# Carbon Flux Distributions at the Glucose 6-Phosphate Branch Point in *Corynebacterium glutamicum* during Lysine Overproduction

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Analyses indicate that the lysine yield in Corynebacterium glutamicum is limited by suboptimal flux partitioning at either the glucose 6-phosphate (Glc6P), phosphoenolpyruvate (PEP), or pyruvate (Pyr) branch points (or a combination thereof), which results in disproportionate production of the required lysine precursors. Suboptimal flux partitioning at a metabolic branch point can result from the inadequate enzyme activity of a subordinate branch (a weakly rigid branch point) or from the active feedback regulation of the flux partitioning (strongly rigid branch point). In order to examine branch-point characteristics, we have utilized metabolite balances, constrained by biochemistry, to estimate flux distributions in the primary metabolic network of C. glutamicum from measured, extracellular, metabolite accumulation rates. These flux distributions, in combination with local metabolic perturbations, were used to infer branch-point characteristics. This study examines the flexibility of the Glc6P branch point, which could limit lysine yield via inadequate NADPH production, by perturbations induced from attenuation of Glc6P isomerase activity (first branch-point enzyme of glycolysis) and by fermentation on gluconate, which effectively bypasses the Glc6P branch point. Results from the analyses of these perturbations, as well as the flux distributions at the Glc6P branch point during the different phases of a control lysine fermentation, indicate that the Glc6P branch point is neither weakly nor strongly rigid, so that limitations in lysine yield must result from rigidity at either the PEP or Pyr branch point.

## Introduction

Improving product yield in many bacterial fermentations necessitates the modification of primary metabolic pathways due to suboptimal flux partitioning at principal metabolic branch points, which results in the suboptimal synthesis of required product precursors. Since metabolic networks are optimized for cell biosynthesis, inherent cellular control architectures often compensate for external modifications and render the network rigid (insensitive) to flux alterations (15). Although the applicability of recombinant DNA technology is now commonplace, targeting genes that may result in a more flexible network still remains problematic. To address this targeting issue, several techniques are currently being developed (see reviews in refs 15 and *19*), of which mass balance techniques appear promising due to their wide-spectrum applicability. However, mass balance techniques cannot be used predictively, since they do not incorporate information regarding enzyme kinetics, which accounts for their robustness. Although the techniques only provide a "snapshot" of metabolic flux distributions at a particular instance, this article will demonstrate that the lack of kinetic information can be overcome with the use of experimental metabolic perturbations that focus on the principal branch points (15) for the product of interest.

Our analysis has focused on lysine production by Corynebacterium glutamicum, in which we have shown (15) that significant alterations in flux partitioning at the glucose 6-phosphate (Glc6P), phosphoenolpyruvate (PEP), and pyruvate (Pyr) principal branch points must occur in order to achieve improvements in lysine yield, although improvements in membrane transport (*1*) would be required to improve lysine production rates. Of the three principal branch points identified, this article focuses on experimental perturbations of the Glc6P branch point to elucidate its constraint on lysine yield. Glucose 6-phosphate is the branch point between glycolysis and the pentose phosphate pathway (PPP). It is possible that the flow of glucose reaching this branch point preferentially enters glycolysis over the PPP, thereby limiting lysine yield by constraining NADPH availability. To investigate the flexibility of the Glc6P branch point, two experimental perturbations were conducted. The first perturbation involved the fermentation and flux analysis of a C. glutamicum mutant, NFG068, with the attenuated activity of Glc6P isomerase, the first branch-point enzyme of glycolysis. This mutant was isolated in an attempt to redirect flux into the pentose phosphate pathway. The second perturbation investigated examined the effect of culturing *C. glutamicum* ATCC 21253 on gluconate as the sole carbon source, a metabolite that directly enters the pentose phosphate pathway and effectively bypasses the Glc6P branch point. The conclusions reached from analyses of these experimental perturbations are also corroborated by the shift in carbon flux at the Glc6P branch point in response to varying NADPH requirements during periods of varying biosynthetic and lysine formation rates, as observed during the control fermentation (19).

A similar analysis of the degree of rigidity observed for the pyruvate principal branch point is presented in a companion paper to this article (20).

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## Materials and Methods

**Microorganisms.** The cultivation of Corynebacterium glutamicum ATCC 21253 Homo-, Leu-, used in this study for the gluconate fermentation and as the parent strain for subsequent mutation–selection studies, has previously been described (19). The mutant strain NFG068 was cultivated under similar conditions, except where noted below.

**MutationSelection.** Mutants of *C. glutamicum* ATCC 21253 were derived by exposing cells to *N*-methyl-*N'*-nitro-N-nitrosoguanidine (11) (NTG) (Sigma Chemical Co., St. Louis, MO) as follows. A 50-mL LB5G (19) seed culture of C. glutamicum ATCC 21253 was grown to an optical density (at 660 nm, 1-cm path length) of 4.8 and transferred to five 15-mL centrifuge tubes. These samples were centrifuged for 5 min at 5000*g* and washed once with 10 mL of 0.1 M citrate buffer (11) (pH 6.0). Four of the centrifuge tubes were resuspended in 9 mL of citrate buffer, and the fifth tube (control) was suspended in 10 mL. To the four 9-mL tubes was added 1 mL of 2 mg/mL NTG, dissolved in water and filter-sterilized. All five tubes were incubated at 30 °C for 30 min and then washed three times with citrate buffer and resuspended in 10 mL of LB5G medium. The four samples exposed to NTG were mixed together with 4 mL of sterile glycerol to give a total volume of 44 mL. One-milliliter aliquots of the treated cells were placed in cryovials and stored at –40 °C for future use. Colony counts from the control and NTG-exposed cells indicated a survival rate of 55% and mutation rates in excess of 25%. All materials and liquids exposed to NTG were soaked in 0.1 N HCl to decompose NTG.

Isolation of C. glutamicum ATCC 21253 mutants with attenuated glucose-6-phosphate isomerase activity ($GPI^A$) was based on the work of Fraenkel and Levisohn (*3*), in which they selected GPI-lacking mutants of Escherichia coli from mutants that exhibited impaired glucose assimilation. This isolation procedure is based on the premise that if a strain lacks GPI, then it must catabolize glucose via the pentose phosphate pathway, which will impede glucose uptake. Mutants with impaired glucose assimilation will utilize amino acids (available in tryptone) as a carbon source instead of glucose, which will cause the pH around a colony to increase rather than decrease, as is typically observed in colonies that readily assimilate glucose. In their study, strains with impaired glucose assimilation were identified as red colonies on tetrazolium indicator plates, since the intracellular reduction of tetrazolium to is red precipitate does not occur at low pH. These mutant strains were then streaked on minimal plates with carbon sources that require GPI for good growth (glucose and galactose) and on plates with carbon sources that are not metabolized via GPI (fructose and gluconate). Strains that exhibited normal growth on the latter but attenuated growth on the former were assayed for GPI activity.

It was found, however, that C. glutamicum growth is inhibited by the tetrazolium dye, so that growth on the other fermentation indicator plates was investigated. Two fermentation indicator plates, EMB (8) and PRED (10) (Table 1), were used instead of the tetrazolium plates to identify colonies that increased the pH of their local environment, a trait associated with attenuated glucose assimilation. C. glutamicum ATCC 21253 colonies developed extremely slowly on EMB plates, exhibiting an almost black appearance on the dark purple background, and colony diameter never exceeded 2–3 mm. This attenuated growth was probably due to methylene blue and eosin Y, which combine to form a dark intracellular precipitate at low pH and are known to be inhibitory to Gram-positive organisms (10). However, mutants of C. glutamicum ATCC 21253 that exhibited impaired glucose uptake displayed good growth on EMB plates, taking on a light pinkish color on the dark purple background, and colony size was close to that observed on LB5G plates. Similarly, ATCC 21253 cultured on PRED plates developed as normal, but their growth turned the orange-colored agar (neutral pH) to yellow (low pH), while mutant strains with attenuated assimilation of glucose turned the agar to a red color (high pH).

**Table 1. Fermentation Indicator Plates**

| EMB plates | | PRED plates[a] | |
|---|---|---|---|
| component | g/L | component | g/L |
| glucose | 10 | glucose | 10 |
| tryptone | 8 | tryptone | 10 |
| yeast extract | 1 | yeast extract | 1 |
| NaCl | 5 | NaCl | 5 |
| $K_2HPO_4$ | 2 | phenol red | 0.020 |
| eosin Y | 0.4 | agar | 18 |
| methylene blue | 0.065 | | |
| agar | 18 | | |

[a] pH 7.2.

Glucose-6-phosphate isomerase attenuated mutants of C. glutamicum ATCC 21253 were isolated as follows. NTG-treated stocks of ATCC 21253 were thawed, and 100-μL samples were removed to inoculate 12 10-mL LB5G culture tubes, which contained 2.5 g/L glucose plus 2.5 g/L fructose instead of 5 g/L glucose, grown overnight, and plated on EMB and PRED plates at the appropriate dilutions to obtain 50–200 colonies per plate. Strains that were nonfermentors of glucose (NFG) were isolated from EMB and PRED plates (*i.e.*, colonies that caused local pH increases) and streaked separately on four PMB (*19*) plates that contained either glucose, fructose, gluconate, or maltose as the sole carbon source. Strains were selected for further characterization if they exhibited good colony development on gluconate, but attenuated development on glucose and variable development on fructose or maltose.

**Enzyme Assays.** Cell-free extract preparation and assays for glucose-6-phosphate isomerase (GPI), isocitrate dehydrogenase (ICDH), and malic enzyme (ME) have previously been described (19). The activities of glucose-6-phosphate dehydrogenase (G6PDH) and gluconate-6-phosphate dehydrogenase (GN6PDH) were measured by following the formation of NADPH (*2*, *16*, *17*). The activity of the Entner–Doudoroff (ED) pathway was measured by following the rate of pyruvate formation from gluconate 6-phosphate (Glcn6P) (*5*, *6*). The activity of 6-phosphogluconolactonase in the reverse direction was assayed by incubating cell-free extract (ca. 0.6 mg/mL protein) with 100 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM NADPH, and 10 mM Glcn6P in 1.0 mL of water for 10 min. The reaction was terminated with the addition of 200 μL of 12% (w/v) trichloroacetic acid, and the amount of Glc6P formed was assayed enzymatically (*7*). The presence of a soluble dinucleotide transhydrogenase was assayed by incubating cell-free extract (ca. 0.3 mg/mL protein) in 50 mM Tris·HCl (pH 7.5), 0.19 mM NADPH, and 0.22 mM NAD and monitoring the decrease in absorbance at 340 nm, where any NADH produced by the transhydrogenase would be oxidized rapidly by the naturally high activity of NADH oxidase present in the cell-free extract. For some assays, cells were cultivated in SLB medium consisting of 20 g/L glucose, 20 g/L tryptone, 15 g/L yeast extract, and 5 g/L NaCl. All assays were conducted at 30 °C.

**Table 2. Activities of Selected Enzymes in *C. glutamicum* ATCC *21253* and NFC 68 Cultured on Various Media**

| | | activity (nmol min$^{-1}$ (mg of protein)$^{-1}$) | | |
|---|---|---|---|---|
| strain | medium | *GPI* | G6PDH | *ICDH* |
| *ATCC 21253* | *PMB* | *966* | *107* | |
| NFG068 | *PMB* | *67.6* | *170* | |
| NFG068 | *SLBG*[a] | *180* | *210* | *1194* |
| NFG068 | *SLB* | *37.8* | *155* | *852* |

[a] *SLB* with potassium gluconate instead of glucose.

**Fermentations.** Fermentor instrumentation, general operation, and sample analysis conform with those of the control fermentation (*19*), except as follows. The preculture medium (PMB) (19) was modified to contain 10 g/L glucose, 20 g/L potassium gluconate, and no citrate for the gluconate fermentation. The gluconate fermentation medium (FM9) was identical to the control fermentation medium FM4 (*19*), except that the glucose concentration was reduced to 10 g/L and 140 g/L potassium gluconate was included in part A of the medium; glucose was sterilized separately and no citrate was used. Gluconate was measured via HPLC with a Bio-Rad (Rockville Centre, NY) Aminex HPX-87H reverse-phase column. The C. glutamicum biochemistry used for flux analysis is identical to that previously discussed (*19*), except where noted. No alteration of the metabolic network was necessary with the recent detection of PEP carboxykinase (PPCK) in C. glutamicum (*13*), as PPC and PPCK are indistinguishable mathematically (*i.e.*, PPCK is lumped with PPC).

## Results and Discussion

**Characterization of the GPI Mutant.** Of 11 000 colonies screened, approximately 96 colonies exhibited impaired glucose fermentation. Of these, nine strains were assayed for GPI activity, of which one (NFG068), isolated from a PRED plate, exhibited severely attenuated GPI activity. No strains could be isolated that lacked GPI. NFG068 was reassayed for GPI as well as other NADPH-producing enzymes and compared to ATCC 21253, the results of which are listed in Table 2. Although the activities of GPI, G6PDH, and possibly ICDH appear to be inducible by gluconate, the assays indicate that GPI in NFG068 is only approximately 7% as active as GPI in ATCC 21253. Growth studies of NFG068 have shown that this strain develops normal colonies on gluconate, small colonies on glucose, and almost no colonies on fructose or malate PMB plates. Furthermore, revertants have not been observed, which indicates that the mutation is fairly stable.

**Fermentation of NFG068.** The results of the NFG068 fermentation are depicted in Figure 1A–D. Since gluconate induces the dehydrogenases of the PPP (17) (also see Table 5), two 50-g (~5 g/L) supplements of potassium gluconate were added early in the fermentation (Figure 1B,C) in an attempt to stimulate the attenuated growth rate of the culture ($\mu$ = 0.15 versus 0.31 h$^{-1}$ for ATCC 21253), but the additions seemed to have little to no effect other than on respiration (Figure 1B). Profiles of biomass and lysine (Figure 1A) exhibited phases characteristic of phase I (pure growth) and phase III (little to no growth, but high lysine production) of the control fermentation (19). However, since growth abruptly stopped and entered a stationary phase at 22.0 h, the culture directly entered phase III, bypassing phase II (rapid growth with high lysine production). Similarly, phase IV (decay in biomass and loss of lysine production) was averted since cell lysis and byproduct formation were not observed, although the lysine synthesis rate did decrease. Other notable features that differed from those of the control fermentation included the attenuated specific production and specific consumption rates of all extracellular metabolites during growth, an almost doubling of the fermentation time (100 versus 5 h), an approximately 50% reduction in specific respiration during growth without gluconate, and a lower maximum biomass titer (9 versus 16 g/L DCW). Specific production and consumption rates during the stationary phase of the fermentation approached those observed in the control fermentation during phases III and IV.

Even though the final lysine titer was 25% greater than the control fermentation (25 versus 20 g/L), the instantaneous yield at the start of lysine production was approximately 35% (molar), only slightly increased over the standard yield (30%, molar). Consequently, the increase in lysine titer resulted from an extension of the viability of the culture (extended phase III), not an increase in instantaneous yield. The activities of GPI and three NADPH-producing enzymes, assayed at 31.0 and 57.5 h, are listed in Table 3. The reduced activity of GPI (11% of ATCC 21253 cultured on gluconate) confirms the existence of the mutation. The activities of GPI, G6PDH, and GN6PDH were slightly higher than nominal, which was probably due to induction by added gluconate (also see Table 5).

**Flux Analysis of NFG068.** Metabolite accumulation rates, $r(t)$, required for flux analysis were calculated from measurements taken at 31.0 and 37.5 h ($t_{av}$ = 34.3 h), after gluconate exhaustion (Figure 1C) to avoid flux alterations induced by gluconate consumption. The measured and estimated accumulation rates are listed in Table 4 and show high consistency (*18*, *21*) ($h$ = 0.09) with respect to mass balance constraints. Compared to the control fermentation, the metabolite accumulation rates for the NFG068 fermentation at 34.3 h were approximately 2–3 times smaller, which is largely a result of the reduced biomass titer. The flux distribution map (Figure 2) is remarkably similar to that observed during the control fermentation (*19*) under similar conditions (late phase II and phase III). Consequently, no significant alteration of flux partitioning at the Glc6P branch point resulted from a 90% attenuation of GPI activity, even though the mutation produced alterations in overall growth kinetics. The mutation did not alter the instantaneous lysine yield significantly, but reduced the specific flux through the network during the growth and initial lysine production phases (data not shown). Such results are consistent with the hypothesis of a dependent network (where flux partitioning must be altered at several branch points in unison) that harbors one or more rigid branch points (15) (points where alterations in flux partitioning are not readily achieved).

To classify the Glc6P branch point as either flexible, weakly rigid, or strongly rigid (*15*), it is necessary to compare the expected consequences of the perturbation to the observed results. If the Glc6P branch point were flexible (flux partition readily changes to meet lysine demand), then the lysine yield would be limited by the rigidity of either the PEP or Pyr branch point. Hence, attempts to redirect flux partition at the Glc6P branch point could result in the attenuation of fluxes throughout the metabolic network without significantly altering flux partitioning, as was observed. On the other hand, if the lysine yield were constrained by a weakly rigid Glc6P branch point (flux partitioning control by relative enzyme activities), then the GPI perturbation should have improved the yield, as the attenuation of GPI should have allowed

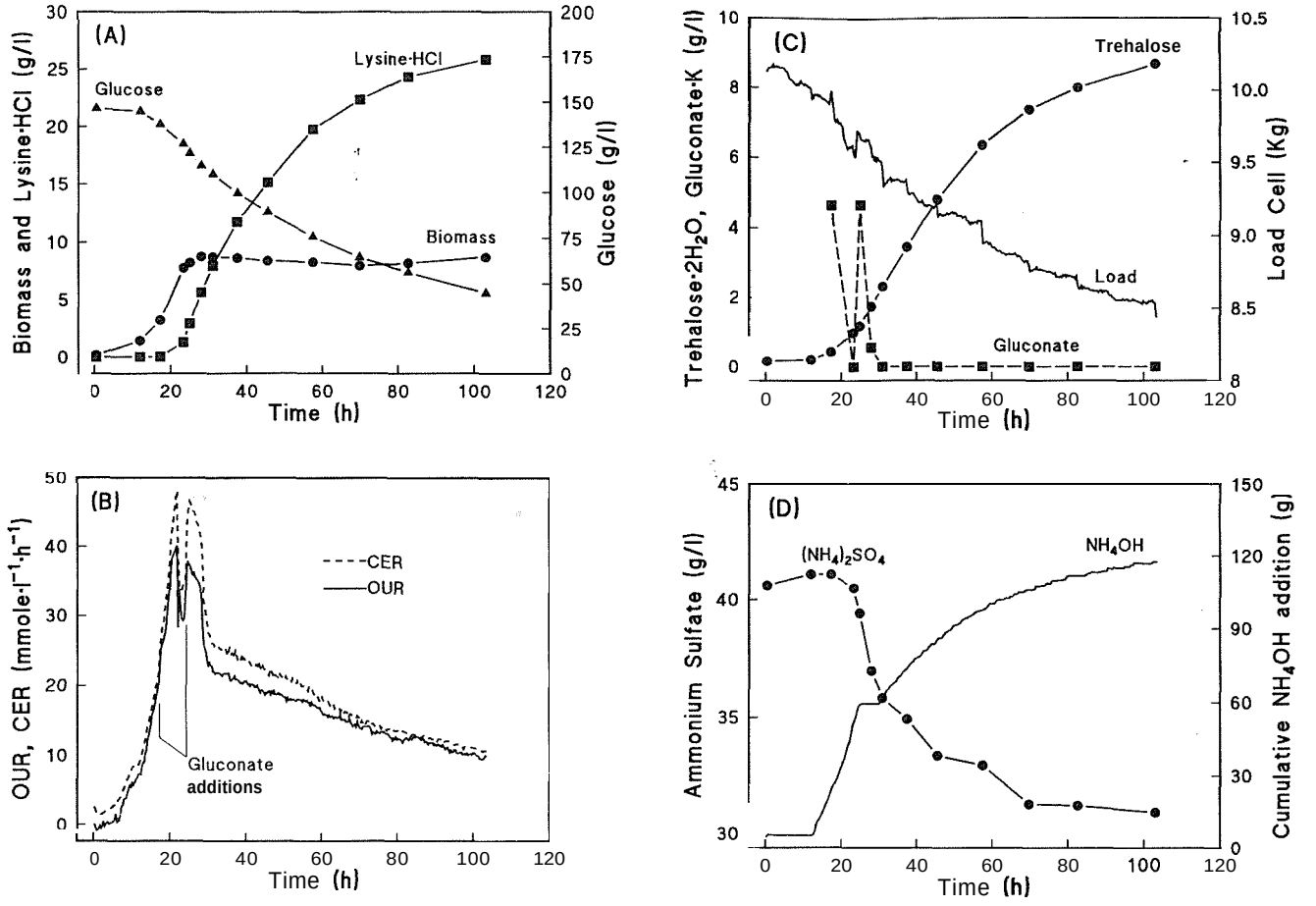


Figure 1. Lysine fermentation of C. *glutamicum* NFG068 (GPI$^{A}$) cultured on glucose medium (FM4): (A) glucose, biomass, and lysine hydrochloride profiles in grams per liter; (B) culture respiration: oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER); (C) profiles of added gluconate, trehalose production, and fermentor volume (load) used to adjust OUR and CER; (D) available ammonium in broth, as ammonium sulfate, and the amount of ammonium hydroxide (26% (w/w) $NH_3$) added to maintain culture at pH 7.0.

Table 3. Activities of Selected Enzymes of **NFG068** during the Fermentation Depicted in Figure 1

| time (h) | protein (mg/mL) | activity (nmol min$^{-1}$ (mg of protein)$^{-1}$) GPI | G6PDH | GN6PDH | ICDH |
|---|---|---|---|---|---|
| 31.0 | 7.84 | 151 | 173 | 448 | 1100 |
| 57.5 | 7.52 | 146 | 154 | 443 | 956 |

Table 4. Measured and Estimated Metabolite Accumulation Rates and Standard Deviations (a) for the **NFG068** Fermentation at 34.3 h from Measurements Taken at 31.0 and 37.5 h (Figure 1)[a]

| metabolites | accumulation rates (mM/h) measured | estimated[b] |
|---|---|---|
| acetate | 0 ± 1 | 0.02 |
| alanine | 0 ± 1 | 0.00 |
| biomass | -0.17 ± 0.9 | -0.15 |
| $CO_2$ | 26.5 ± 2.7 | 26.2 |
| glucose | -9.0 ± 2.5 | -8.7 |
| lactate | 0 ± 1 | 0.03 |
| lysine | 3.16 ± 0.2 | 3.16 |
| $NH_3$ | -5.5 ± 5.8 | -6.3 |
| $O_2$ | -22.7 ± 2.3 | -23.0 |
| pyruvate | 0 ± 1 | 0.00 |
| trehalose | 0.46 ± 1 | 0.57 |
| valine | 0 ± 1 | 0.08 |

[a] Consistency index: h = 0.09. [b] Estimated rates are those that exactly satisfy mass balance constraints and are derived from the estimated fluxes: $\hat{\mathbf{r}}(t) = \mathbf{A}\hat{\mathbf{x}}(t)$.

more Glc6P to enter the PPP, which was not observed. The GPI perturbation, therefore, indicates that the Glc6P branch point is not weakly rigid. As a third possibility, the Glc6P branch point could be strongly rigid (flux partitioning under feedback control). If this were the case, then attenuation of GPI activity would also result in attenuation of the flux in the opposing (*i.e.*, PPP) branch, and the net result would be similar to that actually observed. To investigate the possibility that the Glc6P branch point might be strongly rigid, a gluconate fermentation was conducted.

**Gluconate Metabolism.** The Glc6P branch point is effectively bypassed when gluconate (Glcn) is used as the primary carbon source, since it directly enters the pentose phosphate pathway (Figure 3). Consequently, if the Glc6P branch point is strongly rigid and limits the lysine yield, cultivation of C. *glutamicum* ATCC 21253 on gluconate should improve lysine yield. The basis of this hypothesis and the analysis of a gluconate fermentation are examined below.

Gluconate enters the PPP after phosphorylation to gluconate 6-phosphate (Glcn6P) by gluconokinase (12) and then is subsequently oxidized to ribulose 5-phosphate (Ribu5P) (Figure 3). Gluconate 6-phosphate so formed should not lead to the direct production of Glc6P, since reversal of 6-phosphogluconolactonase is thermodynamically unfavorable ($\Delta G^{\circ} = +5.0$ kcal/mol) (9). Furthermore, it does not appear that the reverse reaction can be driven, since the overall conversion of Glcn6P to Fru6P via 6-phosphogluconolactonase is also thermodynamically unfavorable [$\Delta G^{\circ} = +5.5$ kcal/mol (see Figure 3)], so that the reaction is considered kinetically irreversible. Consequently, all phosphorylated gluconate should be con-

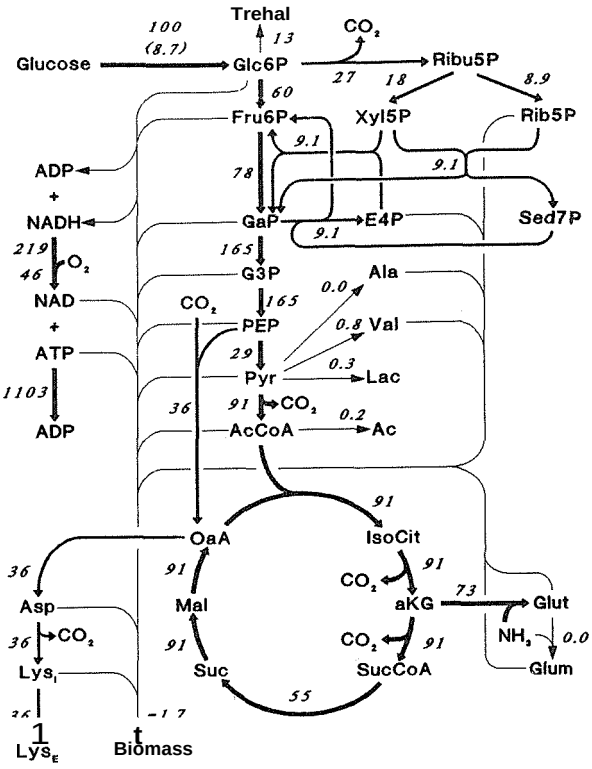


**Figure 2.** Flux distribution map for the NFG068 lysine fermentation at 34.3 h. Fluxes were estimated from measurements taken at 31.0 and 37.5 h (see Figure 1 and Table 4) and normalized by the glucose uptake rate (shown in parentheses, mmol $L^{-1}$ $h^{-1}$).

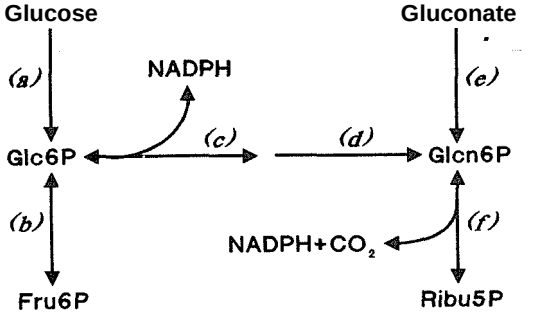


**Figure 3.** Metabolic entry points for glucose and gluconate metabolism: (a) PEP:glucose phosphotransferase system; (b) glucose-6-phosphate isomerase ($\Delta G°$ = +0.4 kcal/mol toward Fru6P formation); (c) glucose-6-phosphate dehydrogenase ($\Delta G°$ = -0.1 kcal/mol toward Glcn6P formation); (d) 6-phosphogluconolactonase ($\Delta G°$ = -5.0 kcal/mol toward Glcn6P formation); (e) gluconokinase; (f) gluconate-6-phosphate dehydrogenase. Reaction $\Delta G°$ values are from Lehninger (9).

verted to Ribu5P by GNGPDH, generating a minimum of 1 mol of NADPH for each mole of gluconate consumed or $[NADPH/gluconate]_{min}$ = 1.0 (this ratio is higher if the PPP operates in a cyclic mode, so that $[NADPH/gluconate]_{max}$ = 5.0). As a result of the minimal NADPH production on gluconate, the minimum theoretical lysine molar yield on gluconate corresponds to 45% (the maximum yield is lower than the maximum yield from glucose, only 69% instead of 75%) (15). For glucose catabolism, $[NADPH/glucose]_{min}$ is 0 and $[NADPH/glucose]_{max}$ is 6.0, corresponding to no glucose entering the PPP and complete oxidation of glucose by the PPP, respectively. However, examination of the flux distributions during phase I, late phase II, and phase III of the control lysine fermentation (19) and the NFG068 fermentation (Figure 2) reveals that the typical PPP flux is 30% of glucose consumption, or

Table 5. **Intracellular** Assays of *C.* glutamicum ATCC 21253 Cultured on **PMB Medium or PMB Medium with** Gluconate as the **Only** Carbon **Source**

| enzyme or pathway | activity (nmol $min^{-1}$ (mg of protein)$^{-1}$) glucose | gluconate |
|---|---|---|
| 6-phosphogluconolactonase, reversal | | 3 ± 3 |
| Entner–Doudoroff | 0 ± 5 | 0 ± 5 |
| malic enzyme | 1 ± 5 | 1 ± 5 |
| glucose-6-phosphate isomerase | 950 ± 50 | 1360 ± 50 |
| glucose-6-phosphate dehydrogenase | 130 ± 20 | 200 ± 20 |
| gluconate-6-phosphate dehydrogenase | 270 ± 20 | 830 ± 20 |
| isocitrate dehydrogenase | 1330 ± 50 | 1270 ± 50 |
| transhydrogenase | ~0 ± 5 | |

$[NADPH/glucose]$ = 0.6, since 2 mol of NADPH is produced for each mole of Glc6P catabolized through the PPP. Since $[NADPH/gluconate]_{min}$ is greater than $[NADPH/glucose]$ typically observed during lysine production on glucose, a fermentation with gluconate as the primary carbon source should exhibit an improved lysine yield if lysine is limited by NADPH production due to the rigidity of the Glc6P branch point.

The above biochemical analysis for gluconate metabolism assumes that (1) alternate pathways for Glcn6P catabolism (such as the Entner–Doudoroff pathway) are not present or induced by gluconate and (2) reactions that consume NADPH similarly are not induced. The presence of a NAD-specific GNGPDH induced by gluconate has already been examined and was not detected (17). To ensure that other enzymes were not induced by gluconate, several intracellular assays were performed on cell-free extracts prepared from glucose- and gluconate-cultured cells, the results of which are presented in Table 5. It is clear from these results that 6-phosphogluconolactonase is not reversible, that the malic enzyme and the ED pathway are not induced by glucose or gluconate, and that a soluble dinucleotide transhydrogenase was not detected in glucose-derived extracts. However, GPI and G6PDH exhibit some induction, and GNGPDH shows a 3-fold increase in activity when cultured on gluconate, which has also been observed by Sugimoto and Shiio (17). No noticeable induction of ICDH occurs. The dramatic increase in GNGPDH activity strongly supports the hypothesis that all gluconate is catabolized through this enzyme and that a gluconate fermentation should produce more intracellular NADPH.

**Gluconate Fermentation of ATCC 21253.** First attempts to grow C. *glutamicum* ATCC 21253 solely on 140 g/L potassium gluconate resulted in linear growth and eventually ended in cell lysis after 45 h (data not presented). Although not assayed, the value of the respiratory quotient ($CO_2$ evolution/$O_2$ consumption) indicated that lysine was not overproduced during the fermentation. However, it was found that if glucose was added to the culture, normal exponential growth was restored (until the glucose was exhausted) even in the presence of high gluconate concentrations. Consequently, it was not the high concentration of gluconate that caused growth inhibition.

To alleviate the linear growth problem, C. *glutamicum* was cultivated on the FM9 medium. The results of the gluconate fermentation are depicted in Figure 4A–D. As illustrated in Figure 4A, the additional 10 g/L glucose allows growth to proceed exponentially even in the presence of 140 g/L potassium gluconate, which does not seem to affect the characteristics or growth rate ($\mu$ = 0.31 $h^{-1}$) of the culture. To ensure that the culture reached the lysine production phase (*i.e.*, consumed all threonine) before

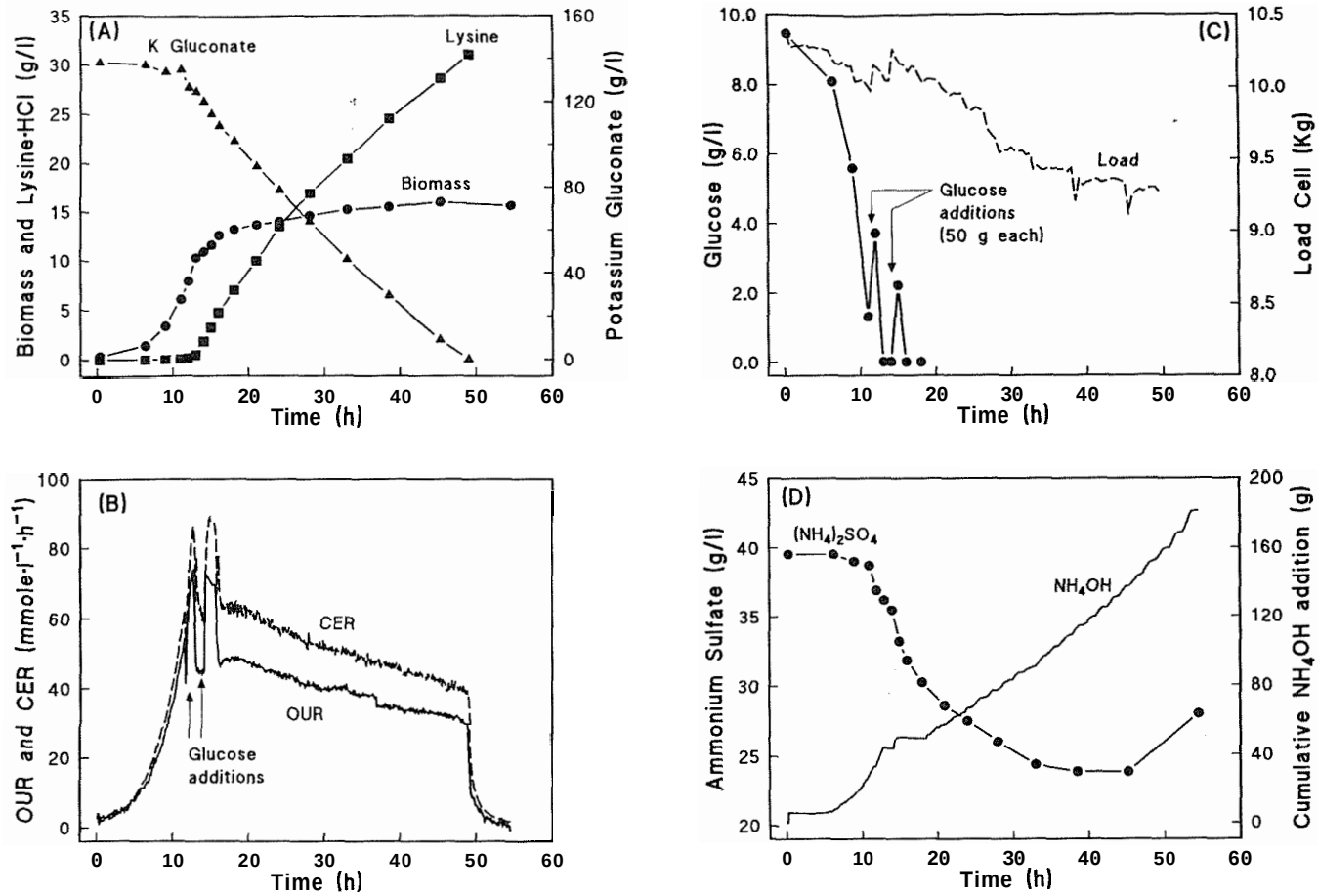


Figure 4. Lysine fermentation of C. *glutamicum* ATCC 21253 cultured an gluconate medium (FM9): (A) potassium gluconate, biomass, and lysine hydrochloride profiles in grams per liter; (B) culture respiration: oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER); (C) glucose concentration profile illustrating points of glucose addition and fermentor volume (from load cells) used to calculate volumetric OUR and CER; (D) available ammonium in broth, as ammonium sulfate, and the amount of ammonium hydroxide (26% (w/w) $NH_3$) added to maintain culture at pH 7.0.

glucose was exhausted, two 50-g (~5 g/L) supplements of glucose were added as depicted in Figure 4C and evident in the culture's respiration (Figure 4B). At 15.7 h (as documented by the drop in respiration and confirmed in Figure 4C), all added glucose was exhausted and 109 g/L potassium gluconate remained as the sole carbon source.

The linear growth characteristic associated with gluconate metabolism becomes evident after the exhaustion of supplied glucose (Figure 4A). Since C. glutamicum does not grow exponentially on gluconate, the culture bypasses phase II (*i.e.*, rapid biomass and lysine synthesis) observed in the control fermentation and directly enters phase III (little to no growth but high lysine overproduction). Here, the growth rate is linear and quite slow, but lasts for an extremely long time (35 h) and is terminated primarily by gluconate depletion. Phase IV conditions (*i.e.*, cell lysis and byproduct formation) are not exhibited. The extension of phase III results in a lysine titer 50% greater than that observed in the control fermentation (31 versus 20 g/L, respectively); however, once again the instantaneous molar yield is not observed to exceed 34%. Other notable features of the fermentation include a higher than normal respiratory quotient (ca. 1.35) and a complete lack of the typically observed byproducts. The respiratory quotient is higher for gluconate catabolism since the degree of reductance of gluconate is lower than that of glucose, yet product formation is similar to that of the control fermentation. Consequently, less oxygen is required to balance substrate and product reducing equivalents (*i.e.*, electron balance) (14).

**Flux Analysis.** The metabolite accumulation rate vector was calculated from measurements taken at 18.0 and 21.0 h ($t$, = 19.5 h), so that gluconate metabolism could be examined separately from glucose consumption. The measured and estimated metabolite accumulation rates are given in Table 6, along with the consistency index. Inspection of Table 6 shows that the metabolism of gluconate is as rapid as the glucose assimilation observed in the control fermentation, and the lack of byproduct formation leads to an extremely good consistency index (h = 0.003). To examine the resulting flux distribution map, the representation of the biochemistry was modified to handle gluconate metabolism.

The glucokinase reaction was represented by

$$\text{Glcn} + \text{ATP} \rightarrow \text{Glcn6P} + \text{ADP} \quad (1)$$

and the oxidative branch of the PPP was broken into the two reactions,

$$\text{Glc6P} + H_2O + \text{NADP} \rightarrow \text{Glcn6P} + \text{NADPN} \quad (2)$$

$$\text{Glcn6P} + \text{NADP} \rightarrow \text{Ribu5P} + CO_2 + \text{NADPH} \quad (3)$$

where 6-phosphogluconolactonase has been lumped with G6PDH in reaction 2. However, when the flux distribution map was calculated from the rates given in Table 6 and the modified biochemistry given above, reaction 2 exhibited a negative flux (data not presented). This occurred because more NADPH was produced than consumed, so that reaction 2 was driven backward in order to counter-

**Table 6. Metabolite Accumulation Rates for the Gluconate Fermentation at 19.5 h from Samples Taken at** 18.0 **and** 21.0 h **(Figure 4)**[a]

| | accumulation rates (mM/h) | |
|---|---|---|
| **metabolites** | **measured** | **estimated** |
| **acetate** | O f 2 | 0.00 |
| alanine | 0 ± 2 | 0.02 |
| **biomass** | 1.67 f 3.2 | 1.72 |
| $CO_2$ | 62.4 ± 6.2 | 62.4 |
| **gluconate** | –17.0 f 3.9 | –17.0 |
| **lactate** | O f 2 | 0.00 |
| **lysine** | 5.48 f 0.4 | 5.48 |
| $NH_3$ | –12.8 ± 11 | –12.3 |
| $O_2$ | –48.0 f 4.8 | –48.0 |
| **pyruvate** | O f 2 | 0.00 |
| **trehalose** | O f 2 | 0.02 |
| valine | O f 2 | 0.03 |

[a] **Consistency index: h** = 0.003. **Also see footnote b to Table** 4.

balance excess NADPH production. Since reversal of reaction **2** is not feasible, primarily due to the irreversibility of 6-phosphogluconolactonase, the biochemistry was modified by deleting reaction 2 and replacing it with direct NADPH oxidation:

$$2NADPH + O_2 \rightarrow 2H_2O + 2NADP \qquad (4)$$

Since NADPH is not known to participate in the respiratory chain, and all other NADPH sinks and alternate reaction paths have been ruled out (Table V), it appears plausible that NADPH could be oxidized nonenzymatically or by an enzyme with poor specificity for NADH, which would have the same net result as the inclusion of reaction 4 in the flux distributions. The flux distribution map with the modified biochemistry is displayed in Figure 5. Other than the PPP, the flux distribution map is fairly similar to that observed in the control fermentation during lysine production and modest growth (phase 111), except that the catabolism of gluconate produces a substantial excess of NADPH, as is evident in Figure 5. This excess NADPH, however, does not result in an increase in lysine yield. These results strongly suggest that lysine yield is not limited by NADPH availability.

The above analysis of gluconate metabolism illustrates some of the power of the metabolite balance technique. Inconsistencies in the flux distributions (*i.e.*, negative flows in irreversible reactions) do not imply that the analysis must be rejected. Since metabolic processes must conform in a manner consistent with mass balance constraints, inconsistencies imply that the biochemistry must be functioning in a manner different from that perceived, which is extremely useful information. It is due to the negative flow in reaction 2 during gluconate catabolism that a search for alternate means of NADPH oxidation was conducted (Table 5). Since reasonable pathways could not be verified to mitigate NADPH overproduction (Table 5), nonspecific oxidation of NADPH appears a valid hypothesis. Although speculative, the excess NADPH production may also explain why C. glutamicum cannot sustain exponential growth on gluconate as the sole carbon source. Since C. glutamicum appears to lack adequate mechanisms for the direct oxidation of NADPH, intracellular accumulation of this reduced metabolite may perturb secondary metabolism (*i.e.*, peripheral reactions associated with biomass synthesis) to the extent that exponential growth is no longer feasible. This may also explain why a GPI-lacking strain of C. glutamicum, which can grow on glucose, could not be isolated (also see ref 16). The increased NADPH synthesis rate associated with gluconate assimilation may also explain the extended

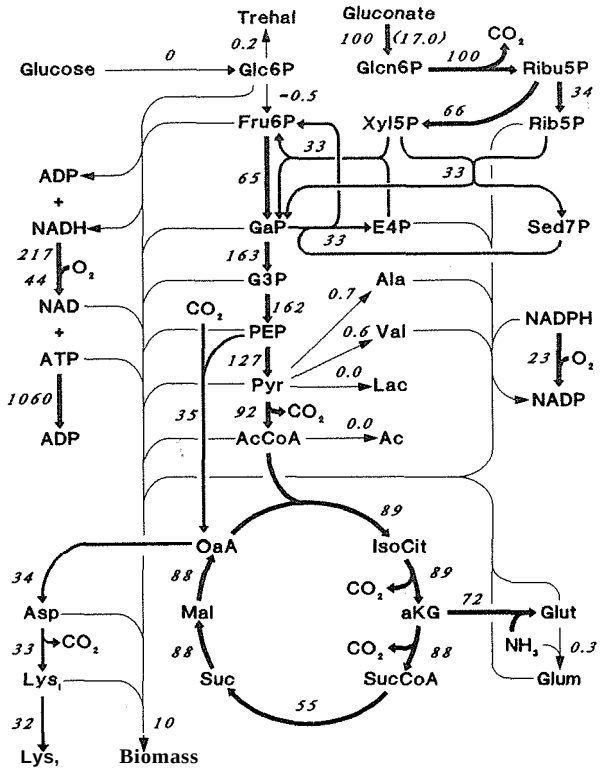


**Figure 5. Flux distribution map for the gluconate fermentation at** 19.5 **h. Fluxes were estimated from measurements taken at** 18.0 **and 21.0 h (see Figure 4 and Table 6) and normalized by the gluconate uptake rate (shown in parentheses, mmol $L^{-1}$ $h^{-1}$). Note that the first enzyme of the pentose phosphate pathway has been removed, and reactions for gluconokinase and direct oxidation of NADPH have been added to eliminate inconsistencies (see text for details).**

viability of the culture compared to that of the control glucose fermentation. It is plausible that the increased NADPH synthesis may increase the intracellular reducing equivalence, which could extend protein stability or enhance protein turnover efficiency and may be similar to the increased lysine titer observed in fed-batch cultures under the restrained-growth control objective (4).

The flexibility of the Glc6P branch point is also supported by alterations in flux partitioning observed at this branch point in the control fermentation during shifts in metabolism from growth to lysine overproduction (19). During pure growth, the majority of glucose entering the Glc6P branch point continues into the glycolysis pathway; however, at the start of lysine overproduction (phase 11), carbon flow is redirected into the pentose phosphate pathway (see Figures 4 and 5 of ref 19). High flux rates in the PPP during phase II of the control fermentation are necessary to meet increased demands for NADPH brought about by high biomass and lysine synthesis rates. This alteration in flux partitioning, caused by increased NADPH demands associated with phase II, indicates that the Glc6P branch point is flexible and that lysine yield does not appear to be constrained by NADPH availability.

## Summary

Results from the fermentation of NFG068 (a $GPI^A$ mutant) indicate that lysine yield is not limited by suboptimal flux partitioning at the Glc6P branch point caused by the dominance of the glycolytic branch. Flux analysis of the gluconate fermentation, and intracellular assays conducted in light of these results, demonstrates that lysine yield is not limited by NADPH production,

since gluconate catabolism should have made NADPH readily available. Therefore, the Glc6P branch point must be flexible, and lysine yield limitations must be attributed to suboptimal flux partitioning (*i.e.*, rigidity) at either the PEP or Pyr principal branch point. The fact that the attenuation of GPI resulted in flux attenuation without affecting flux partitioning at the principal branch points supports the hypothesis regarding perturbations of rigid dependent networks previously discussed (15). It should be stressed that although the Glc6P branch point has been demonstrated to be flexible under the moderate lysine yields observed, it is possible that this branch point could become a limiting point if the lysine yield were dramatically increased by improving flux partitioning at the other principal branch points. That is, the above conclusions regarding the Glc6P branch point cannot be extrapolated to maximum lysine yield, but the same methodology can be applied in a recursive manner on improved strains. It has also been demonstrated that metabolite balances (*i.e.*, flux analysis) can be used to verify the speculated operation of a proposed metabolic network. Analysis of gluconate catabolism, under the currently perceived biochemistry, indicates that C. glutamicum should encounter difficulty in catabolizing gluconate as the sole carbon source, due to the lack of adequate mechanisms to oxidize NADPH.

Although the identification of an alternate pathway for PEP carboxylation, *i.e.*, PPCK (*13*), brings into question the mechanisms governing flux partitioning at the PEP branch point (*15*), its presence (if expressed in the wild-type strain) does not alter estimated flux distributions nor the conclusions drawn regarding the flexibility of the Glc6P branch point.

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## Literature Cited

(1) Broer, S.; Eggeling, L.; Kramer, R. Strains of Corynebacterium glutamicum with different lysine productivities may have different lysine excretion systems. Appl. Enuiron. Microbiol. **1993**, *59*, 316–321.

(2) Deutsch, J. Glucose-6-phosphate dehydrogenase. Methods of enzymatic analysis, 3rd ed.; Bergmeyer, H. U., Ed.; Verlag Chemie: Weinheim, Germany, 1983; Vol. 3, pp 190–197.

(3) Fraenkel, D. G.; Levisohn, S. R. Glucose and gluconate metabolism in an Escherichia coli mutant lacking phosphoglucose isomerase. J. Bacteriol. 1967, 93, 1571–1578.

(4) Kiss, R. D.; Stephanopoulos, G. Metabolic activity control of the L-lysine fermentation by restrained growth fed-batch strategies. Biotechnol. Prog. 1991, **7**, 501–509.

(5) Kovachevich, R.; Wood, W. A. Carbohydrate metabolism by Pseudomonas fluorescens. III. Purification and properties of a 6-phosphogluconate dehydrase. J. Biol. Chem. 1955, 213, 745–756.

(6) Kovachevich, R.; Wood, W. A. Carbohydrate metabolism by Pseudomonas fluorescens. IV. Purification and properties of 2-keto-3-deoxy-6-phosphogluconate aldolase. J. Biol. Chem. 1955, 213, 757–767.

(7) Lang, G.; Michal, G. D-glucose-6-phosphate and D-fructose-6-phosphate. In Methods of enzymatic analysis, 2nd ed.; Bergmeyer, H.U., Ed.; Weinheim, Verlag Chemie: Weinheim, Germany, 1974; Vol. 3, pp 1238–1242.

(8) Lederberg, J. Isolation and characterization of biochemical mutants of bacteria. Methods Med. Res. 1950, 3, 5–22.

(9) Lehninger, A. L. Biochemistry, 2nd ed.; Worth Publishers, Inc.: New York, 1975.

(10) MacFaddin, J. F. Media for isolation-cultivation-identification-maintenance of medical bacteria; Williams & Wilkins: Baltimore, MD, 1985; Vol. 1, pp 294, 620–628.

(11) Miller, J. H. Nitrosoguanidine mutagenesis. In Experiments in molecular genetics; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1974; pp 125–129.

(12) Mori, M.; Shiio, I. Pyruvate formation and sugar metabolism in an amino acid-producing bacterium, Breuibacterium flauum. Agric. Biol. Chem. **1987**, *51*, 129–138.

(13) Peters-Wendisch, P. G.; Eikmanns, B. J.; Thierbach, G.; Bachmann, B.; Sahm, H. Phosphoenolpyruvate carboxylase in Corynebacterium glutamicum is dispensable for growth and lysine production. FEMS Microbiol. Lett. **1993**, *112*, 269–274.

(14) Roeh, J. A. Energetics and kinetics in biotechnology; Elsevier Biomedical Press: New York, 1983.

(15) Stephanopoulos, G.; Vallino, J. J. Network rigidity and metabolic engineering in metabolite overproduction. Science 1991, 252, 1675–1681.

(16) Sugimoto, S.-I.; Shiio, I. Regulation of glucose-6-phosphate dehydrogenase in Breuibacterium flauum. Agric. Biol. Chem. **1987**, *51*, 101–108.

(17) Sugimoto, S.-I.; Shiio, I. Regulation of 6-phosphogluconate dehydrogenase in Breuibacterium flauum. Agric. Biol. Chem. **1987**, *51*, 1257–1263.

(18) Vallino, J. J.; Stephanopoulos, G. Flux determination in cellular bioreaction networks: applications to lysine fermentations. In Frontiers in bioprocessing; Sikdar, S. K., Bier, M., Todd, P., Eds.; CRC Press, Inc.: Boca Raton, FL, 1990; pp 205–219.

(19) Vallino, J. J.; Stephanopoulos, G. Metabolic flux distributions in Corynebacterium glutamicum during growth and lysine overproduction. Biotechnol. Bioeng. **1993**, *41*, 633–646.

(20) Vallino, J. J.; Stephanopoulos, G. Carbon flux distributions at the pyruvate [illegible] during [illegible] overproduction. *Biotechnol. Prog.* **1994**, preceding article in this issue.

(21) Wang, N. S.; Stephanopoulos, G. Application of macroscopic balances to the identification of gross measurement errors. Biotechnol. Bioeng. **1983**, *25*, 2177–2208.

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