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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]