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Growth Rate-Dependent Modulation of Carbon Flux through Central Metabolism and the Kinetic Consequences for Glucose-Limited Chemostat Cultures of *Corynebacterium glutamicum*

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The physiological behavior of *Corynebacterium glutamicum* in glucose-limited chemostat cultures was examined from both growth kinetics and enzymatic viewpoints. Metabolic fluxes within the central metabolism were calculated from growth kinetics and analyzed in relation to specific enzyme activities. At high growth rates, incomplete glucose removal was observed, and this was attributed to rate-limiting capacity of the phosphotransferase system transporter and the probable contribution of a low-affinity permease uptake mechanism. The improved biomass yield observed at high growth rates was related to a shift in the profile of anaplerotic carboxylation reactions, with pyruvate carboxylase replacing malic enzyme. Phosphoenolpyruvate carboxylase, an activity often assumed to be the major anaplerotic reaction during growth of *C. glutamicum* on glucose, was present at only low levels and is unlikely to contribute significantly to tricarboxylic acid cycle fuelling other than at low growth rates.

For several decades, *Corynebacterium glutamicum* and related species have been exploited industrially for the production of various amino acids. Certain of these bacteria excrete glutamic acid under specific nutritional limitations, e.g., biotin limitation or surfactant addition, and this tendency has been progressively accentuated until glutamate concentrations in excess of 100 $g \cdot liter^{-1}$ are now obtained from sugar-based feedstocks (13). Alongside this industrial-scale development of glutamate production, the discovery of other strains has enabled various other amino acids (notably, lysine [36]) to be produced. Such strains have frequently undergone extensive genetic improvements both to broaden the range of amino acids produced and to improve the yields (17). These strategies have often been based upon overturning the natural feedback regulation mechanisms specific to each biosynthetic pathway and have enabled a detailed understanding of these biochemical sequences to be established.

Further improvements will most likely involve metabolic engineering of sugar transport capacity and the central pathways, for which very little relevant information specific to the coryneform group exists. Rates of amino acid production will ultimately depend on the sugar transport capacity, (14) while yield improvements for specific amino acids require a correctly balanced supply of carbon precursor metabolites feeding the specific biosynthetic pathways and the appropriate coenzyme requirements. We do not have an adequate understanding of the control mechanisms that regulate carbon flux through these pathways (glycolysis, the pentose pathway, the tricarboxylic acid [TCA] cycle, and the associated anaplerotic reactions) in *C. glutamicum*. Certain central metabolism enzymes have been purified and characterized (particularly for *Brevibacterium flavum* by Shiio and coworkers [29, 30, 32, 33]), but until recently no coherent model of flux distribution within the catabolic network had been proposed. Pioneering work in this direction from the group of Stephanopoulous, who used matrix-based modelling techniques, has enabled a global overview of central metabolism to be established (35, 36). The predictions obtained by this group, notably, as concerns the carbon distribution between glycolysis and pentose phosphate pathways during batch fermentations of lysine-overproducing strains, are in good agreement with recent nuclear magnetic resonance studies (26, 37). This development opens the perspective for rational improvement of industrial strains to direct carbon flux towards specific pathways, but for this pathway engineering approach to be successful the physiological data used to establish the metabolic models must be solid. Since amino acid production is associated with slow (or stationary-phase) growth, one of the key areas for study is the manner in which the structure of the central pathways is modified in response to growth conditions.

Effort is required to understand which pathways are operative in defined nutritional environments to enable further work to focus on the potentially limiting metabolic nodes. Network analysis from the carbon flux and energetic viewpoints has already enabled identification of the anaplerotic reaction catalyzed by phosphoenolpyruvate (PEP) carboxylase (PPC) as an important locus of flux control (24, 36). However, such studies have overlooked the possible role of other anaplerotic enzymes known to exist in these bacteria under certain growth conditions (11, 20, 28). The growth of *C. glutamicum* ATCC 17965 at various rates in chemostat cultures, limited by the inflowing glucose concentration, was examined in this study. Kinetic analysis and enzymatic activity measurements were used together with energetic-growth-modeling techniques to shed new light on the manner in which sugar transport and the various

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anaplerotic enzymes contribute to the carbon flux pattern over a range of growth rates. The results obtained confirm the importance of the PEP and pyruvate nodes in this species but show that the biochemical reactions involved, particularly those feeding carbon into the TCA cycle, are more numerous than initially believed.

MATERIALS AND METHODS

Organism and cultivation. The microorganism used in this work was *C. glutamicum* ATCC 17965. Cultivation was done at 30°C in a 3.5-liter chemostat (Chemap) whose volume was maintained constant by a weight control system. The pH was maintained at 7.0 by automatic addition of KOH (10 N). The dissolved oxygen concentration never fell below 50% air saturation. During steady states, foaming was controlled by periodic addition of an antifoaming agent (Strucktol J633; Sofraret). The cultures were considered to be at steady state when all culture parameters (biomass, substrate, and product concentrations) reached constant values. Steady-state samples were taken over a period of several residence times, and average values obtained for both kinetic and enzymatic analyses showed less than 5% variation for each regimen.

The medium was prepared in 50-liter reservoirs and sterilized by filtration through membrane cartridges (0.22-μm pore size). Vitamins (sterilized by filtration through cellulose acetate membranes [0.22-μm pore size]) were added separately. The medium, based upon the data of Egli and Fiechter (8), contained nitrilotriacetic acid neutralized with NaOH (0.2 g/liter), deferoxamine (1 mg/liter), $FeSO_4 \cdot 7H_2O$ (30 mg/liter), $CuSO_4 \cdot 5H_2O$ (2 mg/liter), $ZnSO_4 \cdot 7H_2O$ (10 mg/liter), $MnSO_4 \cdot H_2O$ (10 mg/liter), $MgSO_4 \cdot 7H_2O$ (1.0 g/liter), NH_4Cl (4.0 g/liter), $CaCl_2 \cdot 2H_2O$ (50 mg/liter), KH_2PO_4 (3.0 g/liter), biotin (20 μg/liter), thiamine (2 mg/liter), and glucose (15 g/liter).

The composition of the medium used for thiamine limitation was the same as that described above, except for thiamine, whose concentration was diminished to 6 μg/liter.

Analytical methods. Biomass concentration was estimated by a direct gravimetric method following drying of the washed cells for 24 h under a partial vacuum (2×10^4 Pa) at 60°C. In this manner, potential errors in biomass estimation linked to growth rate-associated morphological changes likely to invalidate optical density measurement, were avoided. Glucose was measured enzymatically with an automatic analyzer (YSI 27; Bioblock Scientific). The presence of carbon metabolites within the culture filtrates was verified by high-pressure liquid chromatography (HPX87H column; Bio-Rad) with a refractometer for detection. Gas phase composition was determined by gas chromatography with a Porapak Q column maintained at 40°C with helium as the carrier gas and catharometer detection.

Enzyme assays. All enzymes were assayed by spectrophotometric measurement of variation in $NADH_2$ or $NADPH_2$ concentrations at 340 nm ($\varepsilon = 6.22$ M^{-1} cm^{-1}) or 2-(4-iodophenyl)-3-(4-nitrophenyl-5-phenyltetrazolium chloride (INT), an artificial electron acceptor, at 500 nm ($\varepsilon = 12.4$ M^{-1} cm^{-1}). One unit of enzyme activity was defined here as the amount of an enzyme which converted 1 nmol of the substrate per min. All enzymes were assayed at 30°C and pH 7.8 (intracellular pH during growth on glucose).

Except for the glucose phosphotransferase system (PTS), all enzymes were measured in crude cell extracts obtained by sonication. Crude extracts were prepared as follows. Cells were harvested, washed twice with 0.2% (wt/vol) KCl, and resuspended in Tris-tricarballylate buffer (270 mM, pH 7.8) containing $MgCl_2$ (4.5 mM) and 22% (vol/vol) glycerol. Cells were disrupted by sonication (six cycles of 30 and 60 s spaced out) and kept in ice during the treatment. Cell debris was removed by centrifugation for 15 min at $10,000 \times g$ and 4°C. The supernatant was used for enzyme assays, and the protein concentration of the extract was determined by the method of Lowry et al. (15) with bovine serum albumin as the standard. Endogenous oxidation of $NADH_2$ or spontaneous reduction of INT was systematically quantified to avoid overestimation of certain activities.

PEP carboxylase was assayed by a method based on that of Mori and Shiio (18) in a reaction mixture containing Tris-HCl buffer (pH 7.8, 100 mM), $MnSO_4$ (5 mM), $KHCO_3$ (10 mM), $NADH_2$ (0.15 mM), acetyl coenzyme A (0.1 mM), 10 μg of malate dehydrogenase per ml, and PEP (2 mM). The reaction was started by addition of PEP.

Malic enzyme was assayed by the method described by Mori and Shiio (20) with an optimized reaction mixture consisting of phosphate buffer (pH 7.8, 100 mM), $MgCl_2$ (5 mM), NADP (0.6 mM), and malate (40 mM). Assays were initiated by addition of malate. Malic enzyme was also assayed in the reverse direction in a medium containing phosphate buffer (pH 7.8, 100 mM), $MgCl_2$ (5 mM), $NaHCO_3$ (10 mM), $NADPH_2$ (0.3 mM), and pyruvate (20 mM). This reaction was started by addition of pyruvate.

Pyruvate dehydrogenase (PDH) was assayed by a method based on that of Hinman and Blass (9), in phosphate buffer (pH 7.8, 100 mM)–$MgCl_2$ (5 mM)–bovine serum albumin INT (0.6 mM) (1 g/liter)–lipoamide dehydrogenase (0.1 mg/liter)–dithiothreitol (0.3 mM)–coenzyme A (0.2 mM)–thiamine PP_i (0.2 mM)–NAD (2 mM)–pyruvate (5 mM). The assay was initiated by addition of pyruvate.

Pyruvate kinase was assayed by a method based on that of Ozaki and Shiio (24), in Tris-HCl buffer (pH 7.8, 100 mM)–$MnSO_4$ (5 mM)–KCl (100 mM)–ADP (10 mM)–$NADH_2$ (3 mM)–lactate dehydrogenase (10,000 U/ml)–PEP (2 mM). The reaction was started by addition of PEP.

Oxaloacetate (OAA) decarboxylase was assayed by measuring the formation of pyruvate by an optimized method derived from that described by Mori and Shiio (20). The reaction mixture contained Tris-HCl buffer (pH 7.8, 100 mM), $MnSO_4$ (5 mM), and OAA (10 mM). The reaction was started by addition of OAA and stopped by addition of 20 μl of 40% phosphoric acid to 250-μl aliquots of the mixture removed after various incubation periods. The pyruvic acid formed was measured immediately by using lactate dehydrogenase in a mixture containing Tris-HCl buffer (pH 7.8, 320 mM), $NADH_2$ (0.3 mM), and 10,000 U of lactate dehydrogenase per ml. Because of chemical degradation of OAA into pyruvate, a standard without extract was systematically prepared for this assay. This OAA decarboxylase activity was supposed to be, in reality, a pyruvate-carboxylating enzyme (see Results and Discussion); however, measurement of this reaction in the opposite direction with ATP in the assay was not possible because of the strong instability of OAA. Furthermore, attempts to couple this reaction to $NAD/NADH_2$ oxidoreduction in a continuous manner in either direction were unsuccessful (hence, the use of a stopped reaction procedure).

NADH oxidase was assayed by using a reaction mixture containing Tris-HCl buffer (100 mM), $MnSO_4$ (5 mM), and $NADH_2$ (0.6 mM). No activity was detected when $NADPH_2$ was used instead of $NADH_2$.

The glucose PTS was assayed on toluenized cells by a method based on that of Mori and Shiio (21). Cells were harvested (optical density at 650 nm, 175), washed twice with 0.2% (wt/vol) KCl, and resuspended in 5 ml of Tris-Cl buffer (pH 7.8, 100 mM) supplemented with 10% (wt/vol) polyethylene glycol 6000 and dithiothreitol (30 mM). The cells were permeabilized by addition of 200 μl of toluene followed by vigorous agitation for 1 min at room temperature. The cells were then washed twice with the Tris-HCl buffer and resuspended in 0.8 ml of the same buffer. The optical density at 650 nm of the extract was determined and used in the calculation of enzyme specific activity to avoid experimental errors due to the variable loss of cells during the washing procedure. The activity was assayed immediately under agitation in a thermostat-equipped room at 30°C. The reaction mixture contained Tris-HCl buffer (pH 7.8, 100 mM), PEP (10 mM), $MgCl_2$ (5 mM), dithiothreitol (30 mM), and glucose (10 mM). The assay was started by addition of glucose after incubation of the cells (10 min at 30°C) in the mixture. The reaction was stopped by addition of 40% (vol/vol) phosphoric acid (20 μl) to 250-μl aliquots of the mixture removed after various incubation periods. Pyruvic acid formed was measured after removal of precipitated matter by centrifugation by using the protocol described above for OAA decarboxylase.

Macromolecular cell composition. Washed cells harvested from exponential-phase batch cultures growing on glucose were used to determine the macromolecular composition (as percent mass of dry cell weight) of *C. glutamicum* by using previously published analytical methods (total protein content [15], 52%; RNA [27], 5%; DNA [3], 1%; lipids [2], 13%; cell wall components [2], 19%). Ash content was determined to be 10% by weight measurement after heating of dried cells for 8 h at 500°C. Amino acid composition (expressed as micromoles per gram of dry cell weight) was as follows: alanine, 725; arginine, 190; aspartate, 187; asparagine, 187; cysteine, 1; glutamate, 486; glutamine, 486; glycine, 344; histidine, 63; isoleucine, 175; leucine, 262; lysine, 171; methionine, 5; phenylalanine, 123; proline, 148; serine, 265; threonine, 270; tryptophane, 1; tyrosine, 74; valine, 262. These values were obtained as described by Chang et al., (4) while the method of Anderson (1) was used to assay nucleotide concentrations.

Metabolic fluxes. Metabolic fluxes within the central metabolic network were estimated by using the stoichiometric approach described by Ingraham et al. (10) for *Escherichia coli* and as more recently applied to *C. glutamicum* by Vallino and Stephanopoulos (36). Carbon metabolite requirements for cell growth and the associated coenzyme demand (Table 1) were calculated from the experimentally determined macromolecular cell composition of *C. glutamicum* (see above) by using known anabolic pathways. Experimental kinetic data were used to calculate the specific rates (substrate consumption rates, product accumulation rates, and growth rate) used as inputs for the model. Outputs of biomass constituents were determined by multiplication of the anabolic demand by the specific growth rate. The carbon flux at each step of the pathway was determined for each biochemical reaction by assuming that metabolite pools did not vary within steady-state cultures. Kinetic and stoichiometric data, together with the $NADPH_2$ supply-and-demand balance, were used to determine possible carbon distribution patterns through the various pathways.

Chemicals and commercial enzymes. Chemicals and enzymes were purchased from Sigma Chimie and chosen for maximum purity.

RESULTS AND DISCUSSION

Kinetics of glucose-limited chemostat cultures. The growth of *C. glutamicum* was investigated in carbon-limited chemostat cultures for a range of dilution rates between 0.1 and 0.55 h^{-1} (maximum growth rate, 0.59 h^{-1} in batch cultures). The steady-state regimens and the specific rates and fermentation yields are represented in Fig. 1 and 2. At rates up to 0.33 h^{-1},

TABLE 1. Intermediate metabolites and energy requirements for the formation of 1 g of biomass of the bacterium *C. glutamicum* during exponential growth on glucose[a]

Precursor	Concn (mmol/g)
Glucose 6-phosphate	0.32
Fructose 6-phosphate	0.13
Ribose 5-phosphate	0.68
Erythrose 4-phosphate	0.20
Triose phosphate	0.06
3-Phosphoglycerate	0.87
PEP	0.46
Pyruvate	2.81
Acetyl coenzyme A	3.12
α-Ketoglutarate	1.39
OAA	1.17
ATP	29.20
$NADPH_2$	11.51
$NADH_2$	0.39

[a] Biomass composition is known to vary with respect to growth rate, but the modifications are small over the range of specific growth rates used here and do not significantly modify the precursor requirements.

cell growth was strictly carbon limited with no detectable residual glucose. For such steady states, the biomass concentrations in the fermentor, and hence the fermentation yields, remained constant while specific rates of substrate consumption (both glucose and O_2) and CO_2 production evolved as linear functions of the dilution (growth) rate.

At higher growth rates (>0.33 h^{-1}), incomplete use of glucose (but no proportional decrease of biomass concentration) was observed. The amount of residual glucose remaining in steady-state cultures progressively increased as a function of the growth rate. Supplementing the inflowing medium with various components of the medium did not modify the steady-state parameters, indicating that the appearance of residual glucose could not be attributed to a change in the growth-limiting nutrient. At these growth rates, specific rates of glucose consumption were no longer a direct function of the growth rate and tended towards a limit value. A significant enhancement of the growth yield from 0.55 to 0.61 $g \cdot g^{-1}$ was observed during this period, and as expected, both specific rates of CO_2 production and the proportion of glucose catabolized to CO_2 diminished by a corresponding amount. Oxygen consumption rates showed a similar profile (the respiratory quotient remained constant at 1), leading to increased biomass yields relative to oxygen at high growth rates. Values obtained for exponential growth rate, (0.59 h^{-1}) batch growth are given in Fig. 2 and indicate that biomass yields continued to increase at growth rates higher than those investigated under chemostat conditions.

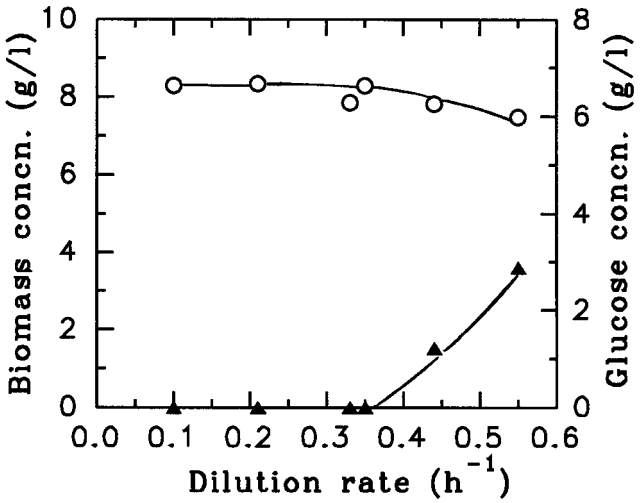

FIG. 1. Residual glucose (▲) and biomass (○) concentrations at various dilution rates glucose-limited chemostat cultures of *C. glutamicum*.

Glucose consumption and uptake system(s). The transport system generally believed to be responsible for glucose uptake in *C. glutamicum* is a PTS which has already been demonstrated in *B. flavum* (21), a microorganism taxonomically close to *C. glutamicum*. The presence of a glucose-transporting PTS was confirmed here, although the activity profile did not follow the observed rates of glucose consumption for the various steady states (Table 2). A constant PTS activity, independent of growth rate, was found for all steady states except that at the lowest growth rate (0.1 h^{-1}), for which PTS activity was significantly higher. Specific PTS transport activity measured in vitro with permeabilized whole cells was higher than actual glucose uptake rates for growth regimens up to (and including) that established at a dilution rate of 0.33 h^{-1}. At higher growth rates, measured levels of PTS activity were lower than the observed specific glucose consumption rates and significant quantities of glucose accumulated in the medium. Such evidence has previously been interpreted as being indicative of the presence of another glucose transporter(s) (22, 23), e.g., glucose permease. Glucokinase activity has been reported for these bacteria (21), and it has been suggested that this might indicate permease activity, although no evidence exists to support this hypothesis. The glucose uptake kinetics presented here support this hypothesis and indicate that such a secondary transport system could represent up to 15% of the global glucose transport in *C. glutamicum* at high growth rates. Since this additional glucose transport mechanism was associated with rapid growth under conditions of significant glucose excess, it would be logical if the permease activity were of the low-affinity type. While mechanistic details require further biochemical study, the results obtained in the present study indicate that at high growth rates, substrate transport is rate limiting.

From an industrial point of view, this rate limitation is unlikely to be important since the production phase for amino acid overproduction is generally at low growth rates or during the stationary phase. Under such conditions, the uptake capacity of the cells is unlikely to be limiting but this potential bottleneck should be borne in mind if growth-coupled production is envisaged. However, the contribution of a permease transporter in addition to the PTS mechanism may have important consequences for the flux distribution at the level of PEP. This metabolic node is considered to be yield limiting for the production of amino acids synthesized from TCA cycle intermediates (37) because of inadequate availability of PEP for carboxylation reactions once the requirements for PTS transport have been allowed for. However, a contribution of only 10% of sugar uptake by the permease system would be sufficient to relax this rigid node and hence modify the site of possible genetic engineering strategies.

If *C. glutamicum* has retained this additional glucose uptake capacity, presumably some benefit is derived. Any significant contribution to sugar uptake can only be obtained in sugar excess conditions, although such conditions will be encountered periodically in natural environments. Selective pressure will be active in glucose-rich niches to favor those strains able to take up and metabolize the available sugar most rapidly. It

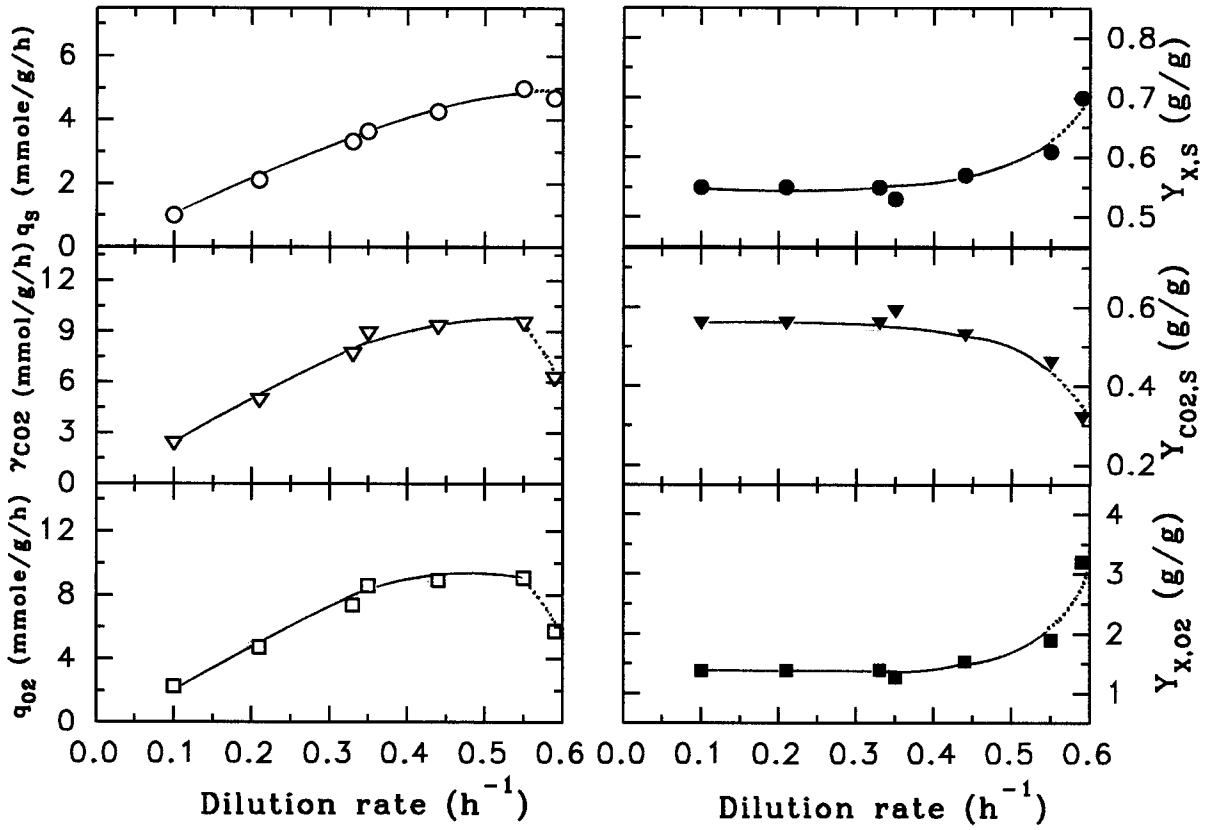

FIG. 2. Yields and specific rates for steady-state glucose-limited chemostat cultures of *C. glutamicum*. Specific rates of glucose consumption (○), CO_2 production (▽), and O_2 consumption (□) and yields of biomass (●) and CO_2 (▼) relative to consumed glucose and of biomass (■) relative to O_2 consumption are shown. The data given for a dilution rate of 0.59 h^{-1} are those obtained during exponential-phase batch cultures growing on identical glucose medium.

is often overlooked that substrate excess conditions stimulate metabolic adaptation to enable efficient exploitation of the available substrate and that bacteria possessing secondary substrate uptake mechanisms have a potential evolutionary advantage (7). In *C. glutamicum*, this additional sugar uptake mechanism has a double effect: not only are rate-limiting reactions improved, but it appears that the additional carbon flux is channelled predominantly into the pentose pathway with an ensuing gain in anabolic reducing power, which enables more efficient carbon substrate transformation into biomass. One might ask why nature has not provoked the development of a more efficient permease to further increase the competitiveness of this strain. When the lactose permease was examined in *E. coli*, this was postulated to be due in part to the general increase in permeability that this may provoke and potentially negative secondary effects (7). An alternative explanation may be that the general metabolic capacity of the central pathways is close to saturation and that additional substrate uptake could not be adequately catabolized.

Anaplerotic reactions. The literature describes various enzymes responsible for (de)carboxylation reactions within *C. glutamicum* and related species, such as malic enzyme and

TABLE 2. Measured enzyme activities and supposed flux through some reactions based on growth kinetic data and stoichiometric modeling during glucose-limited growth of *C. glutamicum*

Enzyme	Activity in mmol/g/h (calculated flux in mmol/g/h)[a] at growth rate (h^{-1}) of:					
	0.10	0.21	0.33	0.44	0.55	0.59
Glucose PTS	6.0 (1.0)	3.9 (2.1)	4.0 (3.3)	4.1 (4.3)	4.1 (5.1)	4.0 (4.7)
Pyruvate kinase	21 (0.3)	16 (1.1)	11 (1.7)	13 (2.2)	13 (2.4)	ND[c] (2.3)
PDH[b]	0.8 (1.0)	0.7 (2.1)	1.6 (3.3)	1.3 (4.1)	0.9 (4.4)	ND (3.2)
PPC	1.0	0.4	0.4	0.4	0.5	0.4
Malic enzyme	0.2	1.4	1.2	0.8	0.5	0.5
OAA decarboxylase (anaplerotic fueling)	8.3 (0.3)	8.8 (0.5)	7.9 (0.84)	11.3 (1.1)	12.4 (1.4)	15.8 (1.5)

[a] Measured activities were the means for at least three independant samples measured in triplicate, and scatter was always less than 5%. Mean values were obtained throughout the exponential growth period under batch conditions with medium identical to that used for chemostat cultures.
[b] Reaction measured in vitro with the artificial electron acceptor INT.
[c] ND, not done.

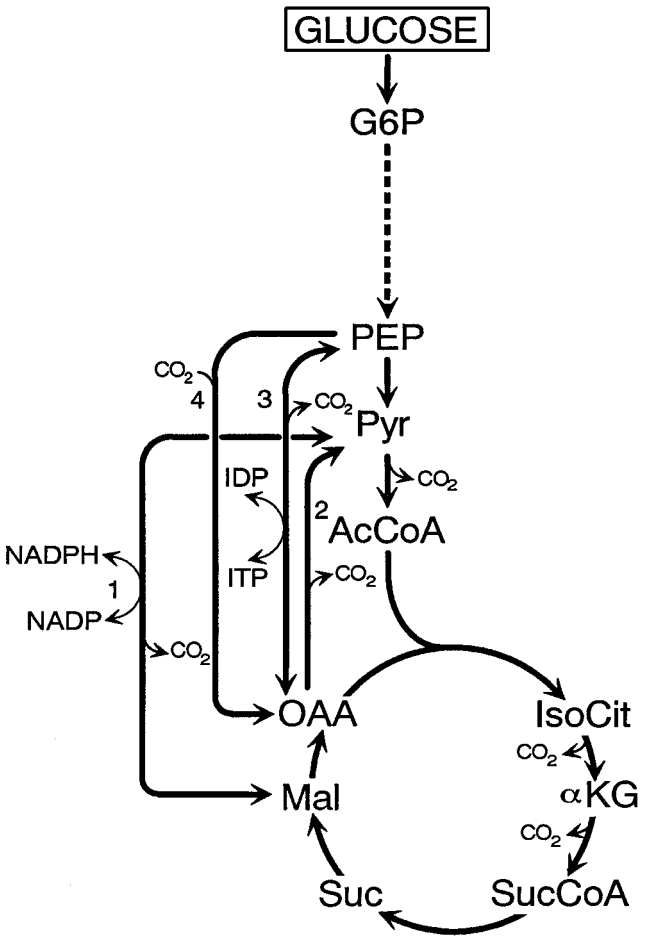

FIG. 3. Enzymes responsible for (de)carboxylation reactions within *C. glutamicum*. 1, malic enzyme; 2, OAA decarboxylase; 3, PEP carboxykinase; 4, PPC; G6P, glucose 6-phosphate; PYR, pyruvate; AcCoA, acetyl coenzyme A; SucCoA, succinyl coenzyme A; αKG, α-ketoglutarate; IsoCit, isocitrate; Suc, succinate; Mal, malate; IDP, inosine diphosphate; ITP, inosine triphosphate.

OAA decarboxylase (19) or PEP carboxykinase (11) (Fig. 3). Despite this, most investigators seem to believe that PPC is the only anaplerotic carboxylation reaction that plays a significant role in TCA cycle refuelling. The PPC activity measured in cell extracts in this study was 0.40 mmol · g^{-1} · h^{-1}, other than at the lowest growth rate examined, for which slightly higher values were obtained (Table 2). This level of specific activity is approximately 10-fold lower than that reported for other strains of *C. glutamicum* (or related bacteria) (19, 36). While it is possible that these groups did not take into account endogenous NADH oxidase activity, which interferes with the assay, and hence overestimated PPC specific activity, it should be noted that these workers were examining strains used for overproducing aspartate or lysine and not glutamate. The net flux through the anaplerotic reactions fuelling the TCA cycle can be precisely estimated from the growth rate and the requirements for OAA and ketoglutarate for macromolecule synthesis (Table 1). The activity of PPC at all but the lowest growth rates was inadequate to meet this anabolic demand (Table 2), even when assayed in the presence of acetyl coenzyme A, an activator of PPC activity (19). Furthermore, recent work using *ppc* mutant strains of *C. glutamicum* has shown that deletion of PPC activity does not modify exponential-growth behavior under batch conditions (25). We conclude from these results that PPC does not play an important role in fuelling the TCA cycle during rapid growth but that this enzyme may play an important role during amino acid production at relatively low growth rates.

In view of this apparent insufficiency as regards PPC activity, the activity profiles of other anaplerotic enzymes were examined (Table 2). Recently, PEP carboxykinase activity has been reported (11), although we were unable to detect this activity in our strain when similar experimental procedures were employed. Furthermore, this enzyme, which is strongly inhibited by ATP, is normally considered to have only a gluconeogenic function and is unlikely to have a role in PEP carboxylation during growth on sugars (11). An NADP-dependent malic enzyme was expressed at high levels in cells obtained at growth rates higher than 0.1 h^{-1} and may replace PPC as the predominant anaplerotic reaction. However, at growth rates above 0.2 h^{-1}, the specific activity of malic enzyme diminished linearly with the growth rate and became insufficient to meet the anaplerotic flux at 0.33 h^{-1}. A further enzyme involved in (de)carboxylation reactions (OAA decarboxylase) was apparently induced to higher levels of expression at this growth rate. It would be more coherent if the increased activity of this enzyme measured as OAA decarboxylase in reality masked a pyruvate-carboxylating enzyme, whose activity replaced malic enzyme as the major anaplerotic enzyme at growth rates higher than 0.33 h^{-1}. Attempts to confirm this in vitro were not successful, although this was believed to be due to technical reasons (see Materials and Methods). However, such a biotin-dependent pyruvate carboxylase activity has been detected in other glutamic acid bacteria (*B. thiogenitalis* [12] and *B. lactofermentum* [34]).

The proposed cascade of anaplerotic reactions from PPC to malic enzyme and then pyruvate carboxylase involved a substrate shift from PEP to pyruvate above 0.1 h^{-1}. The carboxylation reactions fuelling the TCA cycle seem, therefore, to be derived increasingly from pyruvate as the growth rate is increased. The presence of high pyruvate kinase activity in addition to the PTS reaction of pyruvate formation from PEP is largely sufficient to satisfy the additional flux towards pyruvate (Table 2). Flux distribution at the level of pyruvate requires control mechanisms which regulate carbon flux between further catabolism via the PDH and anabolic reactions, which include a variable flux through the pyruvate-carboxylating anaplerotic reactions. Specific activity of PDH diminished at growth rates above 0.33 h^{-1}, although the calculated flux through this enzyme (obtained by metabolic network modelling) continued to increase slightly (Table 2). This fall in specific activity was seen to be due to a maximum rate of PDH synthesis of 10 U · mg^{-1} · h^{-1} at such high growth rates (Fig. 4), as has previously been observed in lactate-grown *C. glutamicum* (5). Furthermore, this maximal rate of enzyme synthesis could not be increased by imposing a thiamine limitation, as has been observed for other microorganisms (6), although increased PDH concentrations were observed under such conditions at lower growth rates. In view of the lack of allosteric effectors (other than $NADH_2$) for the PDH of gram-positive bacteria (16, 31), the catabolic flux will be controlled by the enzyme concentration and the pyruvate pool concentration. The enzyme concentration profile is such that the pyruvate pool would logically increase as a function of the growth rate, possibly inducing the shift in anaplerotic reactions. This would be particularly true for the growth regimens at dilution rates of >0.33 h^{-1} and is consistent with the characteristic odor of diacetyl detected in the effluent gas stream of such cultures.

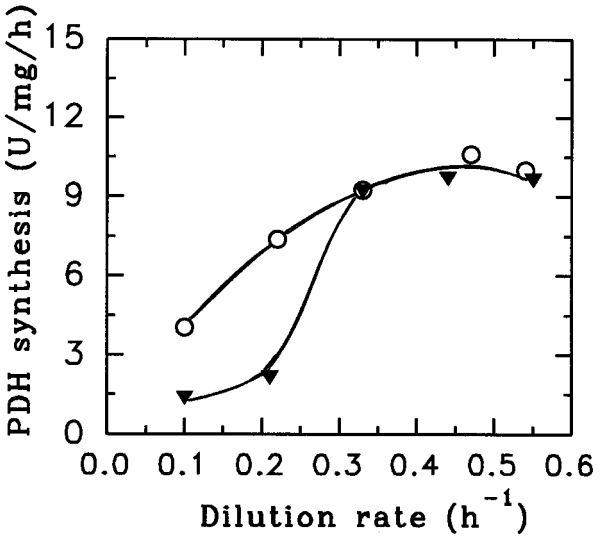

FIG. 4. Specific rates of PDH synthesis as calculated from enzyme specific activities for glucose-limited (▼) and thiamine-limited (○) chemostat cultures at various dilution rates.

Biomass yields and growth energetics. The observed modifications of anaplerotic enzyme concentrations was examined in light of the modified biomass yields. The general consensus is that growth of *C. glutamicum* is limited by the availability of $NADPH_2$ for anabolic reactions. This is believed to be due to the absence of NADH:NADP transhydrogenase (35) in this bacterium and explains the high flux reported through the pentose pathway under conditions of active growth (36). In this context, the progressive increase in biomass yields observed at high growth rates indicates that the metabolism of *C. glutamicum* was modified such as to more efficiently produce the necessary $NADPH_2$. Furthermore, this hypothesis requires that other forms of biochemical energy continue to be produced in adequate amounts.

The proposed cascade of anaplerotic reactions was used to predict carbon flux distribution within the central metabolism. Metabolic fluxes were calculated by matching $NADPH_2$ production to growth rate-dependent requirements (so as to avoid either a deficit or accumulation). A flux of approximately 36% of available glucose 6-phosphate into the pentose pathway was seen to be sufficient for all of the chemostat steady states (Table 2), although a significantly higher flux (58%) was necessary at the maximal rate of exponential growth obtained in batch cultures (Fig. 5). This flux distribution during exponential growth has recently been confirmed with the same strain in batch cultures grown on glucose (26). Furthermore, these values are consistent with those estimated by Stephanopoulos (36), who predicted a value of 54% during exponential growth but 36% during the decelerating growth phase associated with lysine production. Thus, the proposed growth rate-dependent cascade of anaplerotic reactions would enable adequate production of $NADPH_2$ for biomass requirements without necessitating further modifications of the flux distribution model. Under such conditions, high biomass yields observed above 0.33 h^{-1} were directly related to the gain of $NADPH_2$ resulting from the progressive shift from malic enzyme (enzyme $NADPH_2$ dependent) to pyruvate carboxylase. Additional increase of growth yield at the maximum specific growth rate was due to a modified distribution of carbon flux in favor of the pentose pathway. An alternative source of $NADPH_2$ generation involving concerted action of malic enzyme and ensuing carboxylation of either pyruvate or PEP to replenish the TCA cycle at the level of OAA has been proposed during growth of this bacterium on lactate (5). However, the good agreement between predicted flux and nuclear magnetic resonance analysis as regards the distribution of carbon between glycolysis and the pentose pathway, together with the sequential rather than simultaneous increase in expression of the anaplerotic enzymes, argues against such a role for malic enzyme during growth on glucose. It appears that *C. glutamicum* possesses two strategies to generate the $NADPH_2$ necessary for biomass synthesis: substrates entering the central metabolism upstream of glucose 6-phosphate (the glycolysis-pentose pathway branch point intermediate) direct a high proportion of their carbon through the pentose pathway, while substrates feeding carbon into the central pathways downstream of this metabolic node use anaplerotic reactions allowing effective conversion of $NADH_2$ to $NADPH_2$ in the absence of transhydrogenase.

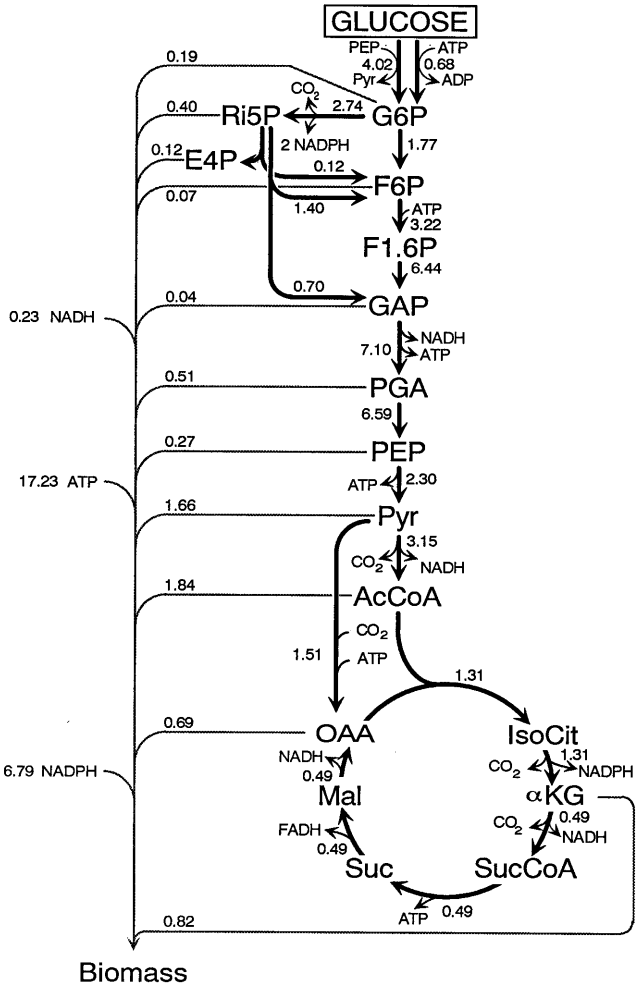

FIG. 5. Carbon flux distribution within central metabolic pathways based on the kinetic data of an exponential-growth batch culture (maximum growth rate, 0.59 h^{-1}). F6P, fructose 6-phosphate; F1.6P, fructose-1,6-diphosphate; GAP, glyceraldehyde-3-phosphate; PGA, phosphoglycerate; Ri5P, ribose 5-phosphate; E4P, erythrose 4-phosphate; FADH, flavin adenine dinucleotide. For other abbreviations, see the legend to Fig. 3.

Of course, such reasoning as regards $NADPH_2$ implies that other forms of biochemical energy are nonlimiting, particularly since a shift of malic enzyme to pyruvate carboxylation would

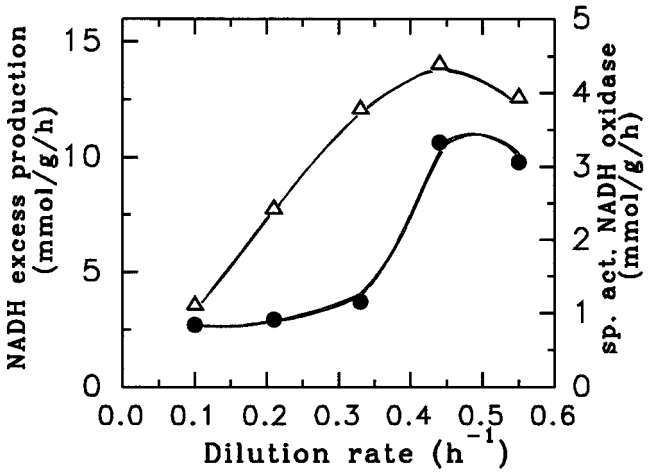

FIG. 6. NADH oxidase specific activity (●) and apparent excess production of $NADH_2$ (△) assuming a P/O ratio of 2 for various glucose-limited chemostat cultures.

increase the ATP demand. Furthermore, the diminished amount of CO_2 produced per unit of glucose consumed would lead to decreased energy production at the same time as biomass yield increased. In view of this, the potential ATP production was examined for all cultures and compared with the theoretical ATP requirement (29.2 mmol · g of cells^{-1}). A large excess was observed, although neither the exact amount of ATP produced per unit of $NADH_2$ via respiration nor the real requirements outside strict anabolic pathways are known. In addition, the specific activity of NADH oxidase, detected in all extracts, varied relative to the dilution rate but was always proportional to the apparent excess of biochemical energy (Fig. 6). It seems logical that organisms unable to interconvert the reserves of anabolic and catabolic reducing equivalents should possess such an enzyme which effectively enables the modulation of respiratory ATP yield, thus ensuring correct coenzyme turnover without unnecessary carbon waste or excessive ATP production.

The descriptive analysis of enzyme levels and growth kinetics on glucose presented here indicates that central metabolism of *C. glutamicum* is considerably more complex than generally believed. Furthermore, this metabolism certainly has little in common with the general textbook overview of bacterial catabolic pathways based mainly on enteric bacteria. While the basic pathways remain intact, the characteristics of the constituent enzymes and the genetic regulation have evolved differently. Future studies should concentrate on exploring the control mechanisms involved so as to further enhance the industrial potential of these unusual bacteria.

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