Anaerobic Growth of *Corynebacterium glutamicum* via Mixed-Acid Fermentation

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Corynebacterium glutamicum, a model organism in microbial biotechnology, is known to metabolize glucose under oxygen-deprived conditions to L-lactate, succinate, and acetate without significant growth. This property is exploited for efficient production of lactate and succinate. Our detailed analysis revealed that marginal growth takes place under anaerobic conditions with glucose, fructose, sucrose, or ribose as a carbon and energy source but not with gluconate, pyruvate, lactate, propionate, or acetate. Supplementation of glucose minimal medium with tryptone strongly enhanced growth up to a final optical density at 600 nm (OD_{600}) of 12, whereas tryptone alone did not allow growth. Amino acids with a high ATP demand for biosynthesis and amino acids of the glutamate family were particularly important for growth stimulation, indicating ATP limitation and a restricted carbon flux into the oxidative tricarboxylic acid cycle toward 2-oxoglutarate. Anaerobic cultivation in a bioreactor with constant nitrogen flushing disclosed that CO_2 is required to achieve maximal growth and that the pH tolerance is reduced compared to that under aerobic conditions, reflecting a decreased capability for pH homeostasis. Continued growth under anaerobic conditions indicated the absence of an oxygen-requiring reaction that is essential for biomass formation. The results provide an improved understanding of the physiology of *C. glutamicum* under anaerobic conditions.

Corynebacterium glutamicum is a Gram-positive soil bacterium belonging to the order *Corynebacteriales* within the *Actinobacteria* [1, 2]. It is used for the industrial production of the bulk products L-glutamate and L-lysine (3.0 and 2.2 million tons per year, respectively) [3] and of several other amino acids with a lower market volume. *C. glutamicum* has become a model organism in microbial biotechnology, and it was shown that a variety of other products besides amino acids can be efficiently synthesized with strains of this species, such as organic acids, diamines, or biofuels [4–7]. Therefore, *C. glutamicum* has become a platform organism for white biotechnology [8–11].

C. glutamicum is nowadays described as a facultative anaerobic organism, based on studies showing that the type strain ATCC 13032 as well as strain R can grow under anoxic conditions when nitrate is present as terminal electron acceptor [12, 13]. Negligible growth was observed in the absence of nitrate. Nitrate is reduced by the membrane-bound nitrate reductase NarGHIJ to nitrite, which accumulates in the medium under strictly anoxic conditions, as *C. glutamicum* does not possess a nitrite reductase [14]. Due to the toxicity of nitrite [15], anaerobic growth of *C. glutamicum* by nitrate respiration in axenic culture is poor [12, 13]. Nitrate is also reduced to nitrite under oxygen-limited conditions, but in this case nitrite can be partially metabolized again [16].

Under oxygen-limited conditions in the absence of nitrate, *C. glutamicum* was found to convert glucose to lactate, succinate, and acetate, which are excreted into the medium [17, 18]. This type of metabolism was reported not to be associated with growth and was used to develop efficient strains for the production of, e.g., succinate [19, 20], D-lactate [21], isobutanol [22], or L-valine [23]. For the corresponding production processes, aerobically grown cells are packed at high cell density under oxygen-deprived conditions, where they remain metabolically active and produce and excrete the products mentioned above. Analysis of the carbon flux under anaerobic conditions in a 100% argon atmosphere by ^{13}C nuclear magnetic resonance (NMR) showed that 95% of the glucose is metabolized in glycolysis and 5% in the pentose phosphate

pathway [24]; 97% of the succinate was formed in the reductive tricarboxylic acid (TCA) cycle and only 3% in the oxidative TCA cycle, with the latter providing reducing equivalents required for the redox balance [24]. Replacement of the argon atmosphere with a 100% CO_2 atmosphere increased the succinate yield about 3-fold to ~0.9 mol/mol glucose and the acetate yield about 2-fold to ~0.3 mol/mol, while the lactate yield was decreased about 3-fold to ~0.4 mol/mol [24]. Gene expression studies revealed that several genes involved in glycolysis, lactate formation, C_3 carboxylation, and the reductive TCA cycle were upregulated under oxygen deprivation, such as *tpi*, *gapA*, *pgk*, *ldhA*, *ppc*, and *mdh*, whereas genes of the oxidative TCA cycle were downregulated [25]. In support of these data, the activities of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), NAD^+-dependent lactate dehydrogenase, and NAD^+-dependent malate dehydrogenase (MDH) were found to be increased under oxygen-deprived conditions compared to aerobic conditions [26].

Recently, we described a mutant of *C. glutamicum* in which the branched aerobic respiratory chain is nonfunctional due to the deletion of the *qcrCAB* genes for the cytochrome bc_1 complex and of the *cydAB* genes for cytochrome *bd* oxidase [27]. This DOOR strain (mnemonic for "devoid of oxygen respiration") showed very poor growth under aerobic conditions in glucose minimal

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
C. glutamicum		
ATCC 13032	Biotin-auxotrophic wild-type strain	75
AtpG-S273P	ATCC 13032 derivative with a point mutation in the *atpG* gene (γ-subunit of the F_1F_o-ATP synthase) leading to a Ser273Pro exchange	This work, based on reference 73
ΔF_1F_o	ATCC 13032 derivative with an in-frame deletion of the *atpBEFHAGDC* genes	31
E. coli DH5α	F⁻ φ80*dlac*Δ(*lacZ*)M15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17* (r_K^- m_K^+) *deoR thi-1 phoA supE44 gyrA96 relA1*	Invitrogen (Karlsruhe, Germany)
Plasmids		
pGA1-AtpG-S273P	Amp^r; pUC derivative containing a 1,006-bp fragment which covers the flanking regions of the *C. glutamicum atpG* gene and a mutation leading to an AtpG-S273P exchange	GeneArt (Regensburg, Germany)
pK19*mobsacB*	Kan^r; vector for allelic exchange in *C. glutamicum* (pK18 oriV_{E.c.}, *sacB*, *lacZ*α)	76
pK19*mobsacB*-AtpG-S273P	Kan^r; pK19*mobsacB* derivative containing a 984-bp SmaI fragment containing the *atpG*-S273P gene and its flanking regions	This work

medium, which could be stimulated by supplementation with peptone, however. Under these conditions, the DOOR strain displayed a fermentative type of catabolism with L-lactate as the major product and acetate and succinate as minor products (27). Based on these results, we reexamined the potential of *C. glutamicum* to grow anaerobically by fermentation.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The bacterial strains used or constructed in the course of this work are listed in Table 1. *Escherichia coli* DH5α was grown aerobically at 37°C on LB agar plates (LB medium with 1.5% [wt/vol] agar) or in 5 ml LB medium at 170 rpm (28). When appropriate, kanamycin was added to a final concentration of 50 µg ml⁻¹. *C. glutamicum* was routinely cultivated at 30°C. For the construction of *C. glutamicum* strain AtpG-S273P, BHIS agar plates (brain heart infusion [BHI] agar [Difco, Detroit, MI, USA] with 1.0 M sorbitol) and BHIS medium were used. When appropriate, kanamycin was added to a final concentration of 25 µg ml⁻¹. For anaerobic growth experiments, *C. glutamicum* strains were aerobically precultivated at 30°C in 5 ml BHIS medium for 8 h (170 rpm). A second precultivation was performed in 100-ml baffled shake flasks at 30°C and 130 rpm overnight in CGXII minimal medium (29) with 100 mM glucose, fructose, sucrose, ribose, sodium gluconate, sodium pyruvate, sodium acetate, sodium lactate, or sodium propionate or 222 mM glucose (4%, wt/vol) as a carbon and energy source. The cells of these precultures were harvested by centrifugation (5,000 × *g*, 20°C, 10 min). For anaerobic cultivations, 50 ml of CGXII minimal medium with the indicated carbon sources and different concentrations of supplements was prepared in 60-ml serum bottles closed air-tight with a butyl rubber stopper, followed by flushing of the medium with sterile nitrogen gas for 30 min. A 0.3-ml portion of a sterile L-cysteine solution (60 g liter⁻¹) was added with a syringe immediately before inoculation of the medium to an optical density at 600 nm (OD₆₀₀) of 1 with aerobically grown cells washed twice with 0.9% (wt/vol) sodium chloride solution. Cells were grown at 30°C with gentle stirring (50 rpm). Sampling was carried out aseptically under constant nitrogen flushing.

For cultivation under controlled conditions, a 1.4-liter bioreactor (Multifors Multi-Fermenter system; Infors, Einsbach, Germany) containing 600 ml morpholinepropanesulfonic acid (MOPS)-free CGXII minimal medium with 4% (wt/vol) glucose was used. The medium was flushed for 60 min with sterile N₂ (0.9 liter min⁻¹) before inoculation with a syringe to an OD₆₀₀ of 1. During cultivation, the medium was flushed with sterile N₂ at a flow rate of 0.3 liter min⁻¹ and agitated with a paddle mixer at 200 rpm. The pH was monitored using a pH controller and maintained at pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 by addition of 3 M KOH or 3 M

HCl. The nitrogen gas was mixed with CO₂ gas as indicated. The serial inoculation experiments were performed in MOPS-free CGXII minimal medium with 4% (wt/vol) glucose and 15 g liter⁻¹ tryptone as a carbon and energy source. The pH was kept at 7.5, and the bioreactor was flushed with 90% N₂ and 10% CO₂ at 0.3 liter min⁻¹. At the indicated time points, 60 ml of culture was withdrawn using a syringe and transferred immediately to another bioreactor with 540 ml of fresh anaerobic medium. When the medium of the main culture was supplemented with tryptone or peptone (BD, Heidelberg, Germany), the preculture was supplemented as well.

Determination of growth parameters and quantification of sugars and organic acids in the culture supernatant. Cell growth was monitored by measuring the OD₆₀₀ using an Ultrospec 500pro spectrophotometer (Amersham Biosciences, Freiburg, Germany) for cultivations in serum bottles or an Ultrospec 3100pro spectrophotometer for cultivations in bioreactors. An OD₆₀₀ of 1 corresponds to 0.25 g cell dry weight (CDW) per liter (30). The culture samples were centrifuged twice (10,000 × *g*, 4°C, 5 min) to remove the cells, and the resulting supernatants were analyzed for the presence of sugars and organic acids by high-pressure liquid chromatography (HPLC) (Agilent Technologies, Waldbronn, Germany) as described previously (31). Sucrose was measured with the Enzytec kit (Biopharm, Darmstadt, Germany) following the manufacturer's manual.

DNA techniques. All enzymes used for restriction and ligation of DNA were purchased from Roche Diagnostics (Mannheim, Germany) or New England BioLabs (Frankfurt am Main, Germany). All oligonucleotides were synthesized by Eurofins MWG Operon. Routine methods such as PCR, restriction, and ligation were carried out according to standard protocols (28). Plasmid DNA was isolated with the QIAprep Spin Miniprep kit from Qiagen (Hilden, Germany). *E. coli* was transformed by the RbCl method (32). Transformation of *C. glutamicum* was performed as described previously (33). All plasmid constructs described below were controlled by DNA sequencing (LGC Genomics, Berlin, Germany).

RESULTS

Fermentative growth of *C. glutamicum* under anaerobic conditions. Motivated by the capacity of *C. glutamicum* for anaerobic growth by nitrate respiration (12, 13) and for aerobic fermentative growth of the oxygen respiration-deficient DOOR mutant (27), we analyzed in more detail the potential of this species for anaerobic growth by fermentation. For this purpose, the wild type was cultured under anoxic conditions in serum bottles containing CGXII minimal medium with a 100 mM concentration of either glucose, fructose, sucrose, ribose, gluconate, pyruvate, acetate, lactate, or propionate as the sole carbon and energy source. All
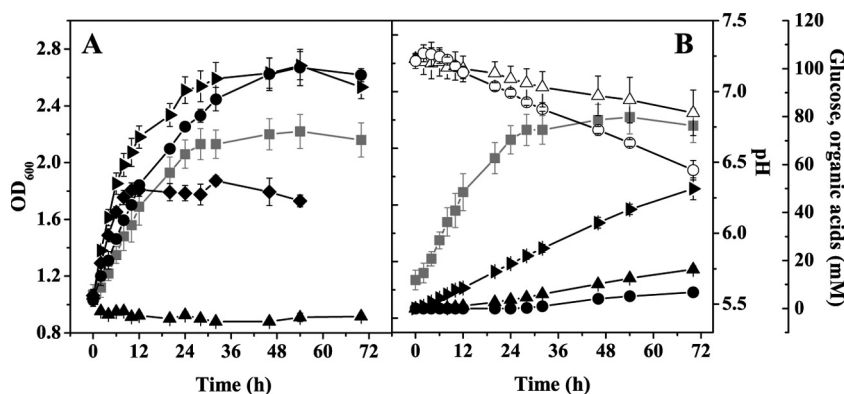
FIG 1 (A) Anaerobic growth of *C. glutamicum* with different carbon sources. The wild type was cultivated in serum bottles containing CGXII minimal medium with 100 mM glucose (■), fructose (▶), sucrose (◆), ribose (●), or gluconate (▲) as the sole carbon and energy source. When pyruvate, lactate, propionate, or acetate was used, the same negative result as with gluconate was obtained. Average values and standard deviations for at least three independent replicates are shown. (B) Biomass formation (■), glucose consumption (○), lactate formation (▶), succinate formation (▲), acetate formation (●), and pH changes (△) during anaerobic growth of the wild type in serum bottles with 100 mM glucose. Average values and standard deviations for four independent replicates are shown.

these compounds can serve as sole carbon and energy sources for aerobic growth of *C. glutamicum* (34–41). During cultivation, the growth, pH, carbon source consumption, and formation of organic acids were followed. On glucose, fructose, sucrose, and ribose, *C. glutamicum* grew linearly from an OD_{600} of 1 to values of between 1.9 and 2.7 (Fig. 1A and Table 2) and excreted lactate, succinate, and acetate, causing an acidification of the medium. This is shown exemplarily for glucose in Fig. 1B. Calculation of the carbon balance showed that 85 to 100% of the consumed carbon sources are metabolized to lactate, succinate, and acetate (Table 2). With gluconate, pyruvate, lactate, propionate, and acetate as carbon sources, no growth was observed, as shown in Fig. 1A exemplarily for gluconate. The absence of growth with these carbon sources makes it very unlikely that the slight growth observed with glucose, fructose, sucrose, and ribose was due to residual amounts of oxygen.

The fact that gluconate did not allow fermentative growth whereas ribose did was puzzling. Both carbon sources are phosphorylated after uptake and then catabolized in the pentose phosphate pathway. The difference is that 6-phosphogluconate first has to be oxidized to ribulose 5-phosphate by 6-phosphogluconate dehydrogenase, with concomitant reduction of $NADP^+$. This enzyme is inhibited by NADPH, which is a key mechanism for controlling cellular NADPH synthesis (42). If NADPH reoxidation was not fast enough under anaerobic fermentative conditions, NADPH could accumulate and cause a complete inhibition of 6-phosphogluconate dehydrogenase activity, which in turn would prevent growth on gluconate. Attempts to achieve anaerobic growth with gluconate by expressing the *E. coli* genes *pntAB* (43) or *udhA* (*sthA*), coding for a membrane-bound and a soluble transhydrogenase, respectively, were not successful (data not shown). Remarkably, anaerobic growth was possible when a combination of gluconate and pyruvate was used, although each substrate alone did not allow growth. When using a combination of

TABLE 2 Biomass formation, growth rate, carbon source consumption, and organic acid formation for wild-type *C. glutamicum* after 46 h of anaerobic cultivation in serum bottles in CGXII minimal medium with the indicated carbon sources or with glucose in the presence or absence of tryptone[a]

Carbon source	Biomass formed (g_{CDW} liter^{-1})	Growth rate (h^{-1})	C source consumed (mM)	C source consumption rate (nmol min^{-1} mg$_{CDW}$$^{-1}$)[b]	Organic acid formation (mM)[c]			Carbon balance (%)[d]
					Lactate	Succinate	Acetate	
Glucose (100 mM)	0.55 ± 0.03	NEG[e]	29 ± 2	19 ± 3	36 ± 2 (0.81)	10 ± 1 (0.34)	13 ± 9 (0.45)	100
Fructose (100 mM)	0.66 ± 0.03	NEG	43 ± 3	24 ± 2	48 ± 5 (1.12)	14 ± 3 (0.33)	9 ± 4 (0.21)	85
Sucrose (100 mM)	0.45 ± 0.02	NEG	37 ± 1	30 ± 2	53 ± 3 (1.43)	10 ± 0 (0.27)	3 ± 0 (0.08)	88
Ribose (100 mM)	0.66 ± 0.03	NEG	46 ± 4	25 ± 2	48 ± 4 (1.04)	11 ± 1 (0.24)	6 ± 1 (0.13)	87
Glucose (222 mM)								
Without tryptone	0.52 ± 0.01	NEG	25 ± 5	17 ± 2	27 ± 10 (1.08)	9 ± 1 (0.36)	10 ± 8 (0.40)	91
With 5 g/liter tryptone	2.06 ± 0.03	0.11 ± 0.01	80 ± 6	14 ± 2	138 ± 2 (1.73)	23 ± 0 (0.29)	9 ± 0 (0.11)	119
With 10 g/liter tryptone	2.76 ± 0.02	0.11 ± 0.01	101 ± 7	13 ± 1	159 ± 4 (1.57)	26 ± 1 (0.26)	10 ± 0 (0.10)	99
With 15 g/liter tryptone	2.93 ± 0.04	0.10 ± 0.01	103 ± 9	13 ± 2	175 ± 3 (1.70)	30 ± 0 (0.29)	7 ± 5 (0.07)	107

[a] Mean values and standard deviations from three independent experiments are shown.
[b] The carbon source consumption rate was calculated for the 46-h period using the final cell dry weight (CDW).
[c] The values in parentheses show the yield of the corresponding fermentation product in moles/mole of carbon source.
[d] The carbon balance was calculated by considering sugars consumed and lactate, succinate, and acetate formed but not biomass formation and consumption of tryptone constituents.
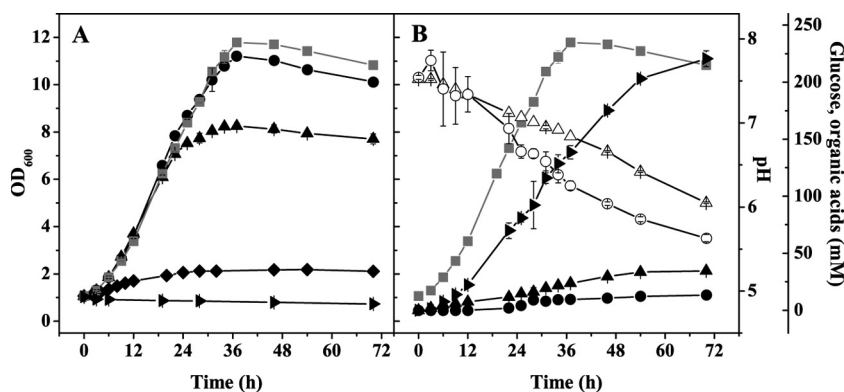[e] NEG, no exponential growth observed.

FIG 2 (A) Influence of different tryptone concentrations (◆, 0 liter^{-1}; ▲, 5 g liter^{-1}; ●, 10 g liter^{-1}; ■, 15 g liter^{-1}) on anaerobic growth of *C. glutamicum* in serum bottles containing CGXII minimal medium with 4% (wt/vol) glucose. In addition, growth in a medium containing only 15 g liter^{-1} tryptone (▶) but no glucose is shown. (B) Biomass formation (■), glucose consumption (○), lactate formation (▶), succinate formation (▲), acetate formation (●), and pH changes (△) during anaerobic growth of wild-type *C. glutamicum* in serum bottles containing CGXII minimal medium with 4% (wt/vol) glucose and 15 g liter^{-1} tryptone. Average values and standard deviations for three independent replicates are shown.

100 mM gluconate and 25 mM pyruvate, 11 mM gluconate and all of the pyruvate was consumed and converted to succinate (13 mM), lactate (7 mM), acetate (14 mM), and in addition L-alanine (2 mM). L-Alanine is synthesized in *C. glutamicum* from pyruvate by the aminotransferases AlaT and AvtA using L-glutamate and L-valine as amino donors, respectively (44). The regeneration of the amino donors from the corresponding 2-oxoacids requires NADPH. Alanine formation is therefore an NADPH-consuming process, which proved to be essential for anaerobic growth with gluconate and pyruvate. An alanine-auxotrophic Δ*alaT* Δ*avtA* mutant (44) was unable to grow anaerobically on this substrate combination in the presence of 3 mM L-alanine but showed growth in L-alanine-supplemented glucose medium (data not shown). Besides L-alanine formation, the high succinate/lactate ratio of 1.9 was a peculiar characteristic of fermentative growth on the mixture of gluconate and pyruvate. Succinate formation requires C$_3$ carboxylation to C$_4$. Carboxylation of pyruvate can proceed not only via the ATP-dependent pyruvate carboxylase (45) but also via malic enzyme, which catalyzes the reversible NADPH-dependent conversion of pyruvate to malate (40). As the latter reaction provides another possibility for NADPH oxidation, we also analyzed a Δ*malE* mutant and found that it was unable to grow anaerobically with gluconate and pyruvate but showed growth with glucose (data not shown). This suggests that both conversion of pyruvate to alanine and reductive carboxylation of pyruvate to malate are necessary for reoxidation of NADPH formed by 6-phosphogluconate dehydrogenase.

Stimulation of anaerobic fermentative growth by peptides and amino acids. With the aim to improve anaerobic growth of *C. glutamicum*, cultivations in serum bottles were performed using CGXII minimal medium with 4% (wt/vol) glucose and various concentrations of BHI, nutrient broth, yeast extract, Casamino Acids, peptone, or tryptone. All supplements improved growth, with tryptone being the most effective one (data not shown). Supplementation with 5, 10, and 15 g liter^{-1} tryptone increased the final OD$_{600}$ from 2 to about 8, 11, and 12, respectively (Fig. 2A). Higher tryptone concentrations did not stimulate growth any further. In the presence of tryptone, cells grew exponentially with a rate of 0.11 h^{-1} (Table 2). A control culture containing 15 g liter^{-1} tryptone but no glucose did not grow at all (Fig. 2A), showing the

strict requirement for sugar. The rate of glucose consumption in the presence of tryptone (24.6 ± 3.5 nmol mg$_{CDW}$$^{-1}$ min^{-1}) was comparable to that in the absence of tryptone (25.8 ± 4.0 nmol mg$_{CDW}$$^{-1}$ min^{-1}) within the first 30 h of cultivation, with lactate, succinate, and acetate as products (Fig. 2B and Table 2). Under aerobic conditions, a minimal medium containing 15 g liter^{-1} tryptone as the sole carbon source enabled growth from an OD$_{600}$ of 1 to an OD$_{600}$ of 4 within 24 h, accompanied by an alkalinization of the medium from pH 7.1 to pH 8.2 (data not shown).

We subsequently tested the influence of different groups of amino acids on anaerobic growth with glucose. In a first series of experiments, three amino acid mixtures were formed based on the ATP demand of their synthesis (46), i.e., mix I (high ATP demand; R, M, H, W, and C), mix II (intermediate ATP demand; I, K, T, Q, F, P, and Y), and mix III (no ATP demand; A, D, E, G, L, S, and V) (Table 3; see Table S1 in the supplemental material). As shown in Fig. S1 in the supplemental material, the simultaneous addition of all three mixtures (each amino acid in a final concentration of 0.8 mM, corresponding to a total amino acid concentration of about 2 g/liter) allowed a growth rate similar to that with 15 g/liter tryptone (Table 3) and a final OD$_{600}$ of 6 (50% of the value obtained with 15 g/liter tryptone). Supplementation with mix I and either mix II or mix III still allowed reasonable growth rates and final OD values of 5.2 and 4.6, respectively. In contrast, supplementation with either mix II plus mix III or with each mix alone improved growth much less than the above-mentioned combinations and led to OD values of between 2.7 and 3.3 (Table 3). According to these results, the best growth was achieved with combinations including mix I (containing the most ATP-demanding amino acids), but surprisingly, mix I alone stimulated growth only weakly, suggesting another limitation. We therefore performed a second series of experiments in which we added all amino acids except one of the five amino acid families (aspartate family, glutamate family, serine family, pyruvate family, and aromatic amino acid family) or L-histidine (Table 3). Only the lack of the amino acids of the glutamate family led to a significant reduction of growth and of the final OD$_{600}$ (by about 50%), whereas the lack of amino acids of the pyruvate family had practically no impact on biomass formation (Table 3). Thus, besides the ATP-demanding amino acids, amino acids of the glutamate family also limit anaerobic growth,

TABLE 3 Growth rate and biomass formed after 46 h of cultivation of wild-type *C. glutamicum* under anaerobic conditions in serum bottles with CGXII minimal medium containing 4% (wt/vol) glucose and different amino acid supplements[a]

Amino acid supplement (0.8 mM each)	μ (h^{-1})	Biomass formed (g_{CDW} $liter^{-1}$)	% biomass[b]
None	NEG[c]	0.52 ± 0.01	35
Mix I (high ATP demand; R, M, H, W, C, N)	0.03 ± <0.01	0.71 ± 0.01	47
Mix II (intermediate ATP demand; I, K, T, Q, F, P, Y)	0.04 ± <0.01	0.67 ± 0.09	45
Mix III (no ATP demand; A, D, E, G, L, S, V)	0.05 ± <0.01	0.83 ± 0.10	55
Mix I + II	0.05 ± 0.01	1.31 ± 0.01	87
Mix I + III	0.06 ± <0.01	1.14 ± 0.08	76
Mix II + III	0.06 ± <0.01	0.67 ± 0.04	45
Mix I + II + III	0.10 ± <0.01	1.50 ± 0.07	100
Glutamate family (E, Q, R, P)	NEG	0.69	46
Serine family (S, C, G)	NEG	0.56	37
Histidine	NEG	0.56	37
Aspartate family (D, M, N, T, I, K)	NEG	0.52	35
Pyruvate family (A, V, L)	NEG	0.53	35
Aromatic amino acid family (F, Y, W)	NEG	0.52	35
All amino acids except:			
Pyruvate family	NEG	1.43	95
Histidine	NEG	1.29	86
Aromatic amino acid family	NEG	1.25	83
Aspartate family	NEG	1.17	78
Serine family	NEG	1.13	75
Glutamate family	NEG	0.79	53

[a] Mean values and standard deviations are shown for experiments with three replicates, and mean values are shown for experiments with two replicates.
[b] Biomass obtained with supplementation of all amino acids set as 100%.
[c] NEG, no exponential growth observed.

which presumably is due to an insufficient carbon flux into the oxidative TCA cycle to form 2-oxoglutarate (24).

pH tolerance range during anaerobic fermentative growth. Under aerobic conditions, *C. glutamicum* shows maximal growth rates at between pH 7.0 and 8.5 and effective pH homeostasis in the pH range between 6.0 and 9.0, in which the internal pH is kept at 7.5 ± 0.5 (47). At pH 6.0, *C. glutamicum* regulates its internal pH by proton extrusion via the respiratory chain and potassium influx via the potassium channel CglK (48, 49). During anaerobic fermentative growth with 100 mM glucose in the absence of tryptone, the pH dropped to 6.9 (Fig. 1B), and during anaerobic growth with 222 mM glucose in the presence of 15 g liter^{-1} tryptone, it dropped to 6.1 (Fig. 2B). pH homeostasis might become a problem for *C. glutamicum* during anaerobic fermentative growth, because organic acids are produced and not consumed later on and because protons cannot be pumped out of the cell by the respiratory chain. We therefore analyzed the pH tolerance of *C. glutamicum* under anaerobic conditions in more detail. For this purpose, cells were cultivated in a bioreactor using MOPS-free CGXII minimal medium with 4% (wt/vol) glucose and, when indicated, 15 g liter^{-1} tryptone. The external pH was kept constant at 6.0, 6.5, 7.0, 7.5, and 8.0 in the absence of tryptone and at 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 in the presence of tryptone. Under both conditions, the highest final optical densities were observed at pH 7.5, which was previously shown to be the preferred internal pH under aerobic conditions (Fig. 3). Higher or lower pH values led to reduced growth. In the absence of tryptone, no growth was observed at pH 6.0 (Fig. 3A), and only weak growth occurred at pH 8.0 after a lag phase of 12 to 24 h (Fig. 3B). In the presence of tryptone, *C. glutamicum* showed weak growth even at pH 5.5 (Fig. 3C) and 8.5 (Fig. 3D). At all tested pH values, growth with tryp-

tone was better than that without. Thus, effective pH homeostasis was possible in the range from pH 6.5 to 8.0 in the absence of tryptone and from pH 5.5 to 8.5 in the presence of tryptone, showing that control of the cytoplasmic pH was still possible and more efficient in the presence of tryptone.

Carbon dioxide demand of anoxic *C. glutamicum* cells. In the course of our experiments we noticed that the final OD$_{600}$ of wild-
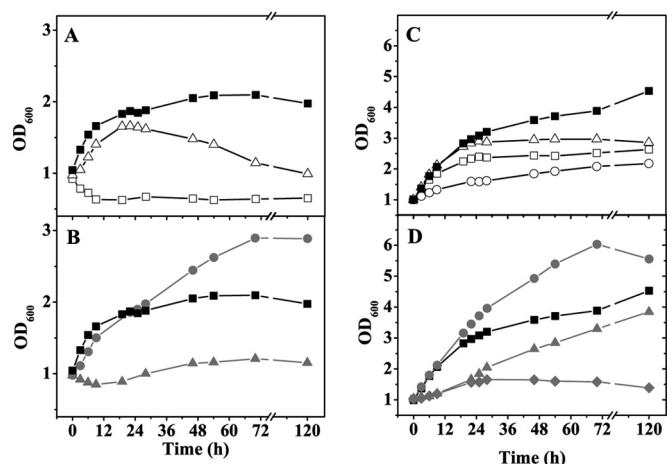

FIG 3 Influence of pH on anaerobic growth of *C. glutamicum* in a bioreactor containing MOPS-free CGXII minimal medium with 4% (wt/vol) glucose and either no tryptone (A and B) or 15 g liter^{-1} tryptone (C and D). The following pH values were tested: 5.5 (○), 6.0 (□), 6.5 (△), 7.0 (■), 7.5 (●), 8.0 (▲), and 8.5 (◆). The bioreactor was flushed with pure N$_2$ at 0.3 liter min^{-1}. The pH was kept constant by addition of 3 M KOH or 3 M HCl. Average values for at least two independent replicates are shown.
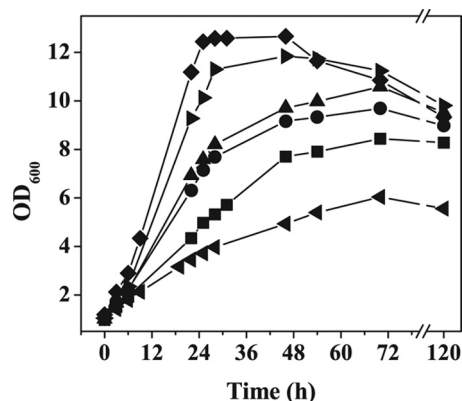
FIG 4 Influence of CO_2 on anaerobic growth of *C. glutamicum* in a bioreactor using MOPS-free CGXII minimal medium with 4% (wt/vol) glucose and 15 g liter^{-1} tryptone at a constant pH of 7.5. The bioreactor was flushed with a gas mixture composed of N_2 and the indicated CO_2 concentrations: 10% (◆), 7.5% (▶), 5% (▲), 2.5% (●), 1% (■), or 0% (◀). Average values for at least two independent replicates are shown.
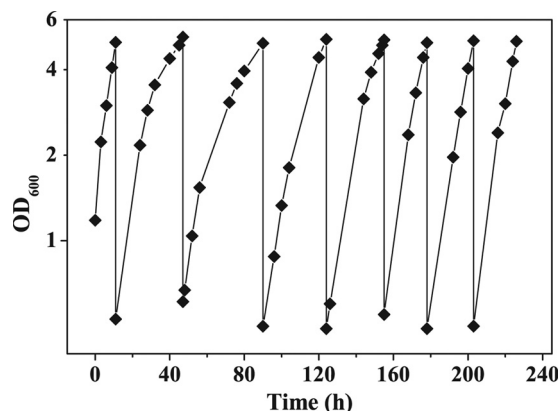

FIG 5 Serial inoculation experiment with wild-type *C. glutamicum*. The cells were cultivated anaerobically in bioreactors containing MOPS-free CGXII minimal medium with 4% (wt/vol) glucose and 15 g liter^{-1} tryptone at a constant pH of 7.5. The bioreactors were flushed with 90% N_2 and 10% CO_2 at 0.3 liter min^{-1}. When the cultures reached an OD_{600} of about 5, 60 ml of the culture was transferred to another bioreactor with 540 ml fresh anoxic medium.

type *C. glutamicum* in CGXII minimal medium with 4% (wt/vol) glucose and 15 g liter^{-1} tryptone under anaerobic conditions in serum bottles was much higher than that in the bioreactor cultivations ([Fig. 2](#) and [4](#)). We assumed that the bioreactor cultures could be limited in carbon dioxide for oxaloacetate biosynthesis via phosphoenolpyruvate (PEP) carboxylase and pyruvate carboxylase and for fatty and mycolic acid synthesis. In contrast to the case in the closed serum bottles, the carbon dioxide formed by the cells in the bioreactor is removed to a large extent by the constant flushing with nitrogen gas. To test this assumption, cultivations were performed in which the bioreactor was flushed with nitrogen gas containing 1%, 2.5%, 5%, 7.5%, or 10% (vol/vol) carbon dioxide. As shown in [Fig. 4](#), both the growth rate and the maximal OD_{600} were significantly improved with increasing CO_2 concentrations. At 10% (vol/vol) CO_2, a maximal OD_{600} of 12.5 and a growth rate of 0.14 h^{-1} was achieved, the latter being 27% higher than in serum bottles. The molar yields of the lactate, succinate, and acetate were not significantly changed at 10% (vol/vol) CO_2 compared to cultivation without CO_2 supplementation (see Table S2 in the supplemental material). Higher CO_2 concentrations did not stimulate growth any further. The observation that anaerobic cultivation in bioreactors with continuous flushing led to a OD_{600} comparable to that for growth in serum bottles argues against the possibility of residual oxygen in the gas flow, as this should have allowed higher cell densities.

Serial growth under anaerobic conditions. In order to test whether anaerobic fermentative growth of *C. glutamicum* is temporally limited due to the existence of an oxygen-dependent reaction that is essential for biomass formation in the long term, we performed serial inoculation experiments in MOPS-free CGXII minimal medium containing 4% (wt/vol) glucose and 15 g liter^{-1} tryptone ([Fig. 5](#)). We transferred 60 ml of the first culture at an OD_{600} of 5 into another bioreactor containing 540 ml of fresh anoxic medium, resulting in a starting OD_{600} of the new culture of about 0.5. This procedure was repeated seven times. During these seven cultivations the cells stayed vital and grew with a growth rate of between 0.09 and 0.13 h^{-1}, suggesting the absence of an oxygen-dependent reaction that is essential for continued biomass formation by *C. glutamicum*.

DISCUSSION

C. glutamicum is currently described as a facultative anaerobe that can grow anaerobically only by nitrate respiration. Here we demonstrate that *C. glutamicum* is also able to grow anaerobically in the absence of nitrate on glucose, fructose, sucrose, and ribose by mixed-acid fermentation with lactate, succinate, and acetate as products. The mentioned carbon sources are catabolized via glycolysis or the pentose phosphate pathway ([Fig. 6](#)) and allow ATP synthesis by substrate-level phosphorylation. No growth was observed on gluconate, pyruvate, lactate, acetate, and propionate. Fermentative growth with acetate and propionate was not expected, as they do not allow net ATP synthesis by substrate-level phosphorylation. Anaerobic growth by lactate fermentation occurs in nature, as shown by propionibacteria that gain net ATP by conversion of lactate to propionate, acetate, and CO_2 either via the methylmalonyl coenzyme A (methylmalonyl-CoA) pathway or via the acrylyl-CoA pathway. However, the key enzymes of these pathways, transcarboxylase, lactyl-CoA dehydratase, and acrylyl-CoA reductase, are not present in *C. glutamicum*. In the case of pyruvate, an exergonic fermentation pathway can be formulated, in which one molecule of pyruvate is converted by pyruvate dehydrogenase, phosphotransacetylase, and acetate kinase to acetate and CO_2 along with formation of NADH and ATP. The NADH could be reoxidized by reduction of a second molecule of pyruvate to L-lactate by the NAD$^+$-dependent lactate dehydrogenase, resulting in the following overall reaction: 2 pyruvate$^-$ + H$_2$O → acetate$^-$ + CO_2 + lactate$^-$ (ΔG° = −95.1 kJ/mol acetate). In contrast to acetate, propionate, and lactate, pyruvate as the sole carbon source was partially metabolized under anaerobic conditions to lactate and acetate, but this conversion was not associated with growth, possibly because the energy budget of the cells did not allow for gluconeogenesis.

Strong evidence was obtained that the inability for to grow anaerobically with gluconate was due to an insufficient capacity for reoxidation of NADPH generated by 6-phosphogluconate dehydrogenase. Supplementation with pyruvate could overcome this problem, as pyruvate allows NADPH reoxidation both by
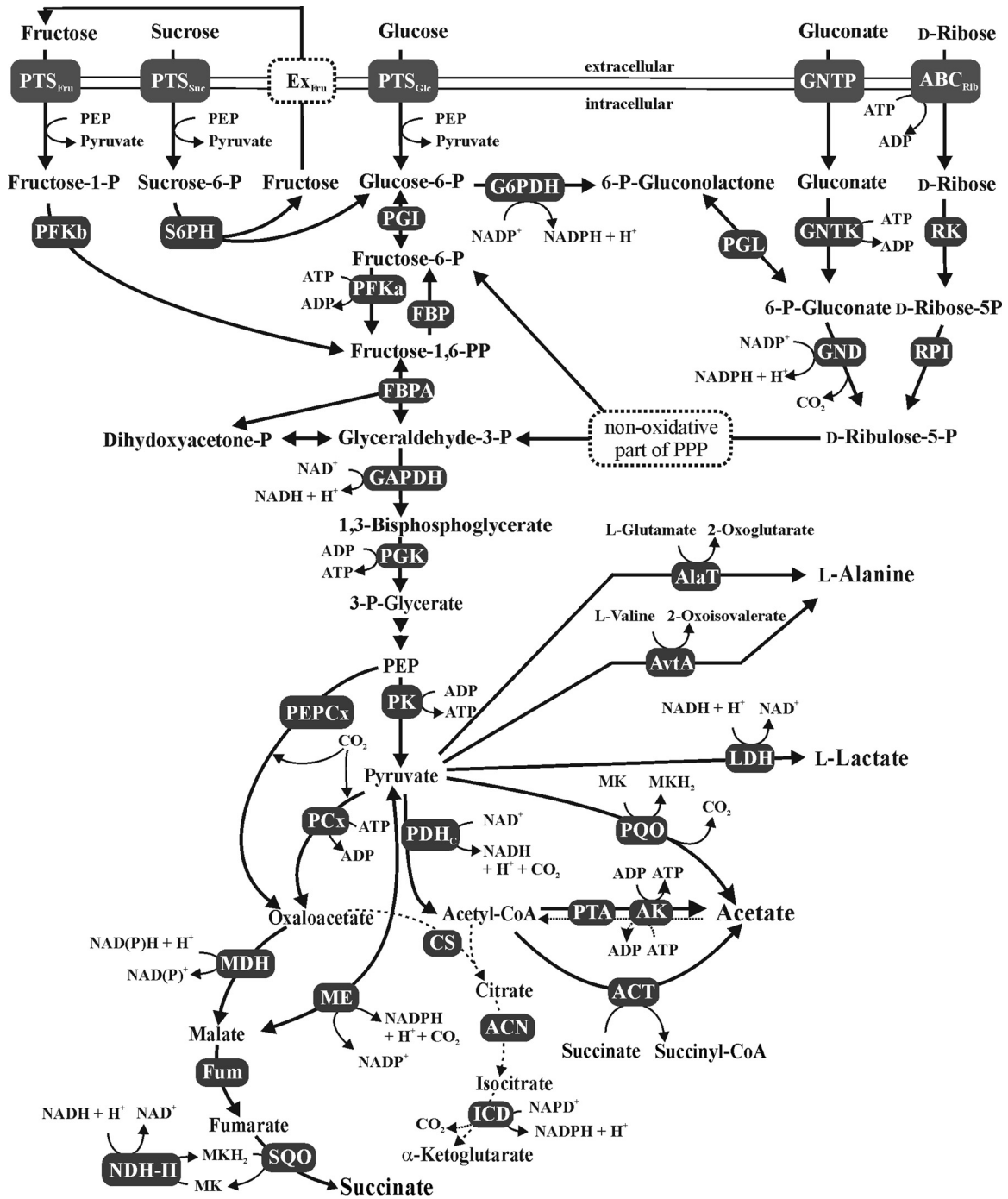
FIG 6 Catabolism of glucose, fructose, sucrose, gluconate, and ribose by *C. glutamicum* and anaerobic central metabolism. Abbreviations: ABC$_{Rib}$, ABC-type transporter for ribose; ACN, aconitase; AK, acetate kinase; CoA, coenzyme A; ACT, acetyl-CoA:CoA transferase; AlaT, alanine aminotransferase; AvtA, valine-pyruvate transaminase; CS, citrate synthase; Ex$_{Fru}$, exporter for fructose; FBPA, fructose-bisphosphate aldolase; FUM, fumarase; Fru, fructose; GAP, glyceraldehyde-3-phosphate; GAPDH, GAP dehydrogenase; Glu, glucose; G6PDH, glucose-6-phosphate dehydrogenase; GND, 6-phosphate-gluconate dehydrogenase; GNTK, gluconate kinase; ICD, isocitrate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; NDH-II, NADH dehydrogenase; P, phosphate; PP, bisphosphate; PPP, pentose phosphate pathway; PCx, pyruvate carboxylase; PDH$_C$, pyruvate dehydrogenase complex; PEP, phosphoenolpyruvate; PEPCx, PEP carboxylase; PFKa, 6-phosphofructokinase; PFKb, 1-phosphofructokinase; PGI, glucose-6-phosphate isomerase; PGK, phosphoglycerate kinase; PGL, 6-phosphogluconolactonase; PK, pyruvate kinase; PQO, pyruvate:quinone oxidoreductase; PTA, phosphotransacetylase; PTS, phosphotransferase system; RK, ribokinase; RPI, ribose 5-phosphate isomerase; S6PH, sucrose 6-phosphate hydrolase; Suc, sucrose; SQO, succinate:menaquinone oxidoreductase. Note that SQO and NADH dehydrogenase are membrane-integral or membrane-associated enzymes, respectively, which is not shown in the figure.

TABLE 4 Overview of putative or verified peptide and amino acid transport systems of *C. glutamicum* based on the genome annotation and available literature

Category	Cg no.	NCgl no.	Gene name	Annotation	Reference(s)
Putative secondary peptide transporters	Cg2884	NCgl2514		Dipeptide/tripeptide permease	77
	Cg3382	NCgl2949		Dipeptide/tripeptide permease	77
Putative peptide ABC transporters	Cg2181	NCgl1915		ABC-type peptide transport system, secreted component	77
	Cg2182	NCgl1916		ABC-type peptide transport system, permease component	77
	Cg2183	NCgl1917		ABC-type peptide transport system, permease component	77
	Cg2184	NCgl1918		ATPase component of peptide ABC-type transport system, contains duplicated ATPase domains	77
	Cg2549	NCgl2238		ABC-type dipeptide/oligopeptide/nickel transport system, secreted component	77
	Cg2550	NCgl2239		ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	77
	Cg2551	NCgl2240		ABC-type dipeptide/oligopeptide/nickel transport system, permease component	77
	Cg2552	NCgl2241		ATPase component of ABC-type transport system, contains duplicated ATPase domain	77
	Cg2675	NCgl2350		ATPase component of ABC-type transport system, contains duplicated ATPase domains	77
	Cg2676	NCgl2351		ABC-type dipeptide/oligopeptide/nickel transport systems, permease component	77
	Cg2677	NCgl2352		ABC-type dipeptide/oligopeptide/nickel transport system, permease component	77
	Cg2678	NCgl2353		ABC-type dipeptide/oligopeptide/nickel transport systems, secreted component	77
Putative orphan periplasmic peptide binding proteins	Cg1249	NCgl1054		ABC-type dipeptide/oligopeptide/nickel transport system, secreted component	77
	Cg1864	NCgl1592	*dciAE*	ABC-type dipeptide/oligopeptide/nickel transport systems, secreted component	77
	Cg2610	NCgl2294		ABC-type dipeptide/oligopeptide/nickel transport system, secreted component	77
Putative or verified secondary amino acid importers	Cg0254	NCgl0203		Putative Na$^+$/alanine symporter (possibly D-alanine)	77
	Cg0555	NCgl0453		Putative D-serine/D-alanine/glycine transporter	77
	Cg0568	NCgl0464	*gapP*	γ-Aminobutyric acid transporter weakly inhibited by asparagine and glutamine	78
	Cg0953	NCgl0799		Putative Na$^+$/proline, Na$^+$/pantothenate symporter	77
	Cg1016	NCgl0856	*betP*	Glycine betaine transporter	79
	Cg1105	NCgl0929	*lysI*	L-Lysine permease	80
	Cg1167	NCgl0985	*metS*	Na$^+$/methionine/alanine transporter, small subunit	81
	Cg1169	NCgl0986	*metP*	Na$^+$/methionine/alanine transporter, large subunit	81
	Cg1257	NCgl1062	*aroP*	Aromatic amino acid and histidine transporter	82, 83
	Cg1305	NCgl1108	*pheP*	Phenylalanine transport system	84
	Cg1314	NCgl1116	*putP*	Na$^+$/proline symporter	85
	Cg2537	NCgl2228	*brnQ*	Branched-chain amino acid uptake carrier	86
	Cg2539	NCgl2230	*ectP*	Ectoine/proline/glycine betaine carrier	87
	Cg2563	NCgl2251	*lcoP*	Ectoine/betaine transporter	88
	Cg3080	NCgl2683	*gltS*	Na$^+$/glutamate symporter	89
	Cg3357	NCgl2925	*trpP*	Putative tryptophan-specific permease, 5-methyltryptophan resistance	90
	Cg3395	NCgl2961	*proP*	Proline/ectoine uptake system	87
Putative or verified amino acid ABC transporters	Cg0737	NCgl0610	*metQ*	ABC-type methionine transport system, secreted lipoprotein component	81
	Cg0736	NCgl0609	*metN*	ABC-type methionine transport system, ATPase component	81
	Cg0735	NCgl0608	*metI*	ABC-type methionine transport system, permease component	81
	Cg2136	Cgl1875	*gluA*	ABC transporter for glutamate uptake, ATPase component	91

(Continued on following page)

TABLE 4 (Continued)

Category	Cg no.	NCgl no.	Gene name	Annotation	Reference(s)
	Cg2137	NCgl1876	*gluB*	ABC transporter for glutamate uptake, secreted glutamate binding protein	91
	Cg2138	NCgl1877	*gluC*	ABC transporter for glutamate uptake, permease component	91
	Cg2139	NCgl1878	*gluD*	ABC transporter for glutamate uptake, permease component	91
	Cg2467	NCgl2168		ABC transporter, ATP-binding protein	77
	Cg2468	NCgl2169		Putative branched-chain amino acid ABC-type transport system, permease component	77
	Cg2470	NCgl2170		ABC transporter, secreted substrate-binding protein	77
Putative or verified amino acid exporters	Cg0183	NCgl0143		Putative threonine efflux protein	77
	Cg2574	NCgl2262		Putative threonine efflux protein	77
	Cg2905	NCgl2533	*thrE*	Threonine export carrier	92
	Cg2941	NCgl2566		Putative threonine efflux protein	77
	Cg0314	NCgl0254	*brnF*	Exporter for branched-chain amino acids and methionine, large subunit	93, 94
	Cg0315	NCgl0255	*brnE*	Exporter for branched-chain amino acids and methionine, small subunit	93, 94
	Cg3412	NCgl2976	*azlD*	Predicted branched-chain amino acid permease (azaleucine resistance)	77
	Cg3413	NCgl2977	*azlC*	Predicted branched-chain amino acid permease (azaleucine resistance)	77
	Cg1424	NCgl1214	*lysE*	Lysine/arginine efflux permease	55
	Cg1434	NCgl1221	*yggB*, *mscCG*	Glutamate exporter	95

conversion to L-alanine and by reductive carboxylation to L-malate. The responsible enzymes, the transaminases AlaT and AvtA and malic enzyme MalE, were shown to be essential for fermentative growth with the gluconate-pyruvate mixture.

Addition of tryptone or other peptide sources or of amino acids strongly enhanced growth of *C. glutamicum* under anaerobic fermentative conditions with the sugars mentioned above, whereas tryptone alone did not allow growth. *C. glutamicum* is known to have efficient peptide uptake systems, although they have not yet been characterized in detail (50–54). At least a fraction of the peptides present in tryptone are taken up and then hydrolyzed by peptidases to amino acids, which then are used for protein synthesis. In agreement, supplementation with amino acids rather than tryptone also improved anaerobic growth. A mixture of all amino acids was required to achieve maximal growth (Table 3). In Table 4, an overview of proposed or experimentally verified peptide and amino acid transport systems of *C. glutamicum* is given. It also includes amino acid exporters, which allow export of amino acids that cannot be degraded and accumulate in the cell when their uptake, e.g., as peptides, is more rapid than their incorporation into proteins, which can lead to toxic effects (55). As proteins account for about 50% of the dry weight of cells (56) and *de novo* amino acid biosynthesis requires a significant fraction of the cellular energy demand, the supplementation of the medium with amino acids or peptides will save a lot of energy. This energy can be used for the synthesis of other building blocks, leading to improved growth, or for maintenance and homeostatic processes.

pH homeostasis is a crucial point for anaerobic fermentative growth of *C. glutamicum*, as acids are formed as end products, causing an acidification of the medium. At the same time, the major components of the respiratory chain involved in proton export, in particular the cytochrome bc_1-aa_3 supercomplex, are inactive. Under aerobic respiratory conditions, the growth optimum of *C. glutamicum* in minimal medium was reported to be between pH 7 and 8.5, and the cells grew at between pH 4 and 10 (47). Under anaerobic fermentative conditions, the growth optimum in minimal medium without tryptone was pH 7.5, and cells grew at between pH 6.5 and 8.0. Thus, the optimal pH range and the pH range allowing growth were drastically reduced under anaerobic conditions, indicating that pH homeostasis is a severe problem. Various possibilities to cope with acid stress have been described in bacteria, such as amino acid decarboxylases like glutamate decarboxylase or lysine decarboxylase (57–59). The protein encoded by cg1261 (NCgl1065) in the genome of *C. glutamicum* was annotated to be a member of the lysine decarboxylase protein family, but it has not been characterized functionally, and no evidence has been found for its involvement in acid stress adaptation in genome-wide transcriptome studies (47, 60). An alternative way to cope with acid stress is the hydrolysis of urea via urease, as found, e.g., in *Helicobacter pylori* (61). A urease is present in *C. glutamicum* and is induced under nitrogen starvation, whereas there is no evidence for an involvement in the acid stress response (62, 63). A limited number of reactions are assumed to be relevant for proton extrusion under fermentative conditions in *C. glutamicum*. (i) The export of lactate (pK_a = 3.90) and succinate (pK_{a1} = 4.16, pK_{a2} = 5.61) by nongrowing *C. glutamicum* cells at an extracellular pH of 5.7 and an intracellular pH of 6.3 was reported to be coupled to the export of 1.4 and 2.3 to 2.7 protons, respectively (24), thereby contributing to the establishment of a proton motive force. The driving force for this proton export is the 10-fold-higher intracellular compared to extracellular concentration of lactate and succinate, as measured by ^{13}C NMR (24). For succinate, the exporter SucE has been identified in *C. glutamicum* (64, 65) but still awaits a detailed biochemical characterization, including the stoichiometry of cotransported ions. For lactate, an exporter has not yet been described for *C. glutamicum*. Uptake of succinate and other C_4-dicarboxylates is catalyzed by the second-

ary transporters DccT and DctA in *C. glutamicum*; however, their expression levels are very low in the wild type (66, 67). Uptake of L-lactate presumably involves a secondary transporter encoded by cg3226 (NCgl2816); however, as the absence of this gene does not prevent growth on lactate, further lactate uptake systems probably exist (68). As in the case of SucE, the putative lactate permease Cg3226 has not yet been characterized biochemically. (ii) The reduction of endogenously formed fumarate to succinate by succinate:menaquinone oxidoreductase (Fig. 6) is presumably coupled with the export of two protons (14, 69) and thus contributes to proton motive force generation. (iii) Proton export driven by ATP hydrolysis via F_1F_o-ATP synthase (4 H^+/ATP) was reported for several Gram-positive bacteria (59, 70, 71), including *Mycobacterium smegmatis* (72). We tested the role of F_1F_o-ATP synthase in the anaerobic fermentative growth of *C. glutamicum* by analyzing AtpG-S273P and ΔF_1F_o mutants, which have a strongly reduced (73, 74) or no (31) F_1-ATPase activity. As shown in Fig. S2 in the supplemental material, fermentative growth of both mutants was improved in glucose medium without tryptone and impaired in glucose medium supplemented with tryptone. A straightforward explanation of these opposing effects cannot be given, but the results demonstrate that F_1F_o-ATP synthase plays a role in anaerobic fermentative growth, either for proton export at the cost of ATP hydrolysis or for ATP synthesis at the cost of proton import. In conclusion, all of the three processes can be assumed to contribute to pH homeostasis under anaerobic conditions.

During anaerobic fermentative growth in bioreactors which were constantly flushed with nitrogen gas, supplementation with 1 to 10% (vol/vol) CO_2 strongly improved growth. CO_2 is required on one hand in the pathway of succinate formation via the reductive branch of the TCA cycle, where oxaloacetate has to be synthesized by carboxylation of PEP or pyruvate, and on the other hand for biosynthetic reactions, in particular fatty and mycolic acid synthesis. In the previous study by Rados et al. (24), the presence of 20% CO_2 reduced the lactate yield from 1.22 to 0.73 mol/mol glucose and increased the succinate yield from 0.29 to 0.59 mol/mol and the acetate yield from 0.13 to 0.19 mol/mol. In contrast, the yields of lactate, succinate, and acetate were changed only moderately in our experiments with 10% CO_2 (see Table S2 in the supplemental material). This difference can be explained by the different conditions used in the experiments. Rados et al. (24) used nongrowing cells resuspended to an OD_{600} of 100 in a buffer without nitrogen, sulfur, or phosphorus sources, whereas we analyzed growing cells at a starting OD_{600} of 1 in a minimal medium supplemented with 15 g/liter tryptone. Therefore, it has to be assumed that in our experiments, CO_2 was used to enhance CO_2-requiring biosynthetic reactions, in particular acetyl-CoA carboxylation for fatty and mycolic acid synthesis, rather than for increasing pyruvate and PEP carboxylation.

In summary, we demonstrate that *C. glutamicum* is able to grow under anaerobic conditions without nitrate by mixed-acid fermentation. In the presence of tryptone or amino acid supplements, cell densities which correspond to 20% of those reached under aerobic conditions can be obtained. Fermentative growth was possible over many generations, supporting the view that none of the reactions involved in biomass formation is strictly dependent on oxygen.

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