Quantification of Intracellular 2-Oxoglutarate Levels in *Escherichia coli*:

Overcoming Fluctuation and Leakage Problems

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ABSTRACT

2-Oxoglutarate is located at the junction between central carbon and nitrogen metabolism, serving as an intermediate for both. In nitrogen metabolism, 2-oxoglutarate acts both as a carbon skeletal carrier and an effector molecule. There have been only sporadic reports of its internal concentrations. Here we describe a sensitive and accurate method for determination of the 2-oxoglutarate pool concentration in *Escherichia coli*. The detection was based on fluorescence derivatization followed by reversed phase HPLC separation. Two alternative cell sampling strategies, both based on a fast filtration protocol, were sequentially developed to overcome both its fast metabolism and contamination from 2-oxoglutarate that leaks into the medium. We observed rapid changes in the 2-oxoglutarate pool concentration upon sudden depletion of nutrients: decreasing upon carbon depletion and increasing upon nitrogen depletion. The latter was studied in mutants lacking either of the two enzymes using 2-oxoglutarate as the carbon substrate for glutamate biosynthesis. The results suggest that flux restriction on either reaction greatly influences the internal 2-oxoglutarate level. Additional study indicates that KgtP, a 2-oxoglutarate-proton symporter, functions to recover the leakage loss of 2-oxoglutarate. This recovery mechanism benefits the measurement of cellular 2OG level in practice by limiting contamination from 2-oxoglutarate leakage.
INTRODUCTION

In enteric bacteria, 2-oxoglutarate (2OG) conjoins the two most important central metabolism pathways, being at the junction between the TCA cycle and central nitrogen metabolism. Unlike central carbon metabolism, central nitrogen metabolism is rather compact and consists of only three enzymes (42): glutamine synthetase (GS, encoded by glnA), glutamate synthase (GOGAT, encoded by gltBD), and glutamate dehydrogenase (GDH, encoded by gdhA); and three metabolites: glutamine (Gln), glutamate (Glu), and 2OG (Fig. S1). NH$_4^+$, the preferred nitrogen source for cell growth, is assimilated through GS and GDH. 2OG from the TCA cycle serves as the sole carbon skeletal substrate, generating Glu through GOGAT and/or GDH and subsequently Gln through GS. Most of the total carbon that 2OG brings into nitrogen metabolism is recycled from the two central nitrogen intermediates: 2OG is regenerated after many transamination reactions from Glu, and Glu is regenerated after various amidotransfer reactions from Gln. Almost all other cellular nitrogen is gained from these two types of nitrogen-transfer reactions. Hence, 2OG has a dual identity: a central carbon intermediate and a nitrogen carrier.

2OG also functions as a regulatory effector. The most notable receptors of 2OG are P$_{II}$ family proteins (31, 38). There are two P$_{II}$ proteins in Escherichia coli, GlnB and its paralog GlnK (6, 53). GlnB is one key player in nitrogen regulation, participating in two branched regulatory cascades that control the activity and expression of GS. In vitro studies have shown that the function of GlnB is regulated directly by 2OG and ATP/ADP and indirectly by Gln (22-24, 50). Mutational and structural studies have further clarified the interaction between GlnB and 2OG (7, 25, 57). The major function of GlnK is to...
interact with and regulate the channel protein AmtB for $\text{NH}_4^+$ uptake (8, 9, 17, 18, 53). This interaction is also regulated by 2OG (13). Numerous reports have documented the regulatory effects of 2OG on P$_\Pi$ proteins targeting a variety of enzymes and transcriptional factors in other bacteria and archaea (31). Beyond P$_\Pi$ proteins, 2OG also acts directly on other diverse proteins (14, 32, 51, 54). Remarkably, they are all nitrogen related, implying 2OG as a sensory metabolite that relates to cellular nitrogen status.

This 2OG nitrogen-sensory view has been supported by several reports with measurements of 2OG pools in organisms other than enteric bacteria (12, 30, 37). On the other hand, Kustu and colleagues have demonstrated that enteric bacteria perceive external nitrogen deficiency as a limitation in Gln pool (21, 44). A similar relationship between internal Gln concentration and external nitrogen deficiency is also found in fungi, algae, and higher plants (4, 16, 40). As 2OG possesses an identity of a central carbon intermediate whereas Gln is a central nitrogen intermediate, there has been a notion that 2OG and Gln respectively serve as the carbon and nitrogen indicators in enteric bacteria to regulate GS activity and expression (38). *In vivo*, there have been several reports of internal 2OG concentrations in *E. coli* concerning aspects other than its role in nitrogen regulation (3, 5, 34, 41, 48). However, the 2OG pool concentrations measured with different methods vary by more than 10-fold: from 0.2 up to 2.3 mM in cells grown in $\text{NH}_4^+$-glucose media under similar batch culture conditions. A recent metabolomics study, employing a unique but unconventional filter-culturing methodology, has shown that the internal 2OG concentration decreases more than 10-fold in response to an $\text{NH}_4^+$-upshift (58).
To clarify the physiological role of 2OG in nitrogen regulation of enteric bacteria, a systematic in vivo study like that used to demonstrate the role of Gln (21) is desired. This demands a reliable and versatile method for determining the internal 2OG concentrations under a variety of growth conditions, which is the main focus of this study. We first adopted a detection method with high sensitivity, and utilized this method in developing a sampling strategy. However, for accurate measurement of the pool concentration of a metabolite, sampling is usually more critical than detection. Thus, fast filtration was chosen as the method of choice for cell harvest. During the development of sampling strategy, we noticed that cells leak 2OG into the medium. This first led to a subtraction strategy. We then observed that 2OG pool is subject to fast depletion and accumulation in response to nutrient availability and cold treatment also triggers loss of 2OG. A wash based sampling strategy was then established as an alternative. Using these strategies, we investigated the cellular leakage of 2OG and found that some strains recycle it through the function of KgpP, a known 2OG transporter (46, 47). Applications of the two alternative, filtration-based sampling strategies under different situations, as well as the physiological role of 2OG as a sensory molecule, will be discussed.
MATERIALS AND METHODS

Bacterial strains and culture conditions. NCM3722, an *E. coli* K-12 prototrophic strain (49), was used as the wild-type control. Mutants were isogenic derivatives of NCM3722: GDH-, FG1113 (ΔgdhA859::FRT); GOGAT-, FG1195 (ΔgltD841::FRT), and KgtP-, FG1602 (ΔkgtP754::kan). All deletion alleles originated from the Keio deletion collection (2). SK2633 was a prototrophic *Salmonella enterica* serovar Typhimurium LT2 strain (10). The base medium N-C was nitrogen- and carbon-free (10). The carbon source was 0.4% glycerol or glucose (w/v), and the nitrogen source was NH₄Cl provided at 20 mM unless specified. All batch cultures were grown aerobically at 37°C. Baffled flask was used for culturing and shaken at 250 rpm, with culture volume no more than 15% of the flask volume.

Sample collection for measurement of internal, external, and total 2OG concentrations. Cell filtration was carried out in a 37°C room, using a 10-place filtration manifold (Hoefer) and nylon membranes (25 mm HNWP disc with 0.45 μm pore size, Millipore). The membrane was pre-wetted with wash medium. Dry membrane was used when cells were not washed during sampling. For cell harvest, 1 to 4 ml of culture containing no more than 1 ml×OD₆₀₀ of cell mass was quickly filtrated under vacuum. 2 ml of wash medium that equilibrated in the 37°C room was pipetted onto the membrane once or twice if wash was required. The membrane was immediately immersed in 1 ml of 0.8 M perchloric acid in an Eppendorf tube. After 30 sec vortexing, the tube was incubated at room temperature for ~20 min, vortexed for another 30 sec, and centrifuged at the maximum speed in a microcentrifuge for 4 min. After discarding the filter and cell debris, the extract was frozen on dry ice and stored at -80°C.
For measurement of 2OG levels in medium, 1 ml of culture was passed through a 0.22 μm syringe filter and the filtrate was collected. 100 μl of filtrate was then mixed with an equal volume of 1.6 M perchloric acid. For total (internal plus external) 2OG amount, 100 μl of culture was mixed with an equal volume of 1.6 M perchloric acid and vigorously vortexed for 30 sec. This direct acid extraction of cell culture served as a true “no-harvest” control, which contains internal cellular plus external medium 2OG. Sample was frozen on dry ice and stored at -80°C.

2OG detection and quantification. We modified a previously reported method developed for measuring 2-oxo acids in human serum (55). The process includes adding external standard and reacting with a fluorescence reagent, followed by HPLC separation for quantification. Before the fluorescence derivatization, all frozen samples were thawed and centrifuged for 1 min. 1 volume of supernatant was mixed with 0.25 volume of external standard, 0.75 volume of HPLC grade water, and 2 volume of reagent mixture. The external standard was a mixture of 2-oxobutyric acid (2OB) and 2-oxovaleric acid (2OV) with 4 μM each in concentration prepared in 0.8 M perchloric acid. The reagent mixture was freshly prepared by dissolving the fluorescence reagent 4,5-methylenedioxy-1,2-phenylenediamine (DMB; 5 mM final concentration; Sigma) into a pre-filtered solution (28 mM sodium dithionite, 1 M 2-mercaptoethanol, and 0.4 M hydrochloric acid). For 2OG quantification, at least one 2OG/2OB/2OV (1:1:1) standard was prepared for each batch of reactions. Reactions were carried out in a sealed tube at 95-100°C for one hour. Derivatized sample was then filtered through an HPLC grade microcentrifuge filter (0.2 μm nylon filter, Costar) and stored at -80°C.
2-oxo acid derivatives were separated by reversed phase HPLC (55), using a Shimadzu Prominence HPLC system (main modules: LC-20AB binary pump, SIL-10AF autosampler, and RF-10AxL fluorescence detector). Normally, 10-20 μl of sample was injected into an HPLC column. The column consisted of a C18 reversed phase main column and its guard module (Dynamax Microsorb AAA, Varian; 100×4.6 mm and 10×4.6 mm, respectively) and was maintained at 40°C during separation. Starting buffer A was 9 volume of 20 mM K+-phosphate buffer (pH 7.0) mixed with 1 volume of buffer B, and the elution buffer B was a mixture of acetonitrile and methanol (v/v, 4:6). The flow rate was 1.0 ml/min. The elution was isocratic first with buffer A for 8 min, followed by a linear gradient to 55% buffer B during the next 12 min, and isocratic at 55% buffer B for another 6 min before column re-equilibration with buffer A. Fluorescence signal was monitored at an excitation wavelength of 370 nm and an emission wavelength of 445 nm.

The 2OB/2OV ratio of peak areas of the 2OG/2OB/2OV standard was used to check for any sign of abnormality of the 2OB/2OV ratio of samples. The standards should all be in equal amounts with a variation no more than 10% if cellular 2OB or 2OV content was negligible (see Results). If so, the 2OG/2OV ratio was applied for normalization of 2OG amounts in samples. 2OG pool concentration in mM internal concentration was converted from the unit of nanomoles per ml×OD600 cells as previously described (39).

Detection of kgtP expression. The primer pair used for PCR was ACATAATAGTGAAGTTGC and AAGGGAATACGCATCCCC. They were from the coding region of Salmonella serovar Typhimurium kgtP. Each primer contained one mismatch (underlined) with the corresponding sequence in E. coli kgtP. RNA
extraction, reverse transcription, and PCR were carried out by using commercial reagents (RNeasy, QIAGEN; random hexamers and ThermoScript reverse transcriptase, Invitrogen; GoTaq Green Master Mix, Promega).

**Other methods.** The NH$_4^+$ concentrations in medium were determined by the method of glutamate dehydrogenase from bovine liver (AA0100, Sigma). For comparison, internal Glu and Gln concentrations were measured as described (21, 39), employing both a standard “no-harvest” protocol and the sampling method detailed above for 2OG pool.
RESULTS

**2OG detection by fluorescence derivatization and HPLC separation.** Except for a couple of cases using mass spectrometry (3, 5), all reported measurements of the 2OG pool have been based on an assay employing glutamate dehydrogenase from bovine liver (34). The enzymatic method demands extraction of sufficient amount of 2OG from cell culture with a sizable volume. This puts a limit on the speed of sampling process that could be a source of inaccuracy for determining 2OG pool concentrations. For a more sensitive detection, a method established for the determination of 2-oxo acid levels in human serum (55) was modified. Cellular extract was reacted with 4,5-methylenedioxy-1,2-phenylenediamine (DMB), resulting in a fluorescent derivative of 2OG. It was then separated by reversed phase HPLC and 2OG amount was quantified. Several 2-oxo acids were tested as external standards for the normalization of 2OG amount. 2-Oxobutyric acid (2OB) and 2-oxovaleric acid (2OV) were chosen, as no other HPLC peak interfered with them. In all tested cell extracts, no detectable level of 2OB and 2OV was ever encountered.

HPLC peak separation and quantification were satisfactory, as examples of chromatogram show (Fig. 1). The detection limit for 2OG, with a signal to “noise” ratio of 10, is ~20 fmol in an injection volume of 10 μl. The “noise” is a tiny 2OG peak that is invisible in the blank at the scale shown in Fig. 1, and is possibly contamination from the reagents and/or water. It should be noted that this “noise” in the blank may increase up to 10-fold if HPLC grade water for dilution of samples was left in a disposable plastic tube for more than a few days. The chemical basis of this phenomenon is unknown.
Cellular leakage of 2OG and a subtraction sampling strategy. A central metabolite is normally subject to a high flux rate so its pool concentration could be artificially changed upon cell manipulation even with a fast sampling process. A preferred practice to avoid any pool fluctuation before extraction is the so called “no-harvest” or “whole broth” method (5, 28), i.e., cell culture is directly mixed with methanol or acid without separating cells from the medium. The Gln and Glu pool measurement we routinely performed is based on such a sampling method (21, 39). However, this strategy is only applicable if no significant excretion occurs for the metabolite of interest. It has been reported that many central carbon metabolites, including 2OG, are present in growth media of *E. coli* and other bacteria (34, 48). We confirmed that indeed *E. coli* cells leak 2OG into growth medium, and subsequently quantified its concentration (Fig. 2A). In an exponentially growing culture, we observed a linear accumulation of 2OG in the medium when cell density was low (OD < 0.2). The external 2OG concentration then reached a plateau at ~1 μM. This leakage could be characteristic of a weak acid such as 2-oxoglutaric acid (pKa = 4.68), a passive phenomenon without involvement of a secretion system. The plateau at high OD may be rationalized by a balance of this passive leakage with an active uptake mechanism (see below).

The leakage made it unsuitable to directly apply the “no-harvest” strategy for the quantification of internal 2OG concentration. We initially reasoned that we might still obtain the internal 2OG concentration using a "no-harvest" approach. First, we would measure the total 2OG amount, which would include both the internal and external concentrations. We would then measure the external 2OG concentration. Finally, we
would subtract this value from the total concentration to obtain the internal concentration. However, there is an error issue associated with the subtraction of two quantifications. Although various reported internal 2OG pool concentrations are in sub-mM to mM ranges, much higher than the external concentration, the relative internal and external amounts are close and even higher for the latter due to the tiny cell volume in comparison to the medium volume. Calculated estimate indicated that up to 90% of the total 2OG amount of a theoretical “no-harvest” sample is from external (Fig. S2; “no-harvest” line). Assuming a mere ±5% assay error, a subtraction may yield, in an extreme scenario, either a 2-fold overestimate or an underestimate with a value near zero: e.g., 100±5% total value minus 90±4.5% external value.

With consideration of the above, a modified subtraction strategy was tested. Rapid filtration with an immediate extraction of filter was performed as a substitute for the “no-harvest”. The process was fast that lasted about 10 to 20 sec and was carried out in a 37°C room, and sufficient medium was remained in contact with the cells on the filter. All of these minimized the disturbance of cellular physiological state. We measured the medium volume retained by the filter by weight. The result was constant, ~70 μl with only a few percentage of variation, even with varying amounts of cells. We limited cell amount per filter at no more than 1 ml×OD600 since too many cells would slow the filtration speed. The process served essentially as a concentrating step that increased the ratio of cell to culture volume. The filter extract gave a total 2OG amount from cells plus 70 μl medium. With a paired measurement of 2OG medium concentration, subtraction led to a pool value. With the increased ratio of cell to medium volume, the medium 2OG fraction became smaller so the potential error from the subtraction was minimized (see...
the filtration line in Fig. S2). Fig. 2B shows the subtraction result of several data points from an exponentially growing culture. The internal 2OG concentration is constant around an average of 0.6 mM and independent of cell density and external 2OG concentration, a characteristic of cells during steady-state growth.

**Depletion and accumulation of 2OG pool upon nutrient shifts.** The subtraction strategy requires paired measurements. To simplify the method and cut assay number in half, we explored a harvest strategy by fast filtration and wash. The wash step may simply eliminate the interference of external 2OG. Although the wash can be carried out in a few sec, it introduces an uncertainty as the manipulation may affect metabolic flux, thus altering the pool concentration. Because of the dual identity of 2OG as an intermediate, carbon and nitrogen contents in the wash solution are of the most concern. Some previous studies of 2OG pools involved a wash step with carbon- and nitrogen-free media or saline solution but their effects were not tested (5, 48).

In order to minimize any potential temperature effect, cells were washed with warmed media (equilibrated in the 37°C culture room). Carbon effect was tested first with a wash-down scheme (Fig. 3A). With different glycerol concentrations in the wash media, the pool value obtained was stable (with an average of 0.5 mM) until glycerol concentration decreased below 0.1 mM. The glycerol-free wash resulted in a ~50% drop in the internal 2OG concentration, even though there was only ~15 sec from the wash medium touching the cells on filter to the step of acid extraction. Cells take up glycerol by a facilitated diffusion mechanism and trap it first by the action of glycerol kinase with a $K_m$ of 10 μM (52). Although 2OG is located many steps down from the glycerol uptake and phosphorylation, the glycerol wash-down result indicates that the change of carbon
influx can be felt almost immediately in the 2OG pool. This reaction could be possibly much shorter than the minimal 15 sec handling time.

Nitrogen (NH$_4^+$) effect was tested next. A wash-down scheme was also applied in the experiment by using a starting material of wild-type cells in exponential growth when ~10 mM NH$_4^+$ was left in the medium (WT in Fig. 3B and 3C). The wash media contained NH$_4^+$ ranging from a near perfectly matched 10 mM down to zero. The resulting 2OG pool concentration from the “perfect” wash was 0.5 mM. As the washing NH$_4^+$ concentration decreased, the pool concentration slowly drifted upwards: increasing by ~40% (0.7 mM) with the 0.5 mM NH$_4^+$ wash, and ~5-fold (2.5 mM) when washed with the NH$_4^+$-free medium. The result is just the opposite of the carbon effect, demonstrating a fast 2OG accumulation upon an NH$_4^+$-downshift.

The same wash-down experiments were performed with GDH$^-$ and GOGAT$^-$ mutants (Fig. 3B and 3C). *E. coli* can synthesize Glu through the action of either the GS/GOGAT cycle or GDH. Both 2OG and NH$_4^+$ are co-substrates for the two pathways. The GDH$^-$ mutant (with only the GS/GOGAT pathway) showed almost the same pattern of 2OG pools as the wild-type, except that the pool concentration was completely flat when using wash medium with >1 mM NH$_4^+$ so that it was slightly higher than that of the wild-type. In contrast, the GOGAT$^-$ mutant (with only the GDH pathway) displayed a 3.5-fold increase in the pool concentration (from 1.1 to 3.9 mM) when NH$_4^+$ concentration in the wash media was changed from 10 mM down to 0.5 mM. Its pool concentration did not increase much further when cells were washed with lower NH$_4^+$ media. There was less than 25% difference between 0.5 and 0 mM NH$_4^+$ washes. A reverse wash-up experiment was carried out using growing GOGAT$^-$ cells when ~2 mM NH$_4^+$ remained in...
the culture (Fig. 3C). The measured 2OG pools traced well among 2 to 10 mM NH$_4^+$ washes with those in the wash-down experiment, and further declined with higher NH$_4^+$ washes. The significance of these results will be discussed.

Temperature and time effects on 2OG pool during filtration and wash. Cold treatment of cells prior to metabolite extraction is a common practice to stop cellular metabolism, presumably “freezing” metabolite pools for accurate measurement. Therefore, we examined the temperature effect on internal 2OG concentration. We utilized the observed NH$_4^+$ effect on 2OG pool concentration of the wild-type (Fig. 3B) and compared washes using either 37°C or 0°C media (Fig. 4A). The results from the 37°C wash were expected (compare gray bars in Fig. 4A with those in Fig. 3B). The 0°C wash prevented 2OG pool from increase in response to low NH$_4^+$ concentrations. However, the treatment did not “freeze” the 2OG pool. The pool values of 0.2 to 0.3 mM from the cold treatment were significantly lower than the undisturbed internal concentration of 0.5 mM obtained from the “perfect” wash with 10 mM NH$_4^+$ (Fig. 3B). Thus, the cold treatment appears to be an error-prone strategy for determination of 2OG pool concentration. The mechanism of 2OG decrease remains unknown.

Another concern was any potential change in internal 2OG concentration during the period between withdrawing cells from the culture vessel and the acid extraction, even though the sampling process was handled with the shortest possible duration of ~15 sec. One such potential change could be caused by the brief stop of culture aeration. We tested this possibility by holding cells in a pipet tip for up to 60 sec before filtration thus reversely increasing the handling duration. The result showed no change of the pool concentration with the prolonged sampling period (Fig. 4B), suggesting that the fast
handling of 15 sec should not cause a significant fluctuation of the internal 2OG concentration.

A simplified and conditional alternative for sample collection. A harvest strategy by fast filtration and wash is desirable because of its simplicity. However, a matched “perfect” wash requires predetermination of the carbon and nitrogen concentrations at the time of sampling, which would be a cumbersome process. This is not necessary under many conditions when both carbon and nitrogen are in sufficient concentrations, as suggested by the results in Fig. 3. For example, the glycerol concentration is of lesser concern if it is in mM range at sampling time. For NH$_4^+$, 10-20 mM is sufficient for the wild-type. Therefore, we did tests of cultures grown in medium with 20 mM NH$_4^+$. By using starting medium for washes, a stable and reproducible 2OG pool value was obtained for exponentially growing wild-type cells (Fig. 5). Even at high cell density when some NH$_4^+$ in the medium had been utilized, e.g., ~16 mM remains at 0.8 OD$_{600}$ as 1 mM yields ~0.2 OD$_{600}$ cell mass, washes with the starting 20 mM NH$_4^+$ medium did not cause noticeable disturbance of the 2OG pool. The data average of 0.5 mM was similar to the subtraction result (Fig. 2B). For GDH$^-$ and GOGAT$^-$ mutants, the pool values obtained were also constant. Their averages of 0.9 and 0.8 mM, respectively, were larger than that of the wild-type). The internal 2OG concentration of wild-type cells using glucose as the carbon source was also determined. The data average of 0.4 mM is comparable to the earliest report on the internal 2OG concentration that is 0.6 mM in an $E. coli$ strain grown in another glucose-NH$_4^+$ medium (34), but not to some reported values when carbon- and nitrogen-free washes were performed (5, 41). In addition, we measured the 2OG pool concentration of wild-type cells grown in a proline/glycerol
medium (Fig. 5). Comparing to NH$_4^+$, proline is a poor nitrogen source and the cells grow slowly (21, 39). The measured 2OG pool value also remained constant during the exponential phase of growth. The data average of 1.4 mM is ~3-fold higher than that of the NH$_4^+/\text{glycerol}$ grown cells. Further testing the filtration and wash strategy on measurements of Glu and Gln pools yielded similar results to those from the “no-harvest” method.

**KgtP function countering the 2OG leakage.** As shown in Fig. 2A, the 2OG level in the medium was first accumulating and then reached a plateau at ~1 μM as cells proliferated. So the weak acid was far from equilibration across the membrane as the plateau concentration was ~500-fold less than the internal concentration of ~0.5 mM. 2OG is chemically stable under the growth condition, as we tested its stability up to 6 hours using a cultured medium that is steriley filtered and with or without addition of extra 2OG standard. Therefore, the data suggest an uptake mechanism to counter the leakage. Otherwise, the external 2OG concentration would have maintained a linear relation with cell density. The external 2OG trend shown in Fig. 2A can be quantitatively explained by a model that balances the leakage with an unregulated function of 2OG uptake (detailed in Fig. S3 and its legend). *E. coli* KgtP, a constitutively expressed proton symporter known as a 2OG permease (46, 47), was then considered to be a candidate for this function. To test this, a *kgtP* deletion mutant was cultured and both external and internal 2OG concentrations were measured during the exponential phase of growth (Fig. 6A). Its 2OG leakage profile was distinctly different from that of the wild-type, showing a near linear increase along the growth. However, lack of KgtP changed
neither the growth rate nor the internal 2OG concentration, whose average of 0.6 mM in
the mutant is comparable to that in the wild-type shown in Fig. 2B.

With the above results, we were puzzled by an early brief test on a *Salmonella*
serovar Typhimurium strain whose culture medium gave a much higher 2OG
concentration than that of the *E. coli* wild-type. For clarification, the experiment was
repeated for a *Salmonella* serovar Typhimurium wild-type strain (Fig. 6B). Its internal
2OG concentration was steady and about 2-fold greater than that of *E. coli*. But its
leakage level and profile appeared much more like those of the *E. coli* kgtP mutant than
the wild-type. The apparent contradiction between these two closely related species was
investigated. We first inspected their kgtP and adjacent sequences (see alignment in Fig.
S4). Although kgtP is annotated in the *Salmonella* serovar Typhimurium LT2 genome, it
appears to have lost its own promoter due to an insertion into ~50 bp of the equivalent
coding region of *E. coli* kgtP. The insertion contains an ORF, encoding a putative
cytoplasmic protein, in the same strand as kgtP. The same is true for each out of 6
genome sequenced *S. enterica* serotypes we inspected, but not for any out of 21 *E. coli*
strains (BioCyc version 14.5; http://biocyc.org; examples shown in Fig. S4). We then
examined transcription of kgtP by RT-PCR in both species (Fig. 6C). The result showed
that kgtP was expressed in *E. coli* but not in *Salmonella* serovar Typhimurium,
suggesting *Salmonella* serovar Typhimurium kgtP is either cryptic or, at least, silent
under the culturing condition.
DISCUSSION

In retrospect, measurement of 2OG pool in *E. coli* is less problematic than in *S. enterica*. The functional KgtP retains a large portion of the leaked 2OG which would otherwise accumulate in the medium at high cell density (Fig. 6) and hinder the accuracy of quantification. The effect of KgtP is to change the internal and external concentration ratio. As illustrated in Fig. S2, the lower the proportion of external 2OG is, the more applicable the subtraction strategy becomes. As 2OG may deplete or accumulate rapidly during sampling (Fig. 3), this strategy is preferred when throughput is less of a concern because it is less intrusive during cell handling than the sampling method with washes. Under certain conditions, the subtraction strategy is the only choice if a near perfect or “harmless” wash can not be easily formulated – e.g., during the transitional phase of nutrient (nitrogen or carbon) depletion when the nutrient is rapidly decreasing due to cell consumption, or during the operation of a nutrient-limited continuous culture.

For quantification of cellular metabolites, such a leakage problem is not limited to 2OG – there are many metabolites that cannot be totally contained by a phospholipid bilayer. For example, there are a number of weak acids in central metabolism such as pyruvate, fumarate, acetate, and lactate whose leakage has been mentioned (34, 48, 56). The quantification of their internal concentrations would depend on the extent of “help” provided by their respective uptake mechanisms similar to what KgtP is to 2OG. Such transporters have been documented in the literature, including a pyruvate transporter (26, 29) and the fumarate/malate/succinate Dct uptake systems (11, 27, 33). Even with a recycle mechanism, however, metabolite leakage presents a challenge for accurate quantification of cellular metabolites. Whether one chooses a “no-harvest” or “whole
broth” protocol, or any form of harvest methods, it is critical to know the excreted level of the metabolite of interest. If a leakage problem was substantial, it is absolute necessary to take external amount into account in order to avoid large errors for internal amount. Our study demonstrated such a challenge in quantification of cellular 2OG, whose leakage problem can be only considered as moderate. There are more severe cases, such as cyclic AMP (15).

From the point of cell growth, the leakage may also be a problem. With excess carbon provided in the culture, the cost of carbon leakage and the benefit of uptake may not be obvious: the loss of KgtP function resulted in some carbon losses, but neither the 2OG pool nor the growth rate was affected (Fig. 6A). However, leakage of multiple metabolites may have a cumulative effect for bacteria in their native habitat when available carbon is scarce, and multiple uptake systems for their retention may collectively serve to conserve the carbon flux for bacterial growth.

We chose filtration over centrifugation for cell harvest because our earlier test using centrifugation was unsatisfactory in terms of data variation. There were several possible reasons. Cells within a tightly packed pellet may have less nutrient/oxygen accessibility than those on a wet membrane disc. Potential temperature shift during centrifugation may also affect metabolite pools, as illustrated by the cold treatment (Fig. 4A). A centrifugation process usually took at least one minute between taking the culture and the acid extraction. In comparison, filtration is a much gentler and faster process. However, in terms of speed, the ~15 sec handling time of the filtration process is still too long compared to the half-life of many central carbon metabolite pools (20). For the 2OG pool, the estimated half-life is 0.5 sec. It is then no surprise that a low glycerol wash can
cause an immediate decline of the 2OG pool (Fig. 3A), even though the reduced flux has to pass through many “buffer” metabolite pools between the glycerol entry and 2OG pool. This illustrates the highly sensitive nature of central metabolite pools; thus their quantification demands great care during cell sampling. In contrast, the central nitrogen intermediates Gln and Glu have much longer half-lives: about half to 1 min. This is mostly due to their larger pool concentrations of 5 mM and 70 mM, respectively, in cells grown with sufficient NH$_4^+$ (39). In addition, temperature effect has to be considered during cell sampling for quantification of metabolites. The commonly exercised cold treatment may not be able to “freeze” cellular metabolic contents and could lead to an inaccurate conclusion.

The responses of 2OG pool to both carbon and nitrogen depletions illustrate its dual identity -- a central carbon metabolite and a carrier for central nitrogen metabolism. Our results show that 2OG rapidly accumulates upon an NH$_4^+$-downshift, while the opposite is observed with a carbon-downshift (Fig. 3). The response to change in NH$_4^+$ is more profound – a 5- to 10-fold increase vs. a 2-fold decrease. The basis of this accumulation, obviously due to a sudden change of NH$_4^+$ assimilation within the central nitrogen metabolism, can be dissected by analyzing the result of mutants. When NH$_4^+$ can only be assimilated upon 2OG by the action of GDH in the GOGAT$^-$ mutant, a shift of NH$_4^+$ concentration down to low mM range is already enough to cause significant 2OG accumulation. By comparison, when NH$_4^+$ can only be incorporated upon 2OG through the action of GS/GOGAT in the GDH$^-$ mutant, the large increase in the 2OG pool does not occur until the NH$_4^+$ concentration is shifted down to the range of ~0.1 mM. The $K_m$ for NH$_4^+$ of GDH and GS are in the range of low mM (36, 43) and 0.1-0.2 mM (1, 35),

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respectively. The agreement of 2OG accumulation in the two mutants and the $K_m$ for
$\text{NH}_4^+$ of the two enzymes indicates that the metabolic fluxes through the two Glu
biosynthesis pathways greatly influence cellular 2OG levels. In the wild-type with both
functional GS/GOGAT and GDH pathways, the 2OG pool changes both in the mM and
sub-mM ranges of $\text{NH}_4^+$ (Fig. 3), more dramatically at the sub-mM (the $K_m$ of GS) range.
The data also suggest that, although GDH contributes, GS/GOGAT is the dominant
pathway for Glu synthesis even though its action costs more energy. This pathway
partitioning has been demonstrated by other means before (19, 58). As for the practical
aspects of 2OG pool measurement, the relationship between the $K_m$ and the pool
disturbance indicates that, if one prefers to perform a “harmless” wash during sample
collection, a near perfectly matched or sufficient $\text{NH}_4^+$ concentration (far above the $K_m$)
has to be present in wash solution to avoid artifacts.

To interpret the physiological role of a sensory molecule such as 2OG, one needs to
understand what it senses and what it regulates. All receptors of 2OG documented so far
are exclusively related to nitrogen metabolism. Among those, the quantitative aspect of
2OG regulation on GS function through *E. coli* GlnB has been well established *in vitro*.
But 2OG is only one of several metabolites affecting GS regulation in enteric bacteria.
Studies have shown that Gln plays a more dominant role than 2OG (23, 24, 39). Internal
Gln limitation is known to be the signal of external nitrogen deficiency in enteric bacteria
(21, 44). Taken recent observations by our group and others (39, 58) and the mutant
analysis after the $\text{NH}_4^+$ shifts (Fig. 3B and 3C), it appears that 2OG is also a nitrogen
signal in *E. coli*. Qualitatively, cellular 2OG and Gln levels sense nitrogen availability in
opposite directions, and their regulatory effects are antagonistic. Quantitatively, the Gln
pool plays a bigger regulatory role in enteric bacteria. In other bacteria, however, the relative importance of the two regulatory metabolites may reverse: 2OG may serve as the only or dominant nitrogen signal when Gln plays no or less role (31).

What makes the scheme complex is that 2OG also responds to external carbon availability (Fig. 3A) and hence could be employed as a carbon signal as well. The earliest 2OG pool data related to GS regulation have shown that internal 2OG concentrations correlate with the dilution rates in a glucose-limited chemostat of *E. coli* (45). As nitrogen assimilation in bacteria costs considerable energy by the action of GS (42), coordinated GS regulation based upon nitrogen and carbon availabilities is suggested through the function of GlnB and hence 2OG is considered to be the best candidate of carbon signal regulating central nitrogen metabolism. With a reliable method established here for quantification of internal 2OG concentration, its precise role from the carbon side for nitrogen regulation in enteric bacteria can be clarified quantitatively through detailed *in vivo* studies. Besides 2OG, there may be other sensory metabolites serving the role. For example, carbon availability may in fact reflect on cellular energy status. It has been shown *in vitro* that ATP/ADP is the third major effector on GS regulation (22), although the physiological implication has yet to be elucidated *in vivo*.
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REFERENCES


flux and indicates that the substrate is an ion. Proc Natl Acad Sci USA 104:18706-18711.


44. **Schmitz, R. A.** 2000. Internal glutamine and glutamate pools in *Klebsiella pneumoniae* grown under different conditions of nitrogen availability. Curr Microbiol **41:**357-362.

45. **Senior, P. J.** 1975. Regulation of nitrogen metabolism in *Escherichia coli* and *Klebsiella aerogenes*: studies with the continuous-culture technique. J Bacteriol **123:**407-418.


FIGURE LEGENDS

FIG. 1. HPLC chromatograms of DMB derivatives of 2-oxo acids. Blank, HPLC grade water (after derivatization reaction); standard, equal amount of 2OG, 2OB, and 2OV; sample, cellular extract with the standard 2OB and 2OV. The two visible peaks in the blank overlap with some of other tested 2-oxo acids, possibly contaminations from the reagents and/or water. Injection of water yields no fluorescence peak.

FIG. 2. 2OG leakage and steady-state pool concentration during exponential growth of E. coli. Wild-type strain NCM3722 was cultured in NH4Cl-glycerol medium. (A) 2OG concentrations in the medium were assayed from immediately after inoculation through the late exponential phase of growth. The growth curve is shown in the inset, with a doubling time of 62 min. (B) Paired filter and medium samples were collected along the exponential phase of growth. Each pool value was the result of subtraction from paired samples.

Fig. 3. 2OG pool is subject to sudden decrease and increase upon nutrient shift. (A) Wild-type strain NCM3722 was inoculated in glycerol-NH4+ medium. Sampling was carried out during the exponential phase of growth. Wash media contained different concentrations of glycerol from 43 mM (0.4% w/v as in the starting medium) down to zero. (B) The wild-type, GDH− (FG1113), and GOGAT− (FG1195) strains were inoculated in glycerol medium with 11.5 mM NH4+. Samples were collected during the exponential phase of growth when NH4+ left in the culture was 10 ± 0.2 mM. Wash media contained different concentrations of NH4+ from 10 mM down to zero. (C) Data of open symbols are the same as those in (B) and plotted in a semi-log scale. The closed symbols are from a wash-up experiment of the GOGAT− mutant. Cells were inoculated

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in glycerol medium with 3.5 mM NH\textsubscript{4}\textsuperscript{+}. Samples were collected during the exponential phase of growth when NH\textsubscript{4}\textsuperscript{+} left in the culture was 2 ± 0.2 mM. Wash media contained different concentrations of NH\textsubscript{4}\textsuperscript{+} from 2 mM up to 50 mM. All inoculations were started at OD\textsubscript{600} ~0.05, and samplings were performed at OD\textsubscript{600} between 0.35 and 0.40. For sample collection, 1 ml of cells was quickly filtered and washed twice with 2 ml medium. Time between wash medium contacting cells on filter and filter immersed into acid for extraction was controlled to be 15 ± 2 sec. The error bars in (A) and (B) are minimal and maximal values obtained in at least two independent experiments.

FIG. 4. Temperature and time effects on 2OG pool during filtration and wash. (A) Wild-type strain NCM3722 was inoculated in glycerol medium with 11.5 mM NH\textsubscript{4}\textsuperscript{+}. Samples were collected during the exponential phase of growth when NH\textsubscript{4}\textsuperscript{+} left in the culture was 10 ± 0.2 mM. Wash media, equilibrated either in a 37°C culture room or in ice water, contained different concentrations of NH\textsubscript{4}\textsuperscript{+}. (B) Strain NCM3722 was inoculated in glycerol medium with 20 mM NH\textsubscript{4}\textsuperscript{+}. Samples were collected during the exponential phase of growth with OD\textsubscript{600} between 0.3 and 0.4. Wash medium was identical to the starting medium, with 20 mM NH\textsubscript{4}\textsuperscript{+} and equilibrated at 37°C. N, normal handling speed of filtration and wash that lasted ~15 sec; N+30'', holding cells in pipet tip for 30 sec after withdrawing culture from vessel and before filtration; N+60'', holding for 60 sec. The error bars are minimal and maximal values for two different OD points.

FIG. 5. Steady-state pool concentration obtained by the filtration and wash strategy. Cells were grown in NH\textsubscript{4}\textsuperscript{+}/glycerol, NH\textsubscript{4}\textsuperscript{+}/glucose, or proline/glycerol medium. Samples were collected at 3-5 different cell densities for each culture during its exponential phase of growth. Wash medium was identical to the starting medium, with 20 mM of either...
NH₄⁺ or proline. The results were from two sets of independent experiments. Average doubling times in min: WT, 63; GDH, 68; GOGAT, 65; WT (NH₄⁺/glucose), 48; WT (proline/glycerol), 99.

FIG. 6. Continuous and near linear accumulation of external 2OG without a functional KgtP. (A) *E. coli* kgtP mutant strain FG1602 and (B) *Salmonella* serovar Typhimurium wild-type strain SK2633 were cultured in NH₄Cl-glycerol medium. 2OG concentrations in medium were assayed from immediately after inoculation through the late exponential phase of growth. Internal 2OG concentrations were also measured at different cell densities. The *E. coli* wild-type external 2OG data in (A) are from Fig. 2A for comparison. (C) kgtP is transcribed in *E. coli* but silent in *Salmonella* serovar Typhimurium. The three strains were cultured identically to those in (A) and (B), cells were collected at OD₆₀₀ ~0.4, and RNA was extracted. cDNA was reverse-transcribed and subjected for PCR detection of kgtP expression. Genomic DNA extracted from colonies served as control templates for PCR.