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Conversion of *Corynebacterium glutamicum* from an aerobic respiring to an aerobic fermenting bacterium by inactivation of the respiratory chain

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ABSTRACT

In this study a comparative analysis of three *Corynebacterium glutamicum* ATCC 13032 respiratory chain mutants lacking either the cytochrome *bd* branch (Δ*cydAB*), or the cytochrome bc_1–aa_3 branch (Δ*qcr*), or both branches was performed. The lack of cytochrome *bd* oxidase was inhibitory only under conditions of oxygen limitation, whereas the absence of a functional cytochrome bc_1–aa_3 supercomplex led to decreases in growth rate, biomass yield, respiration and proton-motive force (pmf) and a strongly increased maintenance coefficient under oxygen excess. These results show that the bc_1–aa_3 supercomplex is of major importance for aerobic respiration. For the first time, a *C. glutamicum* strain with a completely inactivated aerobic respiratory chain was obtained (Δ*cydAB*Δ*qcr*), named DOOR (devoid of oxygen respiration), which was able to grow aerobically in BHI (brain–heart infusion) glucose complex medium with a 70% reduced biomass yield compared to the wild type. Surprisingly, reasonable aerobic growth was also possible in glucose minimal medium after supplementation with peptone. Under these conditions, the DOOR strain displayed a fermentative type of catabolism with L-lactate as major and acetate and succinate as minor products. The DOOR strain had about 2% of the oxygen consumption rate of the wild type, showing the absence of additional terminal oxidases. The pmf of the DOOR mutant was reduced by about 30% compared to the wild type. Candidates for pmf generation in the DOOR strain are succinate:menaquinone oxidoreductase, which probably can generate pmf in the direction of fumarate reduction, and F_1F_O-ATP synthase, which can couple ATP hydrolysis to the export of protons.

1. Introduction

Corynebacterium glutamicum is a Gram-positive soil organism belonging to the order *Corynebacteriales* within the class of *Actinobacteria* [1]. Strains of this species are used since the 1960s for the industrial production of various L-amino acids [2]. In the past years, *C. glutamicum* strains capable of producing various other commercially interesting metabolites such as D-amino acids [3], organic acids [4–7], diamines [8–10], or biofuels [11–13] from renewable carbon sources have been developed. Moreover, the potential of *C. glutamicum* for efficient heterologous protein secretion has been described [14]. Furthermore, as a non-pathogenic relative of *Mycobacterium tuberculosis*, *C. glutamicum* serves to elucidate the synthesis of the complex cell wall common to this group of bacteria [15]. For these reasons, *C. glutamicum* has become a prominent model organism for prokaryotic metabolism and regulation [16,17].

C. glutamicum uses a respiratory type of energy metabolism with oxygen or nitrate as terminal electron acceptors and an F_1F_O-ATP synthase driving ATP synthesis [18]. The branched aerobic respiratory chain of *C. glutamicum* is composed of several dehydrogenases, which reduce menaquinone. These include succinate:menaquinone oxidoreductase (*sdhCAB*) [19], a single-subunit type II NADH dehydrogenase (*ndh*) [20], malate:quinone oxidoreductase (*mqo*) [21,22], pyruvate:quinone oxidoreductase (*pqo*) [23], D-lactate dehydrogenase (*dld*) [18,24], and L-lactate dehydrogenase (*lldD*) [18,25]. Only succinate:menaquinone oxidoreductase includes membrane-integral components, whereas the five other dehydrogenases lack obvious transmembrane helices and are presumably attached to the inner leaflet of the cytoplasmic membrane. Electron transfer from menaquinol to oxygen is catalyzed either by a cytochrome bc_1–aa_3 supercomplex (bc_1–aa_3 branch) [26] or by cytochrome *bd* oxidase (*bd* branch) [27]. In addition, the presence of a cyanide-insensitive third terminal oxidase was suggested [27], but its molecular nature has never been elucidated and the genome sequence gave no evidence for its existence [28]. Anaerobic growth by nitrate respiration is very limited, as nitrate can only be reduced to nitrite by a Nar-type nitrate reductase, but not further to ammonia or dinitrogen. Therefore, nitrite accumulates in the medium and becomes growth-inhibitory [29,30]. Furthermore, it has been demonstrated that under oxygen-deprived conditions *C. glutamicum* converts glucose to L-lactate and succinate without growing [31].

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Several enzymes of the respiratory chain have been studied genetically and biochemically, such as the non-proton pumping type II NADH-DH [20], the succinate:menaquinone oxidoreductase [19], cytochrome *bd* oxidase [27], and the cytochrome bc_1–aa_3 supercomplex [26,32,33]. A distinctive feature of the respiratory chain is that it contains only a single *c*-type cytochrome, which is cytochrome c_1. However, cytochrome c_1 was found to contain two covalently bound heme groups [33,34], both of which are essential for function [26]. The second heme group presumably takes over the function of a separate cytochrome *c* in mediating electron transfer from the first heme group of cytochrome c_1 to the Cu_A center in subunit II of cytochrome aa_3 oxidase, which is a plausible explanation for the formation of a bc_1–aa_3 supercomplex [26].

The two branches of the *C. glutamicum* respiratory chain differ in various aspects. The bc_1–aa_3 branch has a significantly higher bioenergetic efficiency than the *bd* branch. It was proposed that the number of protons formally transported across the membrane per two electrons ($H^+/2e^-$) is six for the cytochrome bc_1–aa_3 supercomplex and two for cytochrome *bd* oxidase [18]. Recent experimental studies are in good agreement with this proposal [35]. Although experimental K_m values are not available, the oxygen affinity of cytochrome aa_3 oxidase is assumed to be lower than that of cytochrome *bd* oxidase [18]. Accordingly, cytochrome *bd* oxidase is presumably required under microaerobic conditions, while the bc_1–aa_3 supercomplex predominates under oxygen-sufficient conditions [18,36]. Another difference between the two branches is that cytochrome *bd* oxidase, in contrast to cytochrome aa_3 oxidase, does not require copper ions for activity.

The role and importance of different respiratory complexes and of F_1F_O-ATP synthase were studied in varying detailedness by mutants of *C. glutamicum* ATCC 13032 [37,38]. Most recently, we characterized a strain lacking the *atpBEFHAGDC* genes for F_1F_O-ATP synthase. Although this strain was completely dependent on ATP synthesis by substrate level phosphorylation, it still reached about 50% of the growth rate and 65% of the biomass of the wild type in glucose minimal medium, proving that oxidative phosphorylation is not essential for growth of this organism [39]. Previously, we reported that deletion of either the *ctaD* gene encoding subunit I of cytochrome aa_3 oxidase or of the *qcrCAB* genes encoding the cytochrome bc_1 complex in the wild type ATCC 13032 resulted in strong growth defects in CGXII glucose minimal medium, which could be largely or completely reversed by complementation of the mutants with expression plasmids for *ctaD* and *qcrCAB*, respectively [33]. In both mutants, the bc_1–aa_3 supercomplex is non-functional.

Besides the Δ*ctaD* and Δ*qcr* mutants, we also analysed the phenotype of *cydAB* deletion mutants lacking cytochrome *bd* oxidase [40]. In glucose minimal medium, the Δ*cydAB* mutant grew like the wild type strain ATCC 13032 in the exponential phase, but growth thereafter was reduced. Functional overproduction of cytochrome *bd* oxidase was possible by overexpression of the entire *cydABDC* cluster, whereas expression of the structural genes *cydAB* alone was not sufficient [40]. The genes *cydC* and *cydD* code for an ABC transporter, which in *Escherichia coli* was shown to be required for the formation of active cytochrome *bd* oxidase [41–44]. Overexpression of *cydABDC* in *C. glutamicum* ATCC 13032 reduced the growth rate by about 50% and the biomass yield by about 35%, which might be explained by a shift of the electron flow from the energetically efficient bc_1–aa_3 branch to the energetically inefficient *bd* branch [40].

The effects of a *ctaD* deletion and of a *cydAB* deletion were also studied in *C. glutamicum* ATCC 13869 with respect to growth and proton translocation [35]. The H^+/O ratio of cells grown in a semisynthetic medium decreased from 3.9 in the parent strain to 2.8 in the Δ*ctaD* mutant, whereas it was increased to 5.23 in the Δ*cydAB* mutant.

As outlined above, until now the *C. glutamicum* respiratory mutants were only analysed with respect to their growth properties and H^+/O ratios. In our present study, a very detailed characterization of the Δ*qcr* and Δ*cydAB* mutants of *C. glutamicum* ATCC 13032 was performed, in which we measured the kinetics of glucose and oxygen consumption, the formation of organic acids as by-products, the proton-motive force, and the maintenance coefficient. These results give a more sophisticated understanding of the role of the two respiratory branches. Moreover, we describe for the first time a strain of *C. glutamicum* which lacks both branches of the aerobic respiratory chain. This strain (Δ*cydAB*Δ*qcr*) was named DOOR, which is mnemonic for "devoid of oxygen respiration". As *C. glutamicum* has not been reported to be able of fermentative growth under anaerobic conditions, the possibility to create such a strain was unexpected and its properties provided novel insights into the bioenergetics of this organism.

2. Materials and methods

2.1. Bacterial strains and culture conditions

C. glutamicum strains and plasmids used in this work are listed in Table 1. For analysis of growth, organic acid production, glucose and oxygen consumption, measurement of internal pH, and membrane potential ($\Delta\Psi$), a 5 ml preculture (BHI medium) was inoculated with colonies from a fresh agar plate (BHI agar + 2% (w/v) glucose) and incubated for 8–16 h at 30 °C and 170 rpm. Cells from the preculture were transferred into 20 ml CGXII minimal medium [45] containing 4% (w/v) glucose and cultivated for 16–24 h at 30 °C and 130 rpm. When indicated, the glucose minimal medium was supplemented with peptone (0.5, 1.0, 2.5 or 5.0 $g l^{-1}$). After washing the cells with 0.9% (w/v) NaCl, the main culture with 50 ml CGXII medium with 4% (w/v) glucose was inoculated to give an optical density at 600 nm (OD_{600}) of 1. The CGXII medium was always supplemented with

Table 1
Strains, plasmids and oligonucleotides used in this study.

Strains, plasmids or oligonucleotides	Relevant characteristics or DNA sequence	Source or reference
Strains		
C. glutamicum ATCC 13032	Wild type, biotin-auxotrophic	[62]
C. glutamicum Δ*cydAB*	ATCC 13032 derivative with a deletion of the *cydAB* genes	[40]
C. glutamicum Δ*qcr*	ATCC 13032 derivative with a deletion of the *qcrCAB* genes	[33]
C. glutamicum DOOR	ATCC 13032 derivative with a deletion of the *cydAB* and *qcrCAB* genes	This work
Plasmids		
pK19*mobsacB*	Kan^R; vector for allelic exchange in *C. glutamicum* (pK18 $oriV_{E.c.}$, *sacB*, *lacZ*α)	[63]
pK19*mobsacB*-Δ*qcr*	Kan^R, pK19*mobsacB* derivative containing a 1062 bp overlap extension PCR product (HindIII/XbaI), which covers the upstream region of *qcrC* gene and the downstream region of *qcrB*	[33]
Oligonucleotides		
Δ*cydAB*-for	5′-TATCACAAGGCTGATGATGTATCC	[40]
Δ*cydAB*-rev	5′-GGTCGCTGAGGCGTTGTTCGG	[40]
Δ*qcr*-for	5′-ACTGTCGACCTCAACGTGCCCTACGCAC	[33]
Δ*qcr*-rev	5′-TGAGTCGACCTGCAATTTCAGGAAACTTCC	[33]

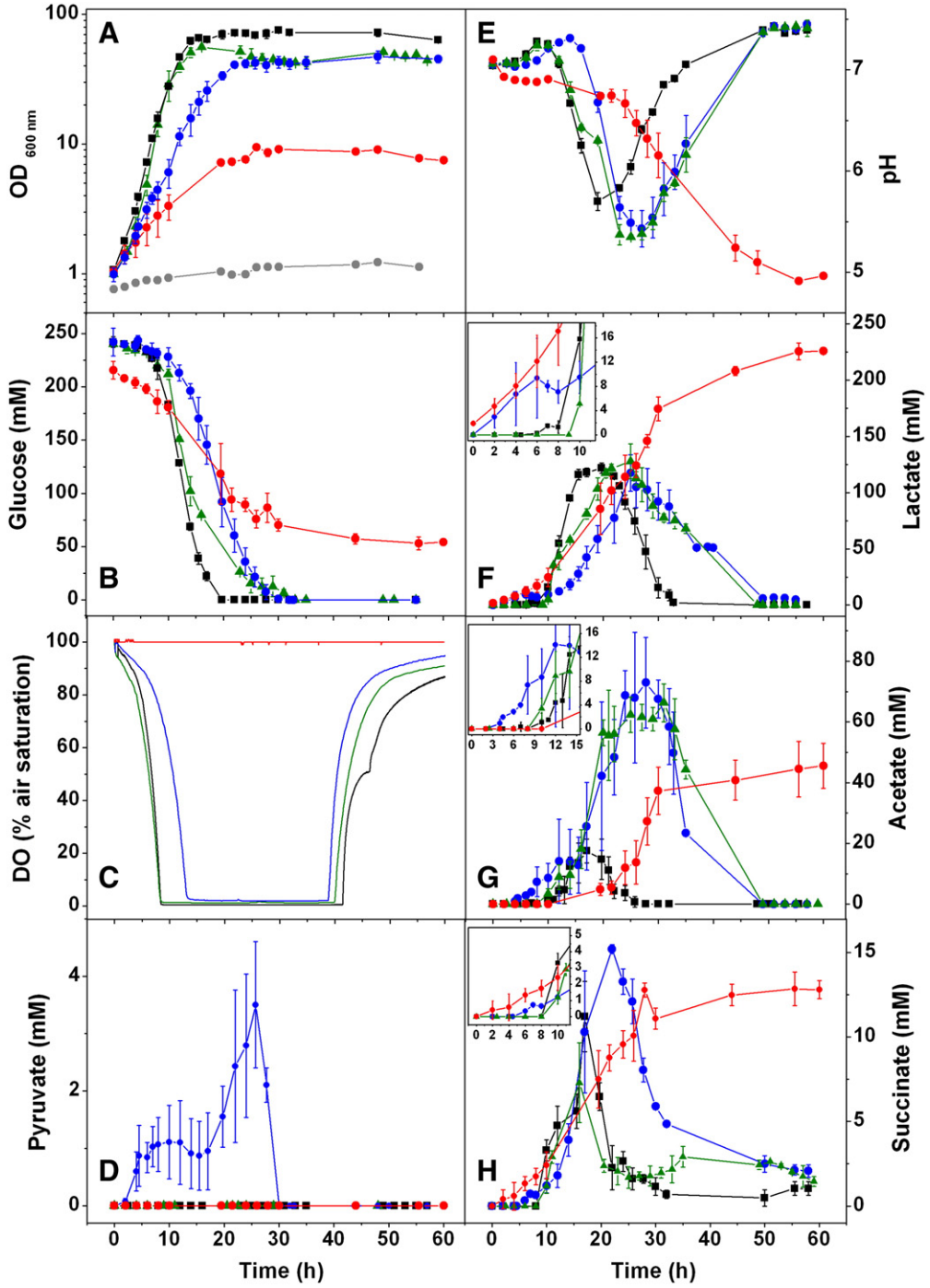

Fig. 1. Comparison of *C. glutamicum* wild type and its Δ*cydAB*, Δ*qcr* and DOOR mutants with respect to growth (A), glucose consumption (B), dissolved oxygen (C), pyruvate formation (D), pH of the supernatant (E), lactate formation (F), acetate formation (G), and succinate formation (H). The strains were cultivated in CGXII minimal medium with 4% (w/v) glucose (A–I) except for the DOOR mutant, which was additionally supplemented with 5 $g l^{-1}$ peptone. The wild type is shown by black squares (-■-) or curves, the Δ*cydAB* mutant by green triangles (-▲-) or curves, the Δ*qcr mutant* by blue circles (-●-) or curves, and the DOOR mutant supplemented with 5 $g l^{-1}$ peptone by red circles or curves (-●-), without peptone by grey symbols (-●-). Mean values and standard deviations from at least three independent cultivations of each strain are shown, except for the DO measurements (panel C), where a representative for three independent experiments is shown.

30 mg l^{-1} 3,4-dihydroxybenzoic acid as iron chelator. When indicated, main cultures were also performed in 50 ml BHI medium supplemented with 4% (w/v) glucose, or in BHI medium supplemented with 4% (w/v) glucose and 21 $g l^{-1}$ MOPS as buffer substance. Main cultures were always grown in baffled 500 ml Erlenmeyer flasks (equipped with a septum for sterile sampling) containing 50 ml medium at 30 °C and 130 rpm. Cells were harvested during the exponential growth phase at an OD_{600} of 3–6 for further analyses.

2.2. Construction of a cydAB–qcrCAB double deletion mutant

The construction of the *C. glutamicum* ATCC 13032 deletion mutants Δ*qcr* and Δ*cydAB* has been described previously [33,40]. For the construction of the DOOR strain lacking both the *cydAB* genes and the *qcrCAB* genes, the plasmid pK19*mobsacB*-Δ*qcr* (Table 1) was transferred by electroporation [46] into competent Δ*cydAB* cells and the transformation mixture was plated on a BHIS agar plate containing

Table 2
Growth parameters, glucose uptake rates (sGUR), pH values and organic acid formation of *C. glutamicum* ATCC 13032 wild type, Δ*cydAB*, Δ*qcr* and the DOOR mutant during cultivation in CGXII minimal medium with 4% (w/v) glucose. The DOOR mutant was supplemented with additionally 5 $g l^{-1}$ peptone and consumed only 160 mM of the 222 mM glucose. Mean values from at least three independent experiments and standard deviation (σ) are given. The values given for lactate, acetate, pyruvate, succinate, fumarate and malate represent the maxima observed during growth; n.d., not detected.

Parameter	Wild type	Δ*cydAB*	Δ*qcr*	DOOR
OD_{600}	75 ± 0.3	56 ± 1.9	47 ± 5.1	9.5 ± 0.5
Cell dry weight (CDW) (g l^{-1})	18.8 ± 0.8	14.1 ± 0.5	11.8 ± 1.3	2.4 ± 0.1
Growth rate (h^{-1})	0.39 ± 0.02	0.37 ± 0.02	0.22 ± 0.03	0.10 ± 0.01
sGUR (nmol min^{-1} mg_{CDW}^{-1})	92 ± 9	94 ± 8	72 ± 12	85 ± 12
pH_{max}	7.43 ± 0.02	7.43 ± 0.01	7.45 ± 0.01	7.10 ± 0.01
pH_{min}	5.70 ± 0.09	5.35 ± 0.02	5.43 ± 0.18	4.91 ± 0.02
$Lactate_{max}$ (mM)	122 ± 5	128 ± 15	118 ± 16	226 ± 1
$Lactate_{max}$ (mmol $(g_{CDW})^{-1}$)	7.4 ± 0.1	10.8 ± 1.5	13.8 ± 2.0	106 ± 4
$Acetate_{max}$ (mM)	17 ± 4	66 ± 6	73 ± 15	46 ± 7
$Acetate_{max}$ (mmol g_{CDW}^{-1})	1.1 ± 0.5	6.2 ± 1.1	7.4 ± 3.4	21 ± 2.9
$Pyruvate_{max}$ (mM)	n.d.	n.d.	3.5 ± 1.1	n.d.
$Pyruvate_{max}$ (mmol g_{CDW}^{-1})	n.d.	n.d.	1.6 ± 1.2	n.d.
$Succinate_{max}$ (mM)	11.1 ± 2.1	7.3 ± 2.4	15.2 ± 0.3	12.9 ± 1.0
$Succinate_{max}$ (mmol g_{CDW}^{-1})	0.70 ± 0.01	0.54 ± 0.03	1.79 ± 0.19	6.03 ± 0.06
$Fumarate_{max}$ (mM)	0.06 ± 0.01	0.95 ± 3.3	1.93 ± 0.34	0.07 ± 0.01
$Fumarate_{max}$ (mmol g_{CDW}^{-1})	0.01 ± 0.01	0.08 ± 0.03	0.19 ± 0.05	0.03 ± 0.01
$Malate_{max}$ (mM)	4.30 ± 1.70	2.43 ± 0.04	3.24 ± 0.41	0 ± 0
$Malate_{max}$ (mmol g_{CDW}^{-1})	0.05 ± 0.01	0.23 ± 0.02	0.31 ± 0.07	0 ± 0

25 μg ml^{-1} kanamycin. After selection for the first and second recombination events, kanamycin-sensitive and sucrose-resistant clones were analysed by colony PCR with the oligonucleotide pairs Δ*cydAB*-for/Δ*cydAB*-rev and Δ*qcr*-for/Δ*qcr*-rev (Table 1) in order to distinguish between Δ*cydAB* and DOOR clones. As expected for a strain which presumably is no longer capable of aerobic respiration, the colonies of the DOOR strain required incubation on the BHIS sucrose plates for about two weeks before they became visible.

2.3. Determination of growth parameters, glucose and organic acids

Growth was followed by measuring the optical density at 600 nm (OD_{600}) with an Ultrospec 500-pro spectrophotometer (Amersham Biotech). The biomass concentration was calculated from OD_{600} values using an experimentally determined correlation factor of 0.25 g_{CDW} l^{-1} for $OD_{600} = 1$ [40]. Quantitative determination of glucose and organic acids in culture supernatants was carried out as described [39].

2.4. Determination of maintenance coefficients

For the determination of maintenance coefficients, chemostat cultivations of *C. glutamicum* ATCC 13032, Δ*cydAB* and Δ*qcrCAB* were performed. The strains were precultivated in shake flasks at 30 °C and 150 rpm in CGXII medium containing 20 g l^{-1} glucose. After 15 h of cultivation 50 ml of the cell suspension was transferred into a 300 ml stirred tank bioreactor (DASGIP, Juelich, Germany) containing 200 ml CGXII medium with 5 g l^{-1} glucose. Cells were grown at 30 °C at a constant pH of 7.0 and a dissolved oxygen concentration of >30%. When glucose was depleted, the chemostat cultivation was started by addition of fresh CGXII medium with 5 g l^{-1} glucose and glucose as the growth-limiting substrate.

In the chemostat experiments, three different dilution rates between 0.05 and 0.3 h^{-1} were applied with a sequential change after 3.5 residence times. The individual dilution rates of the strains were defined based on their maximal growth rates and for each strain at least five different growth rates were performed in biological triplicates. Samples were taken at the end of each dilution rate applied and used for the determination of the cell dry weight. For the determination of the cell dry weight, 2 ml of the culture was transferred into microreaction tubes with known weight (dried for 24 h at 80 °C), and centrifuged for 10 min at 16,000 *g*. After washing the cells with 0.9% NaCl solution, the centrifugation step was repeated and the remaining cell pellet was dried at 80 °C for 24 h, before its dry weight was determined.

The specific glucose uptake rate (q_s) (Eq. (1)) was calculated for each dilution rate, and plotted against the applied growth rate (h^{-1}) for determination of maintenance data, by weighted linear regression function. The intersection of the regression line with the y axis represents the non-growth-associated maintenance coefficient (NGAM), whereas the slope of the regression line represents the growth-associated maintenance coefficient (GAM) [47].

$$q_s = \frac{c_{Glucose} \cdot F_{Medium}}{V \cdot M_{Glucose} \cdot CDW} \quad (1)$$

with

$c_{Glucose}$ glucose concentration in media (g l^{-1})
F_{Medium} feedrate (l h^{-1})
V reactor volume (l)
$M_{Glucose}$ molar mass of glucose (g mol^{-1})
CDW cell dry weight (g l^{-1})

2.5. Measurement of oxygen consumption

Oxygen consumption of growing cultures was determined as described previously [39,48] by following the decrease in dissolved oxygen (DO) using a shake flask reader (SFR) system (PreSens GmbH, Regensburg, Germany). DO (% air saturation) was measured at an interval of 10 min.

Oxygen consumption rates of non-growing cells were measured with a Clarke-type oxygen electrode using a thermostatically controlled,

magnetically stirred 2-ml chamber at 30 °C (Oxygraph, Hansatech Instruments, Germany) and the Oxygraph Plus software. For these experiments, cells growing exponentially in BHI medium with 4% (w/v) glucose were harvested, washed twice with buffer A (250 mM MOPS, 50 mM KCl, 7.35 mM KH_2PO_4, 5.74 mM K_2HPO_4; pH 7.0), and resuspended in the same buffer. These cell suspensions were stored at 4 °C until use. The electrode was calibrated with air-saturated water (100% saturation) and dithionite was added for setting the 0% saturation value. The chamber was filled with 940 µl air-saturated buffer A, and 20 µl air-saturated 500 mM glucose solution (final concentration 10 mM). Oxygen consumption was followed after adding 40 µl of a stock cell suspension in buffer A, to give final OD_{600} of 0.5 to 2.5 in the chamber. Mean values and standard deviations were determined from at least four independent experiments.

2.6. Determination of proton-motive force (pmf)

The determination of the membrane potential ($\Delta\Psi$) and the pH gradient (ΔpH) and the calculation of the proton-motive force (pmf) were performed as described previously [39,49].

3. Results

3.1. Growth, maintenance coefficients and glucose consumption of the ΔcydAB and Δqcr mutants of C. glutamicum

We previously described some growth properties of Δ*qcr* and Δ*cydAB* mutants of *C. glutamicum* ATCC 13032 [24,26,32], however, a detailed analysis of these strains with respect to oxygen and carbon source consumption rates, by-product formation, and bioenergetic parameters was missing and is now presented here. For these experiments, the two mutant strains and the wild type were cultivated in CGXII minimal medium with 4% (w/v) glucose as carbon and energy source. As shown in Fig. 1A and Table 2, the absence of cytochrome *bd* oxidase reduced the growth rate only marginally (0.37 h^{-1} vs. 0.39 h^{-1} for the wild type), whereas the absence of a functional bc_1–aa_3 supercomplex led to an almost 50% decrease (0.22 h^{-1}). The Δ*cydAB* mutant formed 75% of the biomass of the wild type (14.1 ± 0.5 vs. 18.8 ± 0.3 g_{CDW} l^{-1}), whereas the Δ*qcr* mutant reached only 63% (11.8 ± 1.3 g_{CDW} l^{-1}). These results showed that the cytochrome bc_1–aa_3 supercomplex, in contrast to the cytochrome *bd* oxidase, is of major importance for aerobic growth of *C. glutamicum*.

As shown in Fig. 1B, glucose was continuously taken up by all three strains and completely consumed. As expected from the growth rates, the Δ*cydAB* mutant exhibited a similar specific glucose uptake rate (sGUR) as the wild type of around 90 nmol min^{-1} mg_{CDW}^{-1} (Table 2). However, at the end of the exponential growth phase glucose consumption by the Δ*cydAB* mutant slowed down and complete consumption was reached only after about 35 h, whereas the wild type required only 20 h. Chemostat cultivations were used to determine the non-growth-associated maintenance (NGAM) and the growth-associated maintenance (GAM) coefficients (Fig. 2) [47]. The wild type and the Δ*cydAB* mutant showed comparable NGAM coefficients of 0.08 ± 0.02 and 0.07 ± 0.03 $mmol_{glucose}$ g_{CDW}^{-1} h^{-1}, respectively, whereas the GAM coefficient of Δ*cydAB* mutant (10.13 ± 0.23 $mmol_{glucose}$ g_{CDW}^{-1}) was 31% higher than the one of the wild type (7.74 ± 0.16 $mmol_{glucose}$ g_{CDW}^{-1} h^{-1}). The wild type values were comparable to those described for other microbes (Table 3).

The sGUR of the Δ*qcr* mutant was decreased to 72 ± 12 nmol min^{-1} mg_{CDW}^{-1} and complete glucose consumption was reached after 35 h. The NGAM coefficient of the Δ*qcr* mutant was increased almost by a factor of five (0.39 ± 0.08 $mmol_{glucose}$ g_{CDW}^{-1} h^{-1}) and the GAM coefficient by a factor of 1.7 (12.96 ± 0.7 $mmol_{glucose}$ g_{CDW}^{-1} h^{-1}), confirming that the Δ*qcr* mutant has a reduced bioenergetic efficiency with respect to pmf generation and P/O ratio. These results show that the absence of the *bd* branch limits glucose consumption only in the late growth phases, whereas the absence of the bc_1–aa_3 branch has a negative effect already in the exponential growth phase. The increased GAM coefficient of the Δ*cydAB* mutant suggests that also cytochrome *bd* oxidase plays a role in energy conservation during exponential growth.

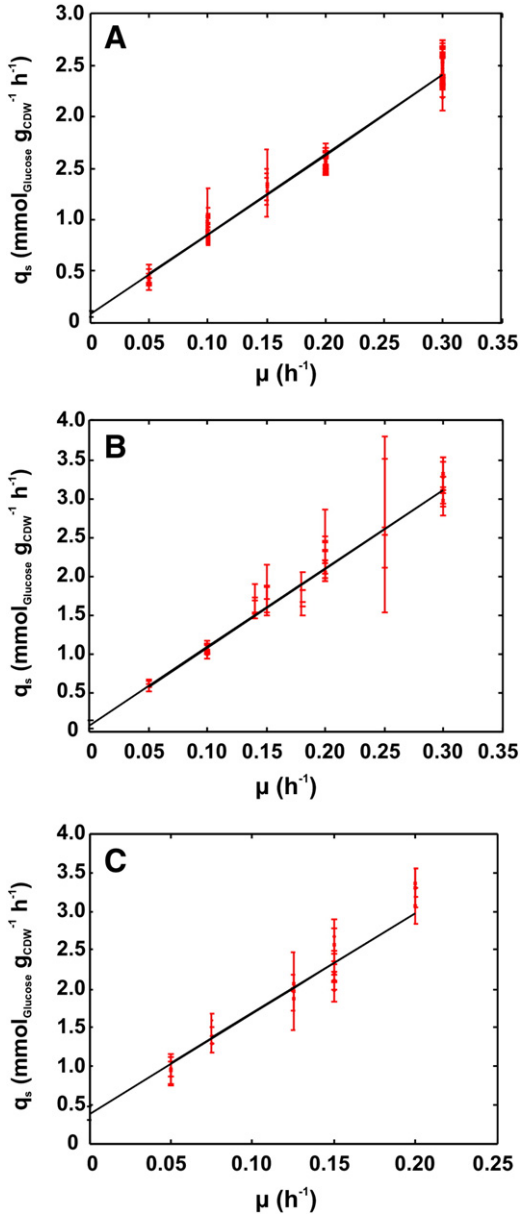

Fig. 2. Determination of maintenance coefficients for *C. glutamicum* wild-type strain ATCC 13032 (A) and its derivatives Δ*cydAB* (B) and Δ*qcr* (C). The strains were cultivated in a chemostat using CGXII minimal medium and glucose as the growth-limiting substrate as described in Materials and methods. For the wild type, Δ*cydAB* and Δ*qcrCAB*, NGAM coefficents of 0.08 ± 0.02, 0.07 ± 0.03 and 0.39 ± 0.08 $mmol_{Glucose}$ g_{CDW}^{-1} h^{-1} and GAM coefficients of 7.74 ± 0.16, 10.13 ± 0.23 and 12.96 ± 0.7 $mmol_{Glucose}$ g_{CDW}^{-1} were determined, respectively.

Table 3
Non-growth and growth-associated maintenance coefficients (NGAM and GAM, respectively) of different microbes. The data for *E. coli* were taken from [64] and [65], the data for *B. subtilis* from [66], and the data for *S. cerevisiae* from [67].

Parameter	*C. glutamicum*	*E. coli*	*B. subtilis*	*S. cerevisiae*
NGAM $mmol_{Glucose}$ g_{CDW}^{-1} h^{-1}	0.08	0.31	0.39	0.07
GAM $mmol_{Glucose}$ g_{CDW}^{-1}	7.74	10.66	11.5	7.41

3.2. Organic acid formation and oxygen consumption by the ΔcydAB and Δqcr mutants

Besides glucose consumption, we also measured the formation of overflow metabolites during growth and observed marked differences between the wild type and the two mutant strains. As shown in Fig. 1D, the Δ*qcr* mutant secreted pyruvate into the medium right from the start of the cultivation, reaching maximal concentrations of about 4 mM after 25 h, after which pyruvate was completely consumed again. In contrast, neither the wild type nor the Δ*cydAB* mutant secreted detectable amounts of pyruvate throughout the cultivation.

As shown in Fig. 1F and Table 2, lactate was the major organic acid secreted by all analysed strains with comparable maximal titers of 122 ± 5 mM (wt after 22 h), 128 ± 15 mM (Δ*cydAB* mutant after 25 h), and 118 ± 16 mM (Δ*qcr* mutant after 25 h). Subsequently, lactate was consumed again by all three strains. The different kinetics of lactate formation and consumption correlated with the differences in growth and also with the decrease and subsequent increase of the pH of the medium (Fig. 1E). Due to reduced growth, the biomass-specific lactate concentration was increased for both deletion mutants compared to the wild type (Table 2).

In order to determine whether lactate secretion is correlated with oxygen availability, oxygen consumption was measured by optical sensing of the dissolved oxygen (DO) concentration (see Materials and methods). As shown in Fig. 1C, oxygen consumption by the Δ*cydAB* culture was comparable to the wild type and after about 7–8 h, the DO concentration of both cultures approached zero and oxygen became limiting. This correlated with the beginning of lactate secretion. The Δ*qcr* culture showed slower oxygen consumption and DO concentrations approached zero after 14–15 h of cultivation. Notably, lactate was secreted by the Δ*qcr* strain right from start of the cultivation, when oxygen was not limited. Besides pyruvate and lactate, also acetate and succinate were found to be excreted. In the case of acetate (Fig. 1G, Table 2), the maximal concentration in the supernatant was 4-fold higher for both the Δ*cydAB* mutant (66 ± 6 mM after 26–30 h) and the Δ*qcr* mutant (73 ± 15 mM after 26–30 h) compared to the wild type (17 ± 4 mM after 15–20 h). As in the case of lactate, the secreted acetate was completely consumed again in the later phases of cultivation. In the case of succinate (Fig. 1H, Table 2), the Δ*cydAB* mutant formed less than the wild type (7.3 ± 2.4 mM vs. 11.1 ± 2.1 mM), whereas the Δ*qcr* mutant formed somewhat more (15.2 ± 0.3 mM), but the differences were small compared to acetate. As shown in Table 2, also some malate and some fumarate were detected in the supernatant of the three strains.

3.3. Construction and growth properties on complex medium of a mutant lacking both the bd and the bc_1–aa_3 branch of the respiratory chain

According to literature, energy metabolism of *C. glutamicum* is restricted to aerobic respiration and anaerobic respiration by nitrate reduction to nitrite. Consequently, a *C. glutamicum* strain lacking both branches of the aerobic respiratory chain should not be viable. Nevertheless, we made attempts to delete the *qcrCAB* genes in the Δ*cydAB* mutant background. Surprisingly, a few tiny colonies were obtained after about 14 days of incubation on the BHIS sucrose plates used for cultivation after the second homologous recombination event. The absence of both the *cydAB* genes and the *qcrCAB* genes in one of the clones was confirmed by PCR and the corresponding strain was named DOOR, which is mnemonic for "devoid of oxygen respiration".

As shown in Fig. 3A, the DOOR mutant was able to grow in BHI medium supplemented with 4% (w/v) glucose from an initial OD_{600} of 0.73 ± 0.06 to a final OD_{600} of 2.44 ± 0.06. In parallel, the pH of the medium decreased from 7.0 to below 5 (Fig. 3B), at which growth of *C. glutamicum* is no longer possible [49,50]. Therefore, the medium was buffered by addition of 21 $g l^{-1}$ MOPS (100 mM). In the presence of MOPS, the DOOR strain grew to a final OD_{600} of 8.66 ± 0.47 and the acidification of the medium was retarded, but not stopped (Fig. 3). The growth rate of the DOOR mutant was 0.18 ± 0.01 h^{-1}, both in the absence and in the presence of MOPS. When the wild type was cultured under the same conditions, it reached a final OD_{600} of about 53 ± 1 and a growth rate of 0.58 ± 0.04 h^{-1}, independent of the absence or presence of MOPS. In the case of the wild type, the pH also initially dropped to about 6, but then increased again to reach a final value of about 8. MOPS delayed the pH increase (Fig. 3B). The results obtained for the DOOR mutant show for the first time that the terminal oxidase activities are not essential for aerobic growth of *C. glutamicum*. The complementation of the DOOR mutant with plasmids for expression of either the *qcrCAB* genes (pJC1-qcrB$_{Strep;}$ [26]) or the *cydABDC* genes (pEKEx2-*cydABDC*, [40]) was successful. In both cases, the growth behaviour on glucose minimal medium could be restored to that of the single mutants Δ*cydAB* and Δ*qcr* (data not shown), showing that the growth defect of the DOOR strain was due to the lack of the respiratory branches rather than to secondary mutations.

3.4. Growth and glucose consumption of the DOOR mutant in glucose minimal medium supplemented with peptone

When the DOOR strain was inoculated into CGXII minimal medium containing 4% (w/v) glucose as sole carbon source, almost no growth occurred (Fig. 1A). However, as shown in Fig. 4, growth was made possible by addition of peptone. Increasing peptone concentrations (0.5, 1.0, 2.5 and 5.0 $g l^{-1}$) led to increases in growth rate and biomass formation, whereas higher concentrations did not stimulate growth any further. In contrast to the DOOR strain, addition of 5 $g l^{-1}$ peptone had only marginal effects on the growth of the wild type, the Δ*cydAB* mutant and the Δ*qcr* mutant (data not shown).

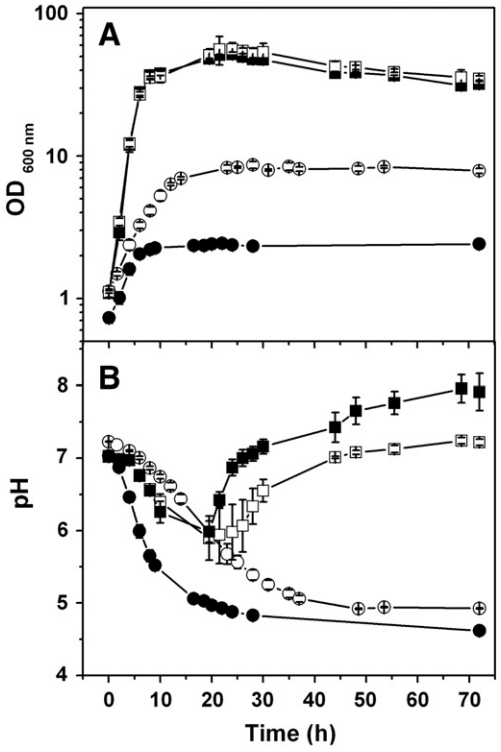

Fig. 3. Comparison of *C. glutamicum* wild type and its DOOR mutant with respect to growth (A) and the pH of the supernatant (B) during cultivation in BHI medium (containing 0.2% w/v glucose) supplemented with 4% (w/v) glucose and either 0 mM MOPS (closed symbols) or 100 mM MOPS (open symbols). The wild type is shown by squares (-■-,-□-) and the DOOR mutant is shown by circles (-●-,-○-). Mean values and standard deviations from at least three independent cultivations are shown.

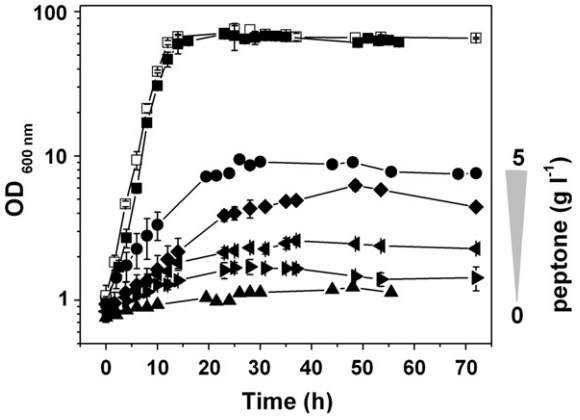

Fig. 4. Influence of peptone on growth of *C. glutamicum* wild type and the DOOR mutant in CGXII medium with 4% (w/v) glucose. Growth of the wild type in the absence and presence of 5 g l^{-1} peptone is shown by filled squares and open squares, respectively. Growth of the DOOR mutant supplemented with different peptone concentrations (g l^{-1}) is indicated as follows: ▲, 0 g l^{-1} peptone; ►, 0.5 g l^{-1} peptone; , 1 g l^{-1} peptone; ♦, 2.5 g l^{-1} peptone; ●, 5 g l^{-1} peptone. Mean values and standard deviation from at least three independent cultivations of each strain are shown.

Furthermore, growth on peptone as sole carbon and energy source was not possible for the DOOR mutant and only minimal growth was observed for the wild type (data not shown).

Growth of the DOOR strain was then analysed in detail during cultivation in CGXII minimal medium containing 4% (w/v) glucose and 5 gl^{-1} peptone, where a final OD_{600} of 9.5 ± 0.5 was reached and a growth rate of 0.10 ± 0.01 h^{-1} (Fig. 1A). In contrast to the strains reported above, glucose was not completely consumed by the DOOR mutant and stopped when about 50 mM glucose was still left (Fig. 1B). This behaviour is presumably caused by the acidification of the medium to a pH of about 5 (Fig. 1E). The sGUR of the DOOR strain was 85 ± 12 nmol min^{-1} mg_{CDW}^{-1} and thus in the range of the wild type (Table 2). Interestingly, although the DOOR mutant did not grow in CGXII glucose medium without peptone, it consumed the glucose with a rate only 30% below that of the wild type (63 ± 38 nmol min^{-1} mg_{CDW}^{-1}) in such a medium (data not shown). Hence one can conclude that the absence of the aerobic respiratory chain influences glucose uptake by *C. glutamicum* only slightly.

3.5. Fermentative metabolism of the DOOR mutant during aerobic growth in glucose minimal medium supplemented with peptone

In order to analyse if the DOOR mutant shows indeed no oxygen-dependent respiration, the dissolved oxygen concentration (DO) was measured throughout growth. As shown in Fig. 1C, no decrease of the DO concentration was observed over a period of 60 h, indicating that there is no alternative terminal oxidase activity left in the DOOR strain. As shown in Fig. 1F and H, the DOOR mutant started to secrete lactate and succinate immediately after inoculation and, after about 10 h, also acetate (Fig. 1G). In contrast to the wild type, the Δ*cydAB* mutant and the Δ*qcr* mutant, these acids were not consumed again, but accumulated to final concentrations of 226 ± 1 mM lactate, 13 ± 1 mM succinate, and 46 ± 7 mM acetate (Fig. 1F–H and Table 2). Neither pyruvate nor malate and only negligible amounts of fumarate were found in the supernatant of the DOOR culture (Table 2). These results show that the DOOR mutant performs a type of mixed acid fermentation under aerobic conditions. When calculating the carbon balance, the carbon present in the products lactate, succinate, acetate, and CO_2 (CO_2 was calculated as the difference of the acetate concentration minus the succinate concentration) corresponded to 89% of the carbon consumed as glucose. The residual 11% could be converted to biomass (maximally about 9%) or yet unidentified minor products. The consumption of carbon from peptone was not quantified and therefore neglected in this calculation.

3.6. Oxygen consumption rates of the DOOR mutant and reference strains

The fact that no decrease of the DO concentration was observed during aerobic growth of the DOOR strain suggested that its oxygen consumption is very low or even absent. Nevertheless, we also determined oxygen uptake rates (OUR) of resting cell suspensions prepared from cells in the exponential growth phase using a Clark-type oxygen electrode (see Materials and methods). The results are summarized in Table 4.

In the presence of 10 mM glucose, the wild type showed an OUR of 138 ± 8 nmol min^{-1} $mg_{Protein}^{-1}$, the Δ*cydAB* mutant of 165 ± 6 nmol min^{-1} $mg_{Protein}^{-1}$, the Δ*qcr* mutant of 109 ± 20 nmol min^{-1} $mg_{Protein}^{-1}$, and the ΔF_1F_O mutant described previously of 370 ± 46 nmol min^{-1} $mg_{Protein}^{-1}$. The highly increased OUR of the ΔF_1F_O mutant correlates with a highly increased glucose uptake rate under oxygen excess [39]. In contrast to the strains reported above, the OUR of the DOOR strain was found to be only 3 ± 1 nmol min^{-1} $mg_{Protein}^{-1}$, which corresponds to 2% of the OUR of the wild type. The residual oxygen consumption is probably caused by oxygen-consuming enzymatic reactions not related to respiration, such as monooxygenases or dioxygenases. All strains except DOOR showed relatively high OUR rates also in the absence of glucose in the assay buffer. This endogenous respiration, which was reported before [51], amounted to about 50% of the respiration rate in the presence of glucose (Table 4). By starving the cells for 3–4 h, endogenous respiration could be strongly decreased, indicating that it is due to degradation of a storage compound. In *C. glutamicum*, glycogen [52] is the prime candidate responsible for endogeneous respiration.

3.7. Proton-motive force of the ΔcydAB, Δqcr and DOOR mutants of C. glutamicum

The pH gradient (ΔpH) and the membrane potential (ΔΨ) of the respiratory mutants and the wild type were measured at external pH values of 7.0 and 6.0 and the results are summarized in Table 5. At neutral pH, the Δ*cydAB* mutant showed a slightly increased internal pH and a slightly decreased membrane potential compared to the wild type, resulting in almost identical pmf values (−228 mV vs. −224 mV). At an external pH of 6, the ΔpH of the Δ*cydAB* mutant was the same as for the wild type, but the membrane potential was 20% higher, resulting in a 13% increased pmf (−225 mV vs. −198 mV). The pmf of the Δ*qcr* mutant was 12% lower than that of the wild type at pH 7, which was due to a decreased membrane potential (−168 mV vs. −192 mV). At pH 6, the pmf of the Δ*qcr* mutant was 8% below that of the wild type, due to a more acidic internal pH and a resulting lower ΔpH (0.89 vs. 1.09). In the case of the DOOR strain, the largest differences to the wild type were observed. At pH 7, the internal pH was 0.33 pH units lower (7.18 vs. 7.51) and the membrane potential almost 50 mV. This resulted in a decrease of the pmf by 31% (−154 mV vs. −224 mV). At pH 6, the decrease of the pmf was also 28% (−143 mV vs. −198 mV), due a strongly

Table 4
Oxygen uptake rates of different *C. glutamicum* strains. For experimental details see Materials and methods. Starved cells were obtained by incubating cells at 30°C in the assay buffer without glucose. n, number of independent experiments; n.d., not determined.

Strain	Oxygen uptake rate (nmol min^{-1} $mg_{Protein}^{-1}$)		
	+ 10 mM glucose	− Glucose	Starved
Wild type	138 ± 8 (n = 4)	60 ± 8 (n = 6)	27 ± 4 (n = 3)
Δ*cydAB*	165 ± 6 (n = 4)	82 ± 22 (n = 4)	32 ± 9 (n = 3)
Δ*qcr*	109 ± 20 (n = 4)	53 ± 12 (n = 5)	15 ± 3 (n = 2)
ΔF_1F_O	370 ± 46 (n = 5)	145 ± 17 (n = 2)	39 ± 3 (n = 2)
DOOR	3.0 ± 0.9 (n = 4)	1.6 ± 0.3 (n = 4)	n.d.

Table 5
Bioenergetic parameters (pH gradient ΔpH, membrane potential ΔΨ, and proton-motive force pmf) determined for *C. glutamicum* wild type and the mutant strains Δ*cydAB*, Δ*qcr* and DOOR. Mean values from at least three independent experiments and standard deviations are shown.

Strain	pH_{ex}	pH_{in}	ΔpH	ΔpH (mV)	ΔΨ (mV)	pmf (mV)
Wild type	7.0	7.51 ± 0.07	0.50 ± 0.05	−30 ± 3	−192 ± 2	−224 ± 3
Δ*cydAB*	7.0	7.72 ± 0.01	0.72 ± 0.01	−43 ± 1	−184 ± 9	−228 ± 9
Δ*qcr*	7.0	7.46 ± 0.03	0.46 ± 0.06	−28 ± 4	−168 ± 1	−197 ± 2
DOOR	7.0	7.18 ± 0.12	0.18 ± 0.11	−11 ± 7	−143 ± 2	−154 ± 9
Wild type	6.0	7.09 ± 0.04	1.09 ± 0.04	−65 ± 3	−131 ± 7	−198 ± 9
Δ*cydAB*	6.0	7.10 ± 0.09	1.10 ± 0.16	−66 ± 6	−158 ± 6	−225 ± 11
Δ*qcr*	6.0	6.89 ± 0.06	0.89 ± 0.06	−53 ± 3	−128 ± 4	−182 ± 1
DOOR	6.0	6.51 ± 0.24	0.51 ± 0.24	−30 ± 15	−113 ± 4	−143 ± 13

decreased internal pH (6.51 vs. 7.09) and a 14% lowered membrane potential (−113 mV vs. −131 mV).

4. Discussion

In this study, three defined respiratory mutants of *C. glutamicum* were analysed in detail with respect to growth, formation of organic acids, oxygen consumption rates, maintenance coefficients and bioenergetic parameters. Based on the properties of the mutants Δ*cydAB* and Δ*qcr*, the roles of cytochrome *bd* oxidase and the cytochrome bc_1–aa_3 supercomplex for aerobic metabolism will be discussed. In contrast to these mutants, the DOOR mutant (Δ*cydAB* Δ*qcr*) cannot perform aerobic respiration anymore and the consequences of this defect for energy metabolism will be discussed.

4.1. Distinct roles of cytochrome bd oxidase and the cytochrome bc_1–aa_3 supercomplex

The Δ*cydAB* mutant showed lowered glucose consumption in the late exponential phase and required 15 h longer than the wild type for complete consumption. Furthermore, four times more acetate was secreted (and subsequently metabolized again) by the Δ*cydAB* mutant, corresponding to a 6-fold increase in biomass-specific acetate formation (6.2 ± 1.1 vs. 1.1 ± 0.5 mmol g_{CDW}^{-1}). These phenotypes hint at a limited respiratory capacity of the Δ*cydAB* mutant in the late stages of growth, where oxygen is limited under the cultivation conditions employed in our study. A restricted respiratory capacity could result from the lack of the *bd* branch and the presumably lower oxygen affinity of the aa_3 oxidase compared to the *bd* oxidase. Our interpretation is in accord with studies of a *cydAB* mutant of *Mycobacterium smegmatis*, which showed the same growth behaviour as the wild type when cultivated at constant DO values of 21% and 5%, but inhibited growth at 1% and 0.5% DO [53]. As mentioned in the Introduction, corynebacteria and mycobacteria are closely related phylogenetically and the respiratory chains bear many similarities.

Depending on the fraction of electrons that are transferred by cytochrome *bd* oxidase to oxygen in the wild type, an increased energetic efficiency might be expected for a Δ*cydAB* mutant in which all electrons should be transferred to oxygen via the bc_1–aa_3 supercomplex, which is supported by the increased H^+/O stoichiometry of the Δ*cydAB* mutant of strain ATCC 13869 [35]. The observation that the non-growth-associated maintenance coefficient of the Δ*cydAB* mutant was comparable to that of the wild type argues against an increased energetic efficiency of this strain and suggests that only a minor fraction of the reducing equivalents is transferred to oxygen via this oxidase. On the other hand, the growth-associated maintenance coefficient was 30% higher in the Δ*cydAB* mutant (Fig. 2), supporting a role of cytochrome *bd* oxidase in energy conservation during exponential growth. A convincing explanation for this discrepancy cannot be given at present.

The growth properties of Δ*qcr* mutant confirmed that the cytochrome bc_1–aa_3 supercomplex is of major importance for aerobic growth of *C. glutamicum*. Again, this is in accordance with studies in *M. smegmatis*, where deletion of *qcrCAB* or of *ctaC* resulted in severe growth defects under oxygen-saturated culture conditions [54]. A peculiar feature of the *C. glutamicum* Δ*qcr* mutant was that it excreted pyruvate and lactate right from the start of the cultivation, although its specific glucose uptake rate was 22% lower than for the wild type. This suggests that the mutant had a limited respiratory capacity already when oxygen was in excess, causing a metabolic blockage at the pyruvate dehydrogenase complex. As a consequence, pyruvate accumulated and was either secreted, or reduced to lactate. The reduced glucose uptake rate of the Δ*qcr* mutant was reflected by a 21% reduced oxygen uptake rate compared to the wild type (Table 4). In our previous study of a ΔF_1F_O mutant of *C. glutamicum*, we also observed pyruvate secretion under conditions of oxygen excess. In this case, a highly increased glucose uptake rate caused pyruvate accumulation [39], even though the oxygen uptake rate of the ΔF_1F_O mutant was 2.7-fold higher than for the wild type (Table 4). Thus, the pyruvate dehydrogenase complex appears to be an important bottleneck when flux through glycolysis is very high or when the respiratory capacity is limited. In agreement with the expectation that the lack of the major coupling site of the aerobic respiratory chain should lead to a reduced bioenergetic efficiency, the pmf of the Δ*qcr* mutant was 8–12% lower than in the wild type and the maintenance coefficients were much higher than for the wild type.

4.2. The DOOR mutant of C. glutamicum behaves like a fermenting bacterium and confirms the absence of a third terminal oxidase

To our surprise, we were able to create a *C. glutamicum* mutant (strain DOOR) that lacks both the cytochrome bc_1 complex and the cytochrome *bd* oxidase. Growth in glucose minimal medium required supplementation with peptone. Whereas the DO concentration decreased to zero in the exponential growth phase of the wild type and the Δ*cydAB* and Δ*qcr* mutants, it remained at 100% in the case of the DOOR strain. In agreement, the DOOR strain showed an oxygen uptake rate of only 2% of the wild type rate (138 nmol min^{-1} $mg_{Protein}^{-1}$; Table 4). Thus, *C. glutamicum* does not possess a third terminal oxidase besides cytochrome aa_3 and cytochrome *bd*, as suggested in a previous study [27].

The measurement of organic acids in the supernatant revealed that the DOOR strain performed a fermentative type of catabolism. The consumed glucose (100% carbon) was converted to lactate (70%), acetate (9.4%), succinate (5%), and carbon dioxide (3.4%). The residual carbon (12%) is incorporated into biomass and/or converted into yet unidentified products. Succinate formation clearly indicates that a partially reductive tricarboxylic acid cycle is operative in the DOOR strain, by which oxaloacetate is reduced to succinate via malate dehydrogenase, fumarase, and succinate:menaquinone oxidoreductase (succinate dehydrogenase). The observation that *C. glutamicum* forms lactate and minor amounts of succinate under oxygen-limited growth conditions was made almost two decades ago [55]. Later it was shown that under oxygen-deprived conditions *C. glutamicum* did not grow, but converted glucose to lactate, succinate, and acetate with a yield of 90% [31]. Whereas in these two studies oxygen limitation or the absence of oxygen was the reason for the conversion of glucose to organic acids, in the case of the DOOR strain it is the absence of a functional respiratory chain in the presence of plenty of oxygen. When the DOOR strain was incubated under anaerobic conditions, it metabolized glucose like the wild type (data not shown).

The fact that the DOOR strain did not grow in glucose minimal medium unless peptone was added points to an energy limitation which can be overcome by the provision of amino acids and peptides. As about 50% of the cell dry weight is made up of proteins and protein synthesis is a major energy-consuming process within the cell, significant energy and carbon savings are made possible by uptake of peptides and amino acids compared to de-novo synthesis. When considering energy conservation in the DOOR strain, ATP can be formed by substrate

level phosphorylation in glycolysis by 3-phosphoglycerate kinase and pyruvate kinase as well as during acetate formation from acetyl-CoA by acetate kinase. Succinate formation on the other hand consumes an ATP equivalent as it requires carboxylation of PEP or pyruvate to oxaloacetate. The reduction of fumarate to succinate by menaquinol via the dihaem succinate:menaquinol oxidoreductase of *C. glutamicum* [19] is presumably coupled to the generation of a proton-motive force, as discussed previously [18] and shown for the related enzymes from *Bacillus subtilis* [56] and *Bacillus licheniformis* [57].

The only alternative to succinate:menaquinone oxidoreductase for building up proton-motive force in the DOOR mutant is to our knowledge F_1F_O-ATP synthase, which could couple ATP hydrolysis to proton extrusion. It is known for *Enteroccous hirae* and related streptococci, which possess a fermentative, non-respiratory type of catabolism in which glucose is converted to lactic acid, that the ATP produced by substrate-level phosphorylation is hydrolyzed by F_1F_O-ATP synthase to generate proton-motive force [58]. However, whereas these organisms have evolved as fermentatives and probably have adapted the rate of ATP hydrolysis by F_1F_O-ATP synthase to their requirements, *C. glutamicum* strongly prefers an aerobic respiratory mode of energy metabolism and its F_1F_O-ATP synthase is used to drive ATP synthesis by proton import rather than proton export by ATP hydrolysis. If the rate of ATP hydrolysis is too high, it might be inhibitory for growth. The issue of proton export by F_1F_O-ATP synthase is also closely related to pH homeostasis (for a recent review see [59]). The *C. glutamicum* DOOR mutant, which rapidly acidifies the medium due to acid formation, has severe problems to maintain the cytoplasmic pH near neutral, as indicated by the ΔpH measurements, in which it showed much lower internal pH values than the other strains (see Table 5). Thus, proton export by F_1F_O-ATP synthase might be essential for pH homeostasis (and generation of the membrane potential), but on the other hand limit growth.

To our knowledge, a deletion of all known branches of the aerobic respiratory chain was reported before only for *E. coli*, which in contrast to *C. glutamicum* is capable of anaerobic growth by mixed acid fermentation. In the *Escherichia coli* ECOM3 mutant (*E. coli* cytochrome oxidase mutant), the genes *cydAB*, *cyoABCD* and *cbdAB* for the three known terminal oxidases were deleted [60]. The strain was initially unable to grow in glucose minimal medium unless supplemented with a full mixture of amino acids, similar to the *C. glutamicum* DOOR strain. The ECOM3 strain still showed significant oxygen consumption, which was reported to be due to the activity of the *ygiN* gene product, annotated as quinol monooxygenase [61]. *C. glutamicum* does not possess a YgiN homolog and the DOOR strain showed almost no oxygen uptake. By adaptive evolution, derivatives of the ECOM3 strain were obtained (ECOM31, 32, and 33) that were able to grow in glucose minimal medium without amino acid supplementation and with a growth rate comparable to that observed for the wild type cultured under anoxic conditions (0.45 h^{-1}). The phenotypes of the adapted strains differed, with ECOM31 producing D-lactate as sole fermentation product, while ECOM32 and ECOM33 exhibited mixed acid fermentation under aerobic conditions with lactate remaining the major product. It remains to be tested whether a similar type of evolution is possible for the DOOR strain of *C. glutamicum*.

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