK2633. The resulting SK3111 ($gdh-515$

$zch-1436$::Tn10) was confirmed by crossing back into SK3062 ($\Delta gltB824$) and obtaining Tet^r transductants ($\Delta gltB824$ $gdh-515$ $zch-1436$::Tn10) whose phenotypes were identical to those of SK3074 ($\Delta gltB824$ $gdh-515$).

Strains bearing wild-type $gdhA$, $gdh-515$, and the null $gdh-51$ alleles were subjected to Western blot analysis with antibodies against GDH. $gdh-515$ caused a substantial overexpression of GDH (Fig. 3A). It was ≈5- to 10-fold higher than that of the wild type (estimated from band intensities), regardless of whether cells were grown in rich medium (Fig. 3A) or minimal medium (data not shown). By sequencing the $gdhA$ promoter region, I found that the early suppressor mutation $gdh-515$ was a 1-bp deletion between the −10 and −35 motifs (Fig. 3B).

As mentioned above, I expected the late suppressor SK3075 to have a mutation(s) in addition to $gdh-515$. The $glnA$ locus was of primary interest because the most significant difference between SK3075 and its predecessor SK3074 was the decreased glutamine pool (Table 1). Glutamine auxotroph (Gln$^-$) SK3106 ($\Delta gltB824$ $gdh-515$ $glnA120$::Tn10) was transduced to glutamine prototrophy (Gln$^+$) with P22 phage grown on SK3075. Most transductants were phenotypically identical to SK3075, indicating that the additional mutation (later designated as $glnA424$) was at or closely linked to $glnA$. By using the linked marker $zif-214$::Tn10, $glnA424$ was moved into wild-type SK2633. The
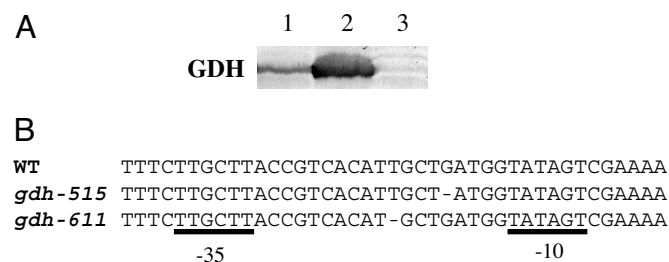
Fig. 3. GDH overexpression with $gdhA$ promoter-up mutations. (A) Isogenic strains SK2633 (wild type, lane 1), SK3111 ($gdh-515$ $zch-1436$::Tn10, chemostat suppressor mutation, lane 2), and SK3128 ($gdh-51$ $zch-1436$::Tn10, null mutation, lane 3) were grown in nutrient broth. Proteins of lysates from equal amounts of cells were separated by 10% SDS/PAGE and examined by Western blot. (B) Sequences of the −10 and −35 motifs of $gdhA$ promoter in wild type, $gdh-515$ (from chemostat early suppressor SK3074), and $gdh-611$ (from plate suppressor FG1143) alleles.

resulting SK3112 (*glnA424 zif-214*::Tn*10*) was confirmed by backcrossing it into the early suppressor SK3074 and phenotypic examination of the transductants. SK3112 is a "leaky" glutamine auxotroph whose internal glutamine pool concentration is limited (6, 14). The strains carrying *glnA424* impair but do not eliminate GS activity and grow slightly more slowly on NH_4^+ as N-source. The growth defect is relieved if glutamine is provided in the growth medium. Sequencing the *glnA* promoter and coding regions revealed that the late suppressor mutation *glnA424* had a 1-bp substitution (C<u>C</u>T to C<u>T</u>T) in the coding region, resulting in a mutant GS^{P95L} enzyme. The basis for the catalytic defect of GS^{P95L} is unknown.

The identification of the mutations and their associated phenotypes helped to understand the sequential events during the chemostat experiment. Overexpressed GDH, as a result of the early *gdhA* promoter-up mutation, accelerated NH_4^+ flux through GDH and primarily refilled the low glutamate pool in $GOGAT^-$ cells under low NH_4^+ conditions (Table 1). Then a partially crippled GS enzyme, caused by the late *glnA* mutation, further pushed the glutamate pool up by cutting the glutamine pool down slightly below that of the wild type. The two-step evolution provided the cells with the ability to use low to trace amounts of NH_4^+ (Fig. 1) even without an intact high-affinity GS/GOGAT cycle. It revealed how the cell endowed with the altered linear pathway, which was disadvantageous in low NH_4^+, could achieve near-optimal growth simply by fine-tuning the pathway. Similarly important is that the bacteria reestablished their preferred relative pool values of high glutamate and low glutamine together with the recovery of growth.

Selection of *S. typhimurium* and *E. coli* $GOGAT^-$ Suppressor Mutants from Low NH_4^+ Plates. Intrigued by the above outcome, I selected suppressors on agar plates, where they would not be in competition. Full-strength minimal medium plates were used to ensure a significant growth defect (Table 1). Both *S. typhimurium* and *E. coli* $GOGAT^-$ strains barely grew on 0.1 mM NH_4^+ plates but did grow with 1 mM NH_4^+. From SK3062, the same $GOGAT^-$ strain used in the chemostat, more than a dozen independent suppressors were isolated from plates with 0.1–0.5 mM NH_4^+. Their *gdhA* loci were examined by the same cross described for the chemostat suppressors. Surprisingly, only one isolate (FG1143) carried a *gdhA*-linked allele that was responsible for the phenotype. Mutation(s) in all others were not linked to *gdhA*. FG1143 grew as well as the chemostat early suppressor SK3074 with low NH_4^+ (Fig. 4*A*). Another representative isolate, FG1158, showed subtle but reproducible growth enhancement over the parent SK3062. Genetic analysis excluded that its *glnA* locus was mutated (data not shown).

Similarly, from *E. coli* $GOGAT^-$ FG1079 (*gltD*::Tn5KAN-I-SceI; *gltD* encodes GOGAT small subunit), eight independent suppressors were isolated. Their growth rates under low NH_4^+ conditions were all between wild-type NCM3722 and the parent FG1079 but hardly distinguishable among themselves. Shown in Fig. 4*B* are two representatives, FG1126 and FG1127. The *gltBD*, *gdhA*, and *glnA* loci in these two suppressors, respectively, were examined genetically. The *gltD* insertion did not change, proved by analyses of (*i*) PCR amplifications of the flanking *gltD* region and (*ii*) P1 transductants by crossing the Kan^r marker back into wild-type NCM3722. By using strains FG1118 (*gltD*::Tn5KAN-I-SceI Δ*gdhA*; Glu$^-$) and FG1134 (*gltD*::Tn5KAN-I-SceI Δ*glnA*; Gln$^-$), respectively, and similar strategies to those described for *S. typhimurium* chemostat suppressors, genetic crosses revealed that neither their *gdhA* nor *glnA* loci were mutated (data not shown). The mutation(s) responsible for their enhanced growth is unknown.

GDH Activities of $GOGAT^-$ Suppressor Mutants. Because only *S. typhimurium* FG1143 carried a *gdhA* mutation, the question remained on the nature of other plate suppressors. Their GDH
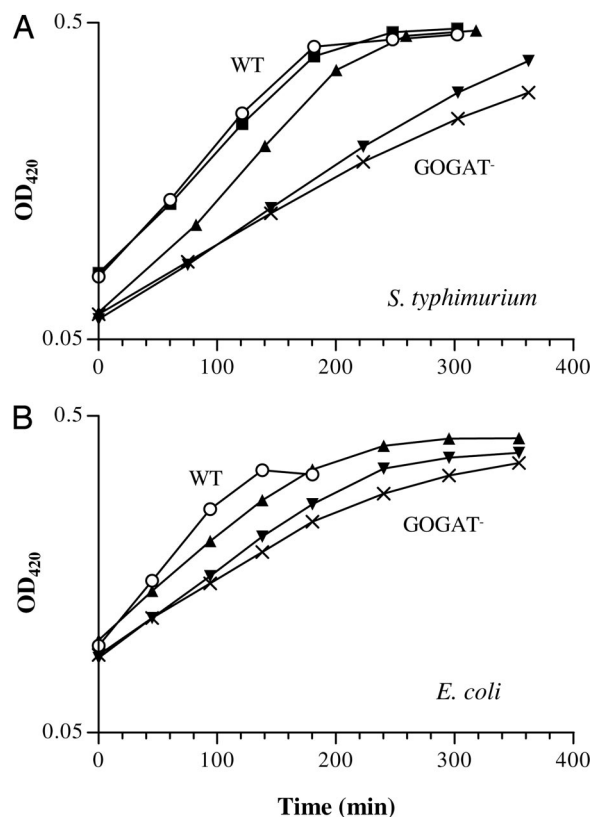
Fig. 4. Growth of $GOGAT^-$ suppressor mutants selected from low NH_4^+ plates. Cells were grown in N^-C^- medium with 1 mM NH_4^+ as N-source and 0.2% glycerol as C-source. Batch cultures were inoculated with overnight cultures grown in the same medium except with 5 mM NH_4^+. (*A*) *S. typhimurium*: SK2633 (wild type, ○), SK3062 (Δ*gltB824*, ×), the chemostat early suppressor SK3074 (Δ*gltB824 gdh-515*, ■), and the plate suppressors FG1143 (▲) and FG1158 (▼). (*B*) *E. coli*: NCM3722 (wild type, ○), FG1079 (*gltD*::Tn5KAN-I-SceI, ×), and the plate suppressors FG1126 (▲) and FG1127 (▼).

activities were measured (Table 2). Compared with the *S. typhimurium* parent SK3062, the chemostat early suppressor SK3074 showed an $\approx$8-fold GDH activity increase, which agreed well with the Western blot result (Fig. 3*A*). The fold increase was about the same for FG1143. This was consistent with their similar growth at low NH_4^+ (Fig. 4*A*). The other *S. typhimurium* plate suppressor, FG1158, showed growth only slightly enhanced over SK3062. Its GDH activity was increased $\approx$30%. For the two *E. coli* plate suppressors, FG1126 and FG1127, GDH activities both doubled over their parent FG1079 (Table 2). Overall, there was a good correlation between GDH activity and the degree of growth enhancement, regardless of whether a lesion in the *gdhA* locus was responsible. Interestingly, in both species, $GOGAT^-$ strains showed $\approx$2.5-fold GDH activity increases over their wild-type counterparts (see *Discussion*).

gdhA Promoter-Up Mutations. The *S. typhimurium* plate suppressor FG1143 carried a new *gdhA* mutation, designated as *gdh-611*. Like *gdh-515* in the chemostat suppressors, *gdh-611* was also a 1-bp deletion between the −10 and −35 motifs of the promoter (Fig. 3*B*). The two deletions are not identical (3 bp apart). Their similar GDH expression levels and growth phenotypes suggested a similar molecular mechanism. Both deletions shortened the native *gdhA* 19-bp spacer between the −35 and −10 motifs to 18 bp, closer to the 17 bp for consensus σ^{70} promoters (16). This could account for GDH overexpression. However, the *gdhA* −10 region belongs to an extended form with an immediate upstream

Table 2. GDH activities

Strain	Genotype	Specific activity, nmol NADPH oxidized/min·mg of protein
S. typhimurium		
SK2633	WT	124
SK3128	GDH⁻	0
SK3062	GOGAT⁻	306
SK3074	Chemostat suppressor	2,350
FG1143	Plate suppressor	2,516
FG1158	Plate suppressor	410
E. coli		
NCM3722	WT	242
FG1113	GDH⁻	2
FG1079	GOGAT⁻	562
FG1126	Plate suppressor	1,132
FG1127	Plate suppressor	1,140

Strains were grown in N⁻C⁻ medium with 2 mM NH_4^+ as N-source and 0.2% glycerol as C-source. Cultures were inoculated with overnight cultures grown in the same medium except with 5 mM NH_4^+. Samples were collected at similar OD_{650} (between 0.38 and 0.39 for *S. typhimurium* and between 0.27 and 0.28 for *E. coli*) after at least two doublings of growth.

TGN (17). The −35 sequence and spacer length in between are more flexible in such σ^{70} promoters. I cannot rule out the possibility of an unidentified *S. typhimurium* regulatory protein acting in trans on this region. A different *gdhA* promoter-up mutation (*gdh-3*, a base substitution mutation also located in the spacer region) was reported in *Klebsiella aerogenes* by Bender and coworkers (18). It was identified from a Glu⁺ revertant of a Glu⁻ strain that lacked GOGAT activity and repressed GDH expression. The authors provided evidence for a likely mechanism of GDH overexpression by this mutated *cis* element. But they also cautioned that the exact nature could be more complex and was yet to be fully understood (18).

Discussion

Growth Defect of GOGAT⁻ Mutants. The direct cause of the growth defect of GOGAT⁻ mutants under low NH_4^+ conditions is insufficient glutamate production through the GDH pathway. However, the intermediate glutamate levels in a GOGAT⁻ strain appear sufficient for cellular biosynthetic needs. For instance, ≈20 mM glutamate, the level present in a wild-type strain at low osmolarity, was able to support maximum growth rate, whereas a GOGAT⁻ strain showed a growth defect at high osmolarity with a similar or even higher pool concentration (Table 1). In terms of biosynthesis, internal glutamate pools of wild type appear to be in excess, especially at high osmolarity. The same is not true for the glutamine pool, which is low especially under N-limiting conditions. The "extra" glutamate is required to maintain and partially balance the K⁺ pool (12). Other hypotheses to explain the growth defect of GOGAT⁻ mutants have been convincingly disproved (19).

Configuration of Internal Glutamate and Glutamine Pools and Their Relationship. Pool measurements in a GOGAT⁻ strain and its suppressors also showed an inverse relationship between glutamate and glutamine pools (Table 1). The glutamine pool in the GOGAT⁻ strain was high because of the blockage of GS/GOGAT cycle: GOGAT is the major glutamine-metabolizing enzyme. As NH_4^+ in the medium was consumed, shrinking NH_4^+ flux through GDH resulted in a declining glutamate pool. This decrease did not limit GS action because glutamate was still saturating GS [$K_m \approx 4$ mM (20, 21)]. On the contrary, the glutamine pool climbed even higher. It has been shown *in vitro*

(widely accepted to be true *in vivo* as well) that 2-oxoglutarate enhances but glutamine limits both GS synthesis and activity as allosteric regulators through the regulatory cascades (2, 22, 23). In both GOGAT⁻ or GDH⁻ strains, the 2-oxoglutarate pool (substrate for both enzymes) was higher than in their parental wild-type strain (unpublished result). The climbing glutamine pool presumably countered the elevated 2-oxoglutarate pool, thus limiting GS action to match the shrinking NH_4^+ flux through GDH. Otherwise, the low glutamate pool would be further depleted. Indeed, monitoring *glnA* expression in a GOGAT⁻ strain with a *glnA-lacZ* fusion showed ≈2-fold repression compared with the wild type (unpublished result).

Pools were reversed in a GOGAT⁻ strain carrying a *gdhA* promoter-up mutation (*gdh-515*): this primary suppressor had a largely refilled glutamate pool by an enhanced GDH flux, a growth recovery, and a lower glutamine pool (Table 1). The last agreed with observations of both a decrease of the 2-oxoglutarate pool and *glnA-lacZ* repression by *gdh-515* (unpublished results). The secondary suppressor mutation (*glnA424*) impaired GS and directly decreased glutamine synthesis, and, as a consequence, levels of the substrate glutamate increased slightly. From GOGAT⁻ through the early suppressor to the late suppressor, the glutamate and glutamine pools showed a series of changes that were inversely coordinated. In the end, the triple mutant (Δ*gltB824 gdh-515 glnA424*) possessed a combination of high glutamate and low glutamine like the wild type, but with a linear two-enzyme pathway instead of the normal three-enzyme circuit. The two remaining reactions were balanced by enhancing NH_4^+ flux through GDH and limiting GS action. The system was impressively resilient.

GDH Regulation. Compared with the vigorous and well studied GS regulation (2), GDH regulation is less understood (1). The only well studied regulatory component is N limitation-induced Nac (the nitrogen assimilation control protein). Nac represses GDH expression in *K. aerogenes* and *E. coli*, but not in the *nac*-free *S. typhimurium* (24–26). Genetic analyses prompted Gowrishankar and coworkers (27, 28) to suggest that *argP* (encoding a LysR-type transcriptional regulator) and *spoT* (encoding ppGpp-3′ pyrophosphohydrolase) were also involved in regulation of GDH synthesis and/or activity. With the requirement for high glutamate, the coordinated pool configuration in the circuit, and the intriguing adaptive responses by the altered GDH–GS pathway, it is conceivable that GDH could be subjected to a multilevel regulation to fine-tune the system. The observed ≈2.5-fold increase of GDH activities in both *E. coli* and *S. typhimurium* GOGAT⁻ strains over the wild types (Table 2) suggests that there is a "built-in" GDH regulatory mechanism to combat glutamate deficiency. Others have previously reported an ≈10-fold increase in GDH activity in GOGAT⁻ strains when high NH_4^+ was replaced with limited glutamine as N-source (19).

The mutations in several *S. typhimurium* and *E. coli* GOGAT⁻ suppressor mutants described in this report have not been identified. They all had elevated GDH activities (Table 2) that appeared responsible for the suppressor phenotypes. Nac cannot be the answer for *S. typhimurium* suppressor mutants and is also unlikely the answer for *E. coli* GOGAT⁻ suppressor mutants, because Nac presumably is not available in the first place (the Ntr system that activates Nac is repressed in GOGAT⁻ with low NH_4^+). Identification of the unknown mutations will likely shed light on GDH control and perhaps even on protection of a high glutamate pool.

Materials and Methods

Strains, Media, and Growth Conditions. All named strains were isogenic of prototrophic *S. typhimurium* SK2633 [derived from strain LT2 (13)] or *E. coli* NCM3722 (29). Phages P22 HT105 and P1*vir* were used for transductions in *S. typhimurium* and *E. coli*,

respectively. *E. coli* allele *gltD*::Tn5KAN-I-SceI was obtained from the Blattner KO collection (30), and alleles Δ*gdhA* and Δ*glnA* were derived from the Keio collection (31).

The full-strength minimal medium was Na^+-based N^-C^- medium plus 1 mM KCl, from which other media with different osmolarities (e.g., diluted and then double-buffered 0.2 × N^-C^-DB) were derived (12). Media were supplemented with NH_4Cl as N-source and glycerol as C-source. When necessary, glutamate or glutamine was provided at 10 mM. Arginine (2.5 mM) was used as the sole N-source for Aut examination. Rich media were nutrient or LB broth. When required, antibiotics were added (25 µg/ml kanamycin and 10 µg/ml tetracycline).

Cell growth experiments were carried aerobically at 37°C. Operations of chemostat and batch culture were performed as described (6, 12). Cell density was measured by absorbance at either 420 or 650 nm (1 OD_{650} ≈ 1.9 OD_{420}). Remaining NH_4^+ in chemostat samples was determined by a GDH-based colorimetric assay (6).

Measurement of Glutamate and Glutamine Pools. Metabolites were extracted from cells by using a "no-harvest" protocol, and fluorescent derivatives of glutamate and glutamine were separated and quantified by a HPLC-based method (6, 13, 32). By using an approximate conversion factor of 1 µl of intracellular water per milliliter per OD_{650} cells (33, 34), pool value in nmol/ml·OD_{650} can be translated to mM internal concentration.

Selection of GOGAT⁻ Suppressor Mutants from Low NH_4^+ Plates. GOGAT⁻ strains were streaked onto full-strength minimal medium plates with 0.2% glycerol as C-source and 0.1–2 mM NH_4Cl as N-source. After 4–7 days of incubation, tiny suppressor colonies appeared on cell streaking lines in plates with 0.1–0.5 mM NH_4^+. They were picked with a sharp-pointed platinum wire under a dissecting microscope, isolated on rich medium plates, and confirmed on minimal medium plates with 0.1 mM NH_4^+.

GDH Assays. A standard Western blot protocol was performed for semiquantitative analysis of GDH protein. *E. coli* GDH antibody was a gift from H. Zalkin (Purdue University, West Lafayette, IN). Assay for GDH activity was mostly carried as described (35). Bacteria were disrupted by passing through a French press cell, and extracts were clarified by centrifugation. The modified assay system contained 50 mM Tris·Cl (pH 7.5), 5 mM 2-oxoglutarate, 5 mM NH_4Cl, and 0.1 mM NADPH. The absorbance of NADPH at 340 nm was monitored for 3 min at room temperature after adding an appropriate amount of extract that resulted in a linear decrease of OD_{340}. Protein amounts in extracts were determined by the Bradford method.

PCR Primers and Sequencing. Primer pair 5′-CGGCAACATT-GAGCGCTATATC and 5′-GTTACGCGTCAGGACATCCG, which flank the Tn5KAN-I-SceI insertion site, was used in confirmation of the *E. coli gltD*::Tn5KAN-I-SceI allele (1,387 bp instead of 148 bp without the insertion). The *S. typhimurium gdhA* promoter region (352 bp) was amplified with 5′-TTTGCCAGC-CCGACCATTGG and 5′-GGTCGCGCTTTTGTACATGGTT, and the *glnA* promoter plus coding region (1,827 bp) was amplified with 5′-CAACTTTGCCTCAGGCATTAGAA and 5′-CCTTG-CAGCAACGCGAAATT. For sequence identification of the *gdh-515*, *gdh-611*, and *glnA424* alleles, the respective PCR products were amplified from the original mutants and sequenced. One point mutation in each case was identified by sequence alignment with the published *S. typhimurium* LT2 genome sequence. Then the mutation was confirmed by sequencing the corresponding region in (*i*) wild-type SK2633 and (*ii*) an independent SK2633-originated strain carrying the allele obtained through a P22-mediated transduction.

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