Carbon Flux Distributions at the Pyruvate Branch Point in *Corynebacterium glutamicum* during Lysine Overproduction

Joseph J. Vallino† and Gregory Stephanopoulos*

Chemical Engineering Department, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

In order to achieve the theoretical maximum yield of lysine from glucose (75% molar), flux partitioning at principal branch points (glucose 6-phosphate, phosphoenolpyruvate, and pyruvate) in the primary metabolic network of Corynebacterium glutamicum must be significantly altered from that observed during balanced growth, so that lysine precursors are synthesized in optimal stoichiometric ratios. In this article, we employ flux analysis, which provides a snapshot of metabolic flux distributions from extracellular measurements, in conjunction with two metabolic perturbations of the pyruvate dehydrogenase complex (PDC) to examine the possibility that flux partitioning at the pyruvate (Pyr) branch point (*i.e.*, pyruvate availability) limits lysine yield. The two perturbation experiments involved (1) isolation and flux analysis of a PDC-attenuated mutant of C. glutamicum and (2) flux analysis following the addition of fluoropyruvate (FP) to a standard fermentation during the initial period of lysine overproduction. No significant alteration in flux partition was observed at any of the three principal nodes in the PDC-attenuated strain. However, the PDC mutation did cause a 70% uniform decrease in carbon flow throughout the network, which indicates that the lysine yield is not limited solely by a weakly rigid Pyr branch point. The addition of FP did not affect the lysine synthesis rate, but did temporarily redirect carbon flow away from the TCA cycle toward pyruvate excretion, which further confirms that the lysine yield is not pyruvate-limited and that the Pyr branch point is not weakly rigid.

Introduction

It has been shown (11) that the yield of lysine, produced aerobically from glucose by deregulated strains of *Corynebacterium glutamicum* (4), is governed by the partitioning of carbon at three principal metabolic branch points: glucose 6-phosphate (Glc6P), phosphoenolpyruvate (PEP), and pyruvate (Pyr). If flux partitioning at any of these three branch points is suboptimal, then the maximum theoretical lysine yield (75% molar) will not be realized. Suboptimal flux partitioning at a branch point can be caused by inequalities in the enzyme activities in competing branches—a weakly rigid branch point—or by enzyme control architectures that actively control flux partitioning—a strongly rigid branch point; otherwise, the branch point is considered flexible and will not limit yield once the end-product regulation is removed (11). One means to ascertain the characteristics of a branch point is to perturb it and observe the transient flux alterations that follow via flux analysis (13, 14). In our previous publications, we have applied this technique to establish the basic metabolic flux distributions during lysine synthesis (14) (the control case) and to examine the Glc6P branch point (15), which has been found to be flexible. In this study, we will use similar techniques to examine the flexibility of the Pyr branch point.

Pyruvate represents the branch point between the pyruvate dehydrogenase complex (PDC), which leads into the TCA cycle and CO_2 production, and dihydrodipicolinate synthase, which catalyzes the condensation of pyruvate with aspartic semialdehyde (ASA) to form lysine. This distribution ignores any pyruvate consumption by an anaplerotic pathway, since no pyruvate carboxylase activity was detected in crude cell extracts of C. *glutamicum*. If such activity is present, then obviously the network structure around pyruvate will have to be modified to include the additional pyruvate carboxylase enzyme. If the Pyr branch point is weakly or strongly rigid, pyruvate will preferentially enter the TCA cycle instead of condensing with ASA, and lysine yield will be limited by pyruvate availability. If the lysine yield suffers from a weakly rigid Pyr branch point, then attenuation of PDC should increase the availability of pyruvate and improve lysine yield. If poor lysine yield is due to rigidity of the PEP branch point or a strong rigidity of the Pyr branch point, then PDC attenuation should result in either the excretion of some intermediate metabolite or overall network flux attenuation due to network structure (11). Although several studies involving PDC attenuation in Brevibacterium *flavum* (6) and B. lactofermenturn (12) have been performed, conclusions regarding the flexibility of the Pyr branch point remain uncertain for two primary reasons: (1) the mutation–selection techniques employed to attenuate PDC (5, 7, 9, 10, 17) often result in alterations of other primary metabolic enzymes, since final strain selection is based on lysine yield improvements and not PDC activity; and (2) the extent of the Pyr branch point perturbation is unknown due to the lack of adequate metabolic monitoring (*i.e.*, often only the overall lysine yield is reported).

To examine the possibility that flux partitioning at the Pyr branch point limits lysine yield, two perturbation experiments were conducted to elucidate the degree of flexibility of the Pyr branch point. The first experiment involved the isolation, fermentation, and flux analysis of a PDC-attenuated (PDC^A) mutant of C. glutamicum

* Author to whom correspondence should be addressed.
† Present address: Marine Biological Laboratory, Ecosystems Center, Woods Hole, MA 02543.

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ATCC 21253. The second experiment involved monitoring flux alterations at the Pyr branch point induced by PDC inhibition caused by the addition of fluoropyruvate (FP) following the start of lysine overproduction.

Materials and Methods

Microorganisms. The cultivation of Corynebacterium glutamicum ATCC 21253, which was used in the fluoropyruvate perturbation study and served as the parent strain for subsequent mutation–selection studies, has been previously described (14). This strain is a homoserine and leucine auxotroph but has the native function of aspartokinase, so that lysine overproduction only occurs when threonine, which is supplied in the medium, becomes exhausted. The mutant strain FPS009, whose selection is described below, was cultivated under the same conditions as ATCC 21253, except where noted below.

Mutation and Selection. The preparation of mutant strains of ATCC 21253 from exposure to *N*-methyl-*N'*-nitro-N-nitrosoguanidine (NTG) has been explained previously (15). Isolation of C. glutamicum ATCC 21253 mutants with attenuated activity of the pyruvate dehydrogenase complex (PDC) is based on the work of Tosaka et al. (12). Since fluoropyruvate (FP) is a strong competitive inhibitor of the PDC, mutant strains that have attenuated activity of PDC are often unable to grow in the presence of FP. Such strains are referred to as FP-sensitive (FP^S) and were isolated as follows.

After an NTG (Sigma Chemical Co., St. Louis, MO) treated stock of C. glutamicum was cultured in 10 mL of LB5G medium (*14*), the cultures were spun down and resuspended in 10 mL of PMB medium (14) (a defined glucose minimal medium) supplemented with 40 μM FP and 500 units/mL penicillin G (both from Sigma) to enrich FP^S strains. These culture tubes were incubated under agitation for 6 h, at which time 10 units of penicillinase (Sigma) were added to each culture tube, which were then incubated for another $^1/_2$ h before resuspension in 10 mL of LB5G medium. These cultures were then plated out on LB5G plates to determine cell density and stored at 4 °C. After the cell density was determined, the cultures were plated on LB5G plates to a density of 50–100 colonies per plate and replica-plated with RepliPlate pads (FMC BioProducts, Rockland, ME) onto PMB plates and PMB plates supplemented with 50 μM FP. Colonies that exhibited growth on PMB plates but not on the PMB plates supplemented with FP were examined for PDC activity.

Fermentations. Fermentor instrumentation, general operation, and sample analysis were the same as those described for the control fermentation, except that acetate and fluoropyruvate were added to the FM4 medium (14) (a defined glucose and ammonium sulfate medium) during the FPS009 fermentation and the PDC inhibition study, respectively. Cell-free extract preparation and the PDC assay have also been previously described (14). All fermentations were run in batch mode and exhibited similar characteristics. The cultures grow exponentially during phase I of the fermentation until the supplied threonine is exhausted, at which time lysine overproduction commences due to the loss of concerted feedback inhibition of aspartokinase. If growth continues along with lysine production, then the culture is defined to be in phase II. Phase III of the culture is marked by lysine production but little to no growth, and phase IV is the death phase.

Flux distributions in the primary metabolism of C. glutamicum, whose biochemistry has been previously reviewed, are determined by flux analysis (*13*, *14*). This technique utilizes measured rates of accumulation and depletion of extracellular metabolites, stoichiometrically derived mass balance constraints, and a pseudo-steady-state approximation for intracellular metabolites to obtain a snapshot of metabolic activity at the time of measurement. A consistency index (16) is calculated to identify the presence of gross errors in the measurements, the pseudo-steady-state approximation, or the biochemistry. No alteration of the metabolic network was necessary with the recent detection of PEP carboxykinase (PPCK) in C. glutamicum (*6*), as PPC and PPCK are indistinguishable mathematically (*i.e.*, PPCK is lumped with PPC).

Table 1. Activity of the Pyruvate Dehydrogenase Complex (PDC) in Selected FP^S Mutants of C. glutamicum ATCC 21253

strain	PDC activity
ATCC 21253	27.9
FPS002	17.9
FPS008	25.4
FPS009	0.45
FPS012	21.2
FPS013	32.8

[a] *In nmol* min^{-1} *(mg of* protein$)^{-1}$.

Results and Discussion

PDC Mutant. Out of 2900 colonies examined, 13 exhibited sensitivity to FP at 50 μM, of which 5 were cultured on SLB medium (15) and assayed for PDC activity (Table 1). Of these strains, FPS009 exhibited a 98% attenuation of PDC activity and was selected for fermentation studies.

Although FPS009 would grow on PMB plates, it did not grow well in PMB liquid medium. However, the growth inhibition was significantly reduced, although attenuated with respect to ATCC 21253, if 5 g/L potassium acetate were added to the PMB suspension medium. Growth stimulation by acetate addition supports the hypothesis that PDC attenuation was the growth-limiting mutation. The mutation is stable since revertants were not observed.

Fermentation of FPS009. Results of the FPS009 fermentation cultured on the FM4 medium are depicted in Figure 1A–D. During the first 70 h of cultivation, the strain exhibited linear growth (not shown). However, upon the addition of 60 g of potassium acetate (*ca.* 6 g/L), the culture began exponential growth, which continued until the biomass reached 8 g/L dry cell weight (DCW) at approximately 124 h (Figure 1A). Growth and accumulation–depletion rates for all extracellular metabolites during this period were severely attenuated as compared to the control fermentation (ca. 65–75% reduction), but followed profiles similar to those observed in the control fermentation (14). At the end of the exponential growth stage (phase I), lysine production commenced and biomass continued to increase to 10.3 g/L DCW (120–124 h), which resembles the phase II (rapid growth and lysine production) characteristics of the control fermentation. At the end of phase II, growth entered a stationary period, during which lysine synthesis continued (phase III, 124–131 h). The culture then progressed into a slow death phase, where lysine synthesis diminished with time (phase IV). During phase III, 50 g of potassium acetate was added to stimulate growth, but it had little effect other than on respiration (Figure 1B). Although a lysine hydrochloride titer only reached 12.6 g/L, the lysine yield averaged 34% during phases II and III (based on glucose), which is a slight improvement over the control fermentation (see flux analysis below). Unlike the control fermentation, trehalose

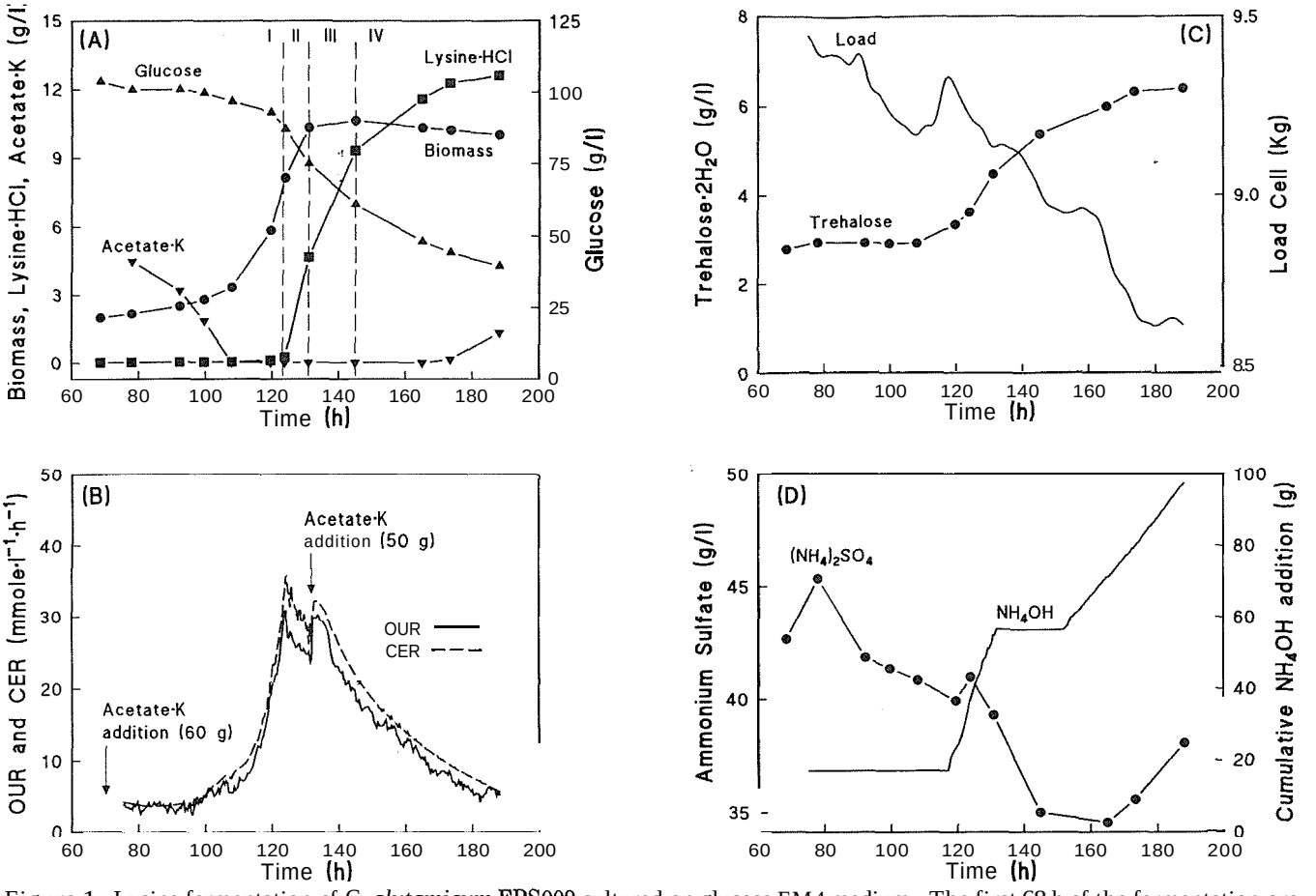

Figure 1. Lysine fermentation of C. *glutamicum* FPS009 cultured on glucose FM4 medium. The first 68 h of the fermentation are not shown (linear growth). At 70 and 132 h, 60 and 50 g, respectively, of potassium acetate were added aseptically. (A) Glucose, biomass, lysine hydrochloride, and potassium acetate concentrations; (B) oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER); (C) medium weight (load) and trehalose·$2H_2O$; (D) available ammonium in medium, as $(NH_4)_2SO_4$, and cumulative amount of NH_4OH (26% w/w NH_3) added to maintain culture at pH 7.0 during the course of the fermentation.

was the only byproduct observed during the fermentation (Figure 1C).

Flux Analysis of FPS009. Although acetate was added twice during the fermentation, flux distributions could still be calculated without the complications associated with the glyoxylate shunt, since glucose has been shown to repress the glyoxylate shunt even in the presence of acetate (14). To examine glucose catabolism uncomplicated by acetate consumption, metabolite accumulation rates were calculated during phase II from measurements taken at 124 and 131 h (t_{av} = 127.5 h), during which time acetate was not present. The measured rates are listed in Table 2 along with the estimated rates and the consistency index. Specific rates are reduced to approximately one-half to one-third of the magnitude of the control fermentation. However, the resulting flux distribution map (Figure 2) is very similar to that observed in the control fermentation during late phase II. Furthermore, flux leading toward lysine synthesis at the Pyr branch point (29% of the Pyr synthesis flux) is the same as that observed in the control fermentation. Therefore, the 98% attenuation of PDC activity did not result in flux redistribution at any of the three principal branch points, but it did result in flux attenuation in the overall network.

If lysine yield were limited solely by a weakly rigid Pyr branch point (*i.e.*, pyruvate preferentially enters the TCA cycle, so that lysine synthesis is, in essence, pyruvate-limited), then PDC attenuation should have increased pyruvate availability and lysine yield. As this was not observed, it can be concluded that lysine yield is not limited solely by a weakly rigid Pyr branch point.

Table 2. Measured and Estimated Metabolite Accumulation Rates and Standard Deviations for the FPS009 Fermentation at 127.5 h from Measurements Taken at 123.9 and 131.0 h (Figure 1)[a]

	accumulation rates (mM/h)	
metabolites	measured	estimated[b]
acetate	0 ± 1	-0.04
alanine	0 ± 1	-0.07
biomass	3.09 ± 0.9	3.00
CO_2	27.5 ± 2.8	28.8
glucose	–9.48 ± 1.8	–9.83
lactate	0 ± 1	-0.05
lysine	3.41 ± 0.1	3.41
NH_3	–8.2 ± 6.1	–8.7
O_2	–26.3 ± 2.6	–25.0
pyruvate	0 ± 1	0.04
trehalose	0.32 ± 1	0.10
valine	0 ± 1	-0.29

[a] Consistency index: h = 0.66. [b] Estimated rates are those that exactly satisfy mass balance constraints as governed by the biochemistry (12).

Since each principal branch point in the lysine network (Glc6P, PEP, and Pyr) contributes a stoichiometrically consumed component for the synthesis of lysine (namely, NADPH, oxaloacetate, and pyruvate, respectively), the network is considered dependent (11). In a dependent network, the flux partition at all principal branch points must vary in unison to prevent the excretion of intermediate metabolites or their intracellular accumulation. If one or more of the principal branch points in a dependent network is rigid, then flux partitioning at all principal

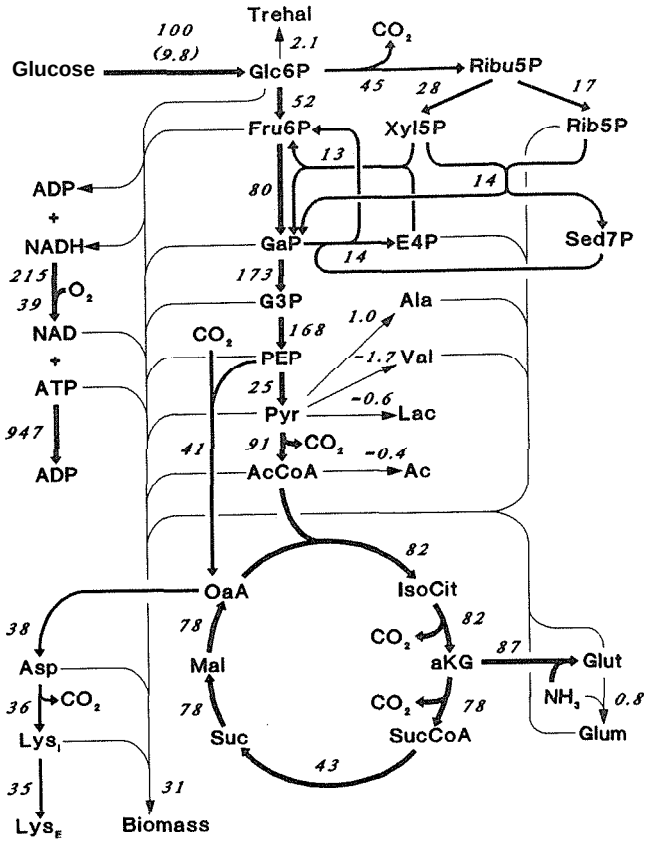

Figure 2. Flux distribution map for the C. glutamicum FPS009 lysine fermentation at 127.5 h. Fluxes were estimated from measurements taken at 123.9 and 131.0 h (phase II, see Figure 1 and Table 2) and normalized by the glucose uptake rate (shown in parentheses, mmol L^{-1} h^{-1}).

branch points cannot vary in unison to meet the stoichiometric needs of the product. Furthermore, if metabolic controls prevent the excretion or accumulation of intermediate metabolites, then the attenuation of any branch in a rigid, dependent network will result in overall flux attenuation in the network. Hence, the observed results from the attenuation of PDC are consistent with a dependent network that harbors a rigid branch point. Since the Glc6P branch point has already been identified as flexible, the rigidity in the network must be due to either a strongly or weakly rigid PEP branch point or a strongly rigid Pyr branch point. This conclusion was confirmed further by the following inhibition study.

FluoropyruvatePerturbation. Fluoropyruvate (FP) is a strong competitive inhibitor of PDC in B. *lactofermentum* (12) and is also found to inhibit PDC in C. glutamicum ATCC 21253, as illustrated in Figure 3. Although 1 mM FP inhibits 83% of PDC activity in the crude extract, it is not a perfect competitive inhibitor as it is slowly catabolized (*12*), which may explain the residual PDC activity at 10 mM FP (Figure 3). Nevertheless, PDC inhibition is certainly evident, and the specificity of the inhibitor allows for precise perturbation of the Pyr branch point without alteration of other enzymes that often occur in mutation studies. The effects of attenuating PDC activity during lysine production were monitored via flux analysis following the addition of FP.

FP Fermentation. A standard lysine fermentation of ATCC 21253 cultured on minimal glucose (FM4) medium was conducted; however, at the onset of lysine production (indicated by the telltale drop in respiration), 20 mmol of FP was added aseptically to inhibit PDC (Figure 4A–E).

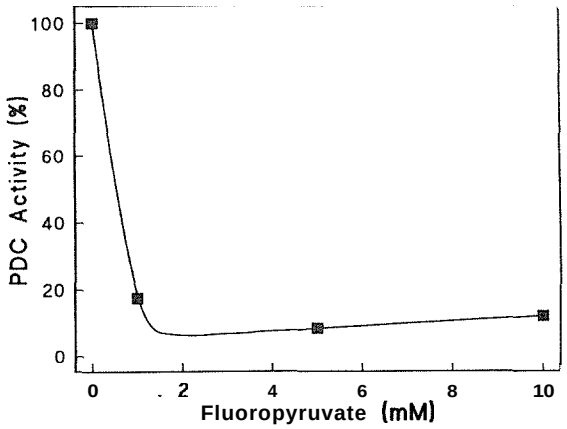

Figure 3. Effect of fluoropyruvate on the activity of the pyruvate dehydrogenase complex (PDC) in the crude cell extract of C. glutamicum ATCC 21253. The activity of PDC at 100% was 27.5 nmol min^{-1} (mg of protein)$^{-1}$. The PDC assay (12) contained 50 mM pyruvate and 2 mg/mL crude extract protein.

The resulting fermentor FP concentration (*ca.* 2.1 mM) was chosen on the basis of in uitro PDC inhibition by FP (Figure 3).

Immediately following the addition of FP, a dramatic drop in respiration occurred (Figure 4B), accompanied by a decrease in the growth rate (Figure 4A) and the accumulation of extracellular pyruvate (Figure 4C). Lysine synthesis and glucose consumption rates immediately following FP addition were unaffected (Figure 4A). However, a few hours after FP addition, respiration resumed, excreted pyruvate was reconsumed, and glucose uptake exhibited a transient decrease, which was probably due to pyruvate reconsumption. The temporary nature of the perturbation is undoubtedly due to the breakdown of FP, as mentioned above. The remainder of the fermentation exhibited characteristics similar to those of the control fermentation, except that extracellular pyruvate accumulation reached higher levels, and the stationary period (phase III) was extended. The excretion of pyruvate also resulted in an elevated ammonium sulfate concentration due to the increase in ammonium hydroxide required to maintain the culture at pH 7.0 (Figure 4E). Although the lysine hydrochloride titer increased by 25% compared to the control fermentation (from 20 to 25 g/L), the instantaneous lysine molar yield during the 13–19-h period (phase II) was 30%, which is approximately the same as the control fermentation. Hence, the increase in lysine titer was due to the extension of lysine production (phase III) and not to an increase in yield, which is similar to what was observed in the fermentations conducted to examine the glucose 6-phosphate branch point (15). Consequently, the FP perturbation of the Pyr branch point did not result in lysine yield improvements.

FP Flux Analysis. Flux distributions were calculated from measurements taken at 13.0 and 14.0 h (phase II, during pyruvate accumulation) in order to capture the flux perturbations induced by the addition of FP. The measured and estimated metabolite accumulation rate vectors and the associated consistency index at t_{av} = 13.5 h are displayed in Table 3, where the high rate of pyruvate accumulation should be noted. The resulting flux distribution map (Figure 5) clearly illustrates the diversion of pyruvate from the TCA cycle (which now supports a reduced flux) to extracellular pyruvate excretion (Pyr_E), while the flux distributions in the remaining pathways appear relatively unaffected as compared to the control fermentation in late phase II. To highlight the effect of

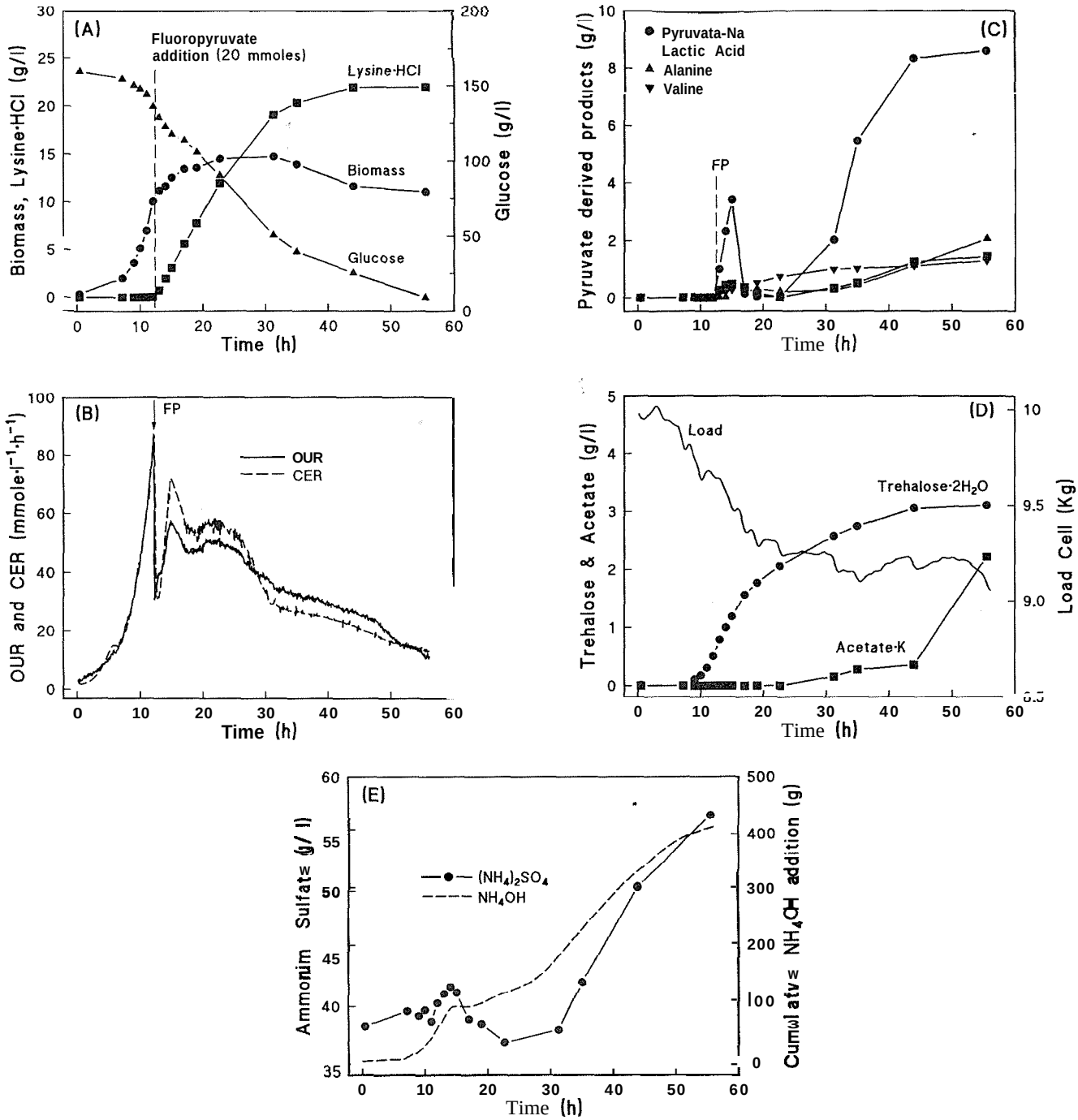

Figure 4. Fluoropyruvate (FP) inhibited lysine fermentation of C. glutamicum ATCC 21253 cultured on glucose FM4 medium. At 12.3 h, 20 mmol of sodium fluoropyruvate, dissolved in 30 mL of water, was added aseptically, as illustrated by the dashed lines. (A) Glucose, biomass, and lysine hydrochloride concentrations; (B) oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER); (C) concentrations of pyruvate-derived byproducts; (D) fermentor load (medium weight), trehalose, and potassium acetate concentrations; and (E) available ammonium in medium, as $(NH_4)_2SO_4$, and cumulative amount of NH_4OH (26% w/w NH_3) added to maintain culture at pH 7.0 during the course of the fermentation.

FP, detailed flux distributions around the PEP and Pyr branch points normalized by the PEP synthesis rate for the control fermentation during late phase II and the FP perturbed fermentation at t_{av} = **13.5** h are illustrated in Figure 6 (A and B, respectively). The inhibition of PDC by FP caused an approximately 50% reduction in the flux supported by PDC at the Pyr branch point, while flux distributions at the PEP branch point remained relatively unaffected.

The fluoropyruvate inhibition experiment can be viewed as the transient analog of the more permanent disruption of the TCA flux exhibited by the PDC-attenuated mutant. In the latter, glucose uptake and glycolytic and TCA fluxes were all reduced to one-third to one-half the wild-type magnitude, and no accumulation or secretion of intermediates was observed. Under fluoropyruvate inhibition, the normally high PDC flux was drastically reduced, forcing the excretion of pyruvate as an outlet for the glycolytic flux, which remained high even in the presence of fluoropyruvate. Changes in flux partitioning to favor the anaplerotic pathway, and hence oxaloacetate formation, were not observed in either perturbation. As a result, the lysine yield was unaffected despite the significantly higher availability of pyruvate for condensation with ASA in the dihydrodipicolinate synthase reaction. One can then conclude that lysine yield is not limited by the preferential

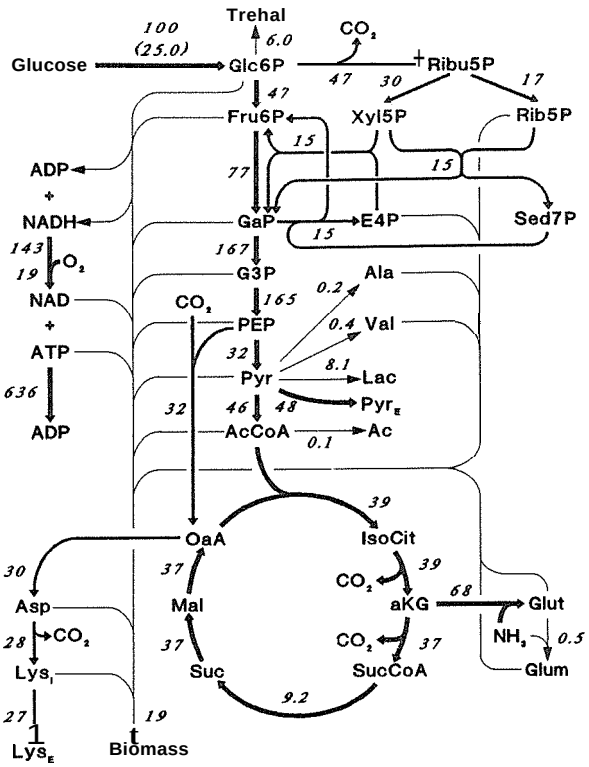

Figure 5. Flux distribution map for the fluoropyruvate-perturbed lysine fermentation of C. glutamicum ATCC 21253 at 13.5 h. Fluxes were estimated from measurements taken at 13.0 and 14.0 h (see Figure 4 and Table 3) and normalized by the glucose uptake rate (shown in parentheses, mmol L^{-1} h^{-1}). The extracellular pyruvate accumulation rate is illustrated by Pyr_E flux.

Table 3. Measured and Estimated Metabolite Accumulation Rates and Standard Deviations for the FP-Perturbed Fermentation at 13.5 h from Measurements Taken at 13.0 and 14.0 h (Figure 4)[a]

	accumulation rates (mM/h)	
metabolites	measured	estimated
acetate	0 f 2	0.02
alanine	0 f 2	0.04
biomass	5.0 ± 8.1	4.70
CO_2	41.0 f 4.1	42.2
glucose	–32.2 f 20	–25.0
lactate	2.0 f 2	2.0
lysine	6.86 ± 0.2	6.86
NH_3	–17.7 ± 44	–17.1
O_2	–41.4 f 4.1	–40.3
pyruvate	11.8 f 2	12.0
trehalose	0.6 f 2	0.7
valine	0.1 f 2	–0.1

[a] Consistency index: h = 0.31.

consumption of pyruvate by the TCA cycle, that is, the Pyr branch point is not weakly rigid.

Summary

Flux analysis of FPS009 (PDC^A mutant) indicates that lysine yield is not limited by pyruvate preferentially entering the TCA cycle over condensation with ASA, since the 98% reduction in PDC activity did not alter flux partitioning at the Pyr branch point. However, the observed reduction of all metabolic fluxes resulting from PDC activity attenuation is consistent with the hypothesis governing a dependent network that harbors one or more rigid branch points. Pyruvate excretion induced by PDC inhibition by FP also indicates that the lysine yield in ATCC 21253 is not limited by pyruvate availability and

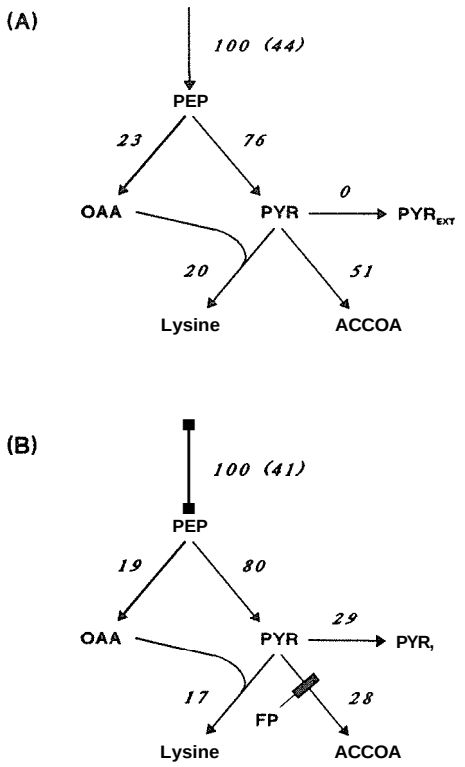

Figure 6. Flux distributions around the PEP and Pyr principal branch points normalized by the PEP synthesis rate (shown in parentheses in mmol L^{-1} h^{-1}) for (A) the control fermentation during late phase II (12) and (B) the fluoropyruvate (FP) perturbed fermentation at 13.5 h (extracted from Figure 5). The solid rectangle notes the sites of FP inhibition, and PYR_{ext} represents extracellular pyruvate. Not all fluxes involving PEP, oxaloacetate (OAA), and/or Pyr are illustrated.

supports the hypothesis that the Pyr branch point is not weakly rigid.

Perturbations of the Glc6P branch point, previously described (*15*), indicate that lysine yield is not limited by NADPH availability which, in conjunction with the results from this manuscript, indicates that oxaloacetate (OAA) is inadequately synthesized with respect to NADPH and Pyr availability. Net OAA synthesis is governed by anaplerotic reactions, of which only PEP carboxylase (PPC) was originally detected in *C. glutamicum*. However, in addition to PPC, the presence of PEP carboxykinase (PPCK) has recently been detected (*3*), and one could speculate about the possible existence of a membrane-associated Pyr carboxylase (PC), a malic enzyme, or a glyoxylate pathway among the possible mechanisms supplying OAA. At this time, it is unknown which of these anaplerotic enzymes supports the dominant flux to OAA during lysine overproduction, so that it is not possible to associate a particular branch point (PEP or Pyr) or mechanism to OAA synthesis or its regulation. But we can speculate. Although we have also recently detected PPCK in *C. glutamicum* ATCC 21253 (*3*), it is found to be active only near the end of a fermentation, which is to be expected for an enzyme typically associated with the gluconeogenesis pathway. This is true even for a strain in which PPC is deleted from the genome (*2*, *6*). If PC is present and supports a significant flux during lysine synthesis, then our results would indicate that it should have a control architecture similar to that of PPC (*11*), since attenuation of PDC would otherwise result in increased PC flux and lysine yield. Of course, restrictions in lysine excretion (*1*) can enhance metabolic rigidity if intracellular metabolites accumulate and feed back on the

primary metabolism, and such restrictions will certainly cap the lysine production rate.

We emphasize that the methodology of flux analysis coupled with metabolic perturbation, exemplified in both this article and its companion (*15*), is intended as a tool to facilitate the rational identification of enzymes, branch points, or subnetworks that may limit product synthesis. Due to the inherent complexity of metabolic networks and their incomplete and changing knowledge base, this approach, by itself, cannot identify metabolic limitations with 100% certainty. Nevertheless, it has been found by the authors to be quite useful as a tool to guide more detailed studies of flux partitioning at metabolic branch points.

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