Role of Cytochrome *bd* Oxidase from *Corynebacterium glutamicum* in Growth and Lysine Production[▽]

Armin Kabus, Axel Niebisch, and Michael Bott*

Institut für Biotechnologie 1, Forschungszentrum Jülich, D-52425 Jülich, Germany

Received 2 August 2006/Accepted 19 November 2006

Corynebacterium glutamicum possesses two terminal oxidases, cytochrome *aa*₃ and cytochrome *bd*. Cytochrome *aa*₃ forms a supercomplex with the cytochrome *bc*₁ complex, which contains an unusual diheme cytochrome *c*₁. Both the *bc*₁-*aa*₃ supercomplex and cytochrome *bd* transfer reducing equivalents from menaquinol to oxygen; however, they differ in their proton translocation efficiency by a factor of three. Here, we analyzed the role of cytochrome *bd* for growth and lysine production. When cultivated in glucose minimal medium, a *cydAB* deletion mutant of *C. glutamicum* ATCC 13032 grew like the wild type in the exponential phase, but growth thereafter was inhibited, leading to a biomass formation 40% less than that of the wild type. Constitutive overproduction of functional cytochrome *bd* oxidase in ATCC 13032 led to a reduction of the growth rate by ~45% and of the maximal biomass by ~35%, presumably as a consequence of increased electron flow through the inefficient cytochrome *bd* oxidase. In the L-lysine-producing *C. glutamicum* strain MH20-22B, deletion of the *cydAB* genes had only minor effects on growth rate and biomass formation, but lysine production was increased by ~12%. Thus, the respiratory chain was shown to be a target for improving amino acid production by *C. glutamicum*.

Corynebacterium glutamicum is an aerobic gram-positive soil bacterium (8) that is used for the large-scale biotechnological production of amino acids, in particular L-glutamate (17) and L-lysine (12, 16). Several studies have indicated the importance of proper oxygenation conditions under manufacturing operations to attain high amino acid yields (2, 29, 38). In addition, overexpression of the *glbO* gene encoding a hemoglobin-like protein was shown to improve lysine synthesis (21). Biochemical and genetic studies revealed that *C. glutamicum* possesses a branched respiratory chain with two terminal oxidases (for review see references 3 and 4). The reducing equivalents formed by the oxidation of substrates are initially transferred to menaquinone by several dehydrogenases, including a type II NADH dehydrogenase, succinate dehydrogenase, malate:quinone oxidoreductase, and pyruvate:quinone oxidoreductase. Reoxidation of menaquinol is catalyzed either by the cytochrome *bc*₁ complex, which passes the electrons to the terminal oxidase cytochrome *aa*₃, or by cytochrome *bd* oxidase (Fig. 1). The cytochrome *bc*₁ complex is encoded by the *qcrCAB* genes encoding cytochrome *c*₁, Rieske iron-sulfur protein, and cytochrome *b*, respectively (22). Cytochrome *c*₁ is unusual in that it contains two covalently bound heme groups (22, 34) and represents the only *c*-type cytochrome in *C. glutamicum*. In accord with our suggestion that the second heme group of cytochrome *c*₁ takes over the function of a separate cytochrome *c* in electron transfer to cytochrome *aa*₃, we recently demonstrated that the *bc*₁ complex and cytochrome *aa*₃ oxidase form a supercomplex (23), which presumably allows a high rate of electron transfer between the two complexes. It was also shown that

cytochrome *aa*₃ oxidase consists of four subunits encoded by the genes *ctaD* (subunit I), *ctaC* (subunit II), *ctaE* (subunit III), and *ctaF* (subunit IV) (23). The last three genes are located immediately upstream of *qcrCAB*, whereas *ctaD* is located separately 345 kb upstream of *ctaC*, as revealed by the genome sequence of *C. glutamicum* (13). The cytochrome *bd* oxidase consists of two subunits encoded by *cydA* (subunit I) and *cydB* (subunit II) (18), which are located upstream of *cydD* and *cydC* (Fig. 2). In *Escherichia coli* the last two genes are required for the formation of active cytochrome *bd* (11, 27) and encode an ABC transporter which was reported to catalyze the export of L-cysteine (25) and glutathione (26). Since *C. glutamicum* does not possess a proton- or sodium ion-pumping NADH dehydrogenase, only the cytochrome *bc*₁-*aa*₃ supercomplex and cytochrome *bd* oxidase couple electron transfer to the generation of an electrochemical proton gradient. Besides being required for ATP synthesis and various secondary transport processes, the electrochemical proton gradient appears to be required as a driving force for succinate dehydrogenase, which probably catalyzes a reversed electron transfer when oxidizing succinate to fumarate with menaquinone as electron acceptor (31).

Mutants of the *C. glutamicum* wild-type strain ATCC 13032 which are unable to synthesize or assemble the *bc*₁ complex or cytochrome *aa*₃ oxidase showed a severe growth defect on brain heart infusion agar plates as well as in glucose minimal medium, indicating that the *bc*₁-*aa*₃ branch of the respiratory chain is of major importance for aerobic respiration (22, 23). Cytochromes of the *a*, *b*, and *c* types are spectroscopically detectable under all growth conditions and in all growth phases tested hitherto. In this study, we analyzed the role of cytochrome *bd* oxidase for growth of *C. glutamicum* and lysine production. To this end, we deleted and overexpressed the cytochrome *bd* oxidase genes in *C. glutamicum* ATCC 13032 and its lysine-producing derivative MH20-22B.

* Corresponding author. Mailing address: Institut für Biotechnologie 1, Forschungszentrum Jülich, D-52425 Jülich, Germany. Phone: 49 2461 615515. Fax: 49 2461 612710. E-mail: m.bott@fz-juelich.de.

[▽] Published ahead of print on 1 December 2006.

FIG. 1. Model of the branched respiratory chain of *C. glutamicum*. As indicated by the number of protons presumably translocated to the exterior per two electrons transferred to oxygen, the branch composed of the cytochrome bc_1 - aa_3 supercomplex has a threefold-higher bioenergetic efficiency than the cytochrome bd branch. Since *C. glutamicum* does not possess a proton- or sodium ion-pumping NADH dehydrogenase, only the bc_1 - aa_3 supercomplex and cytochrome bd oxidase couple electron transport to the generation of an electrochemical proton gradient.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *C. glutamicum* strains and plasmids used in this work are listed in Table 1. For analyzing growth and lysine production, a first preculture was grown in brain heart infusion medium with 2% (wt/vol) glucose for 8 h and an aliquot of cells was transferred either to CGXII minimal medium (15) containing 4% (wt/vol) glucose or to modified CGXII minimal medium (32) with 10% (wt/vol) glucose to give an optical density at 600 nm (OD_{600}) of 1. The CGXII medium was supplemented with 30 mg/liter 3,4-dihydroxybenzoic acid as iron chelator and, if appropriate, with 0.3 g/liter leucine. After overnight incubation, cells of the second preculture were harvested, washed three times with 0.9% (wt/vol) NaCl, and used for inoculation of either CGXII medium with 4% (wt/vol) glucose or CGXII medium with 10% (wt/vol) glucose to an OD_{600} of 1. Cultivations were done in baffled 500-ml Erlenmeyer flasks with 60 ml medium at 30°C and 150 rpm. *E. coli* DH5 α , which was used as a host for cloning, was cultivated in LB medium or on LB agar plates at 37°C. When appropriate, kanamycin was used at a concentration of 25 μ g/ml (*C. glutamicum*) or 50 μ g/ml (*E. coli*) and isopropyl- β -D-thiogalactopyranoside (IPTG) was used at a concentration of 1 mM.

DNA manipulations. Enzymes used for DNA restriction, ligation, or dephosphorylation were obtained either from Roche Diagnostics (Mannheim, Germany) or from New England Biolabs (Frankfurt am Main, Germany). For Southern hybridization (35), 10 μ g genomic DNA isolated as described previously (10) was completely digested with appropriate restriction enzymes, size fractionated on a 1% agarose gel, and transferred onto a nylon membrane (Hybond-N⁺; Amersham Pharmacia Biotech) by vacuum-supported diffusion. Labeling of the probes with digoxigenin (DIG), hybridization, washing, and detection were carried out with the DIG Chem-Link labeling and detection kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). For size determination, DIG-labeled DNA molecular weight markers II and III (Roche

Diagnostics, Mannheim, Germany) were used. Plasmid DNA from *E. coli* or *C. glutamicum* was isolated with the QIAprep Spin Miniprep kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany).

Construction of *cydAB* deletion mutants. In-frame *cydAB* deletion mutants of *C. glutamicum* ATCC 13032 and MH20-22B were constructed as described previously (22). For this purpose, the *cydA* upstream region including the first six codons of *cydA* and the *cydB* downstream region including the last 12 codons of *cydB* were amplified with the Expand High Fidelity kit (Roche Diagnostics) using the primer pairs $\Delta cydAB$ -1/ $\Delta cydAB$ -2 and $\Delta cydAB$ -3/ $\Delta cydAB$ -4, respectively (Table 2). The resulting PCR products of 561 bp and 574 bp, respectively, were subsequently fused by crossover PCR to a product of 1,114 bp. After digestion with *EcoRI* and *PstI*, this fragment was cloned into pK19mobsacB (30) cut with the same restriction enzymes to yield pK19mobsacB- $\Delta cydAB$. DNA sequence analysis revealed that the cloned PCR product contained no unwanted mutations. Subsequently, plasmid pK19mobsacB- $\Delta cydAB$ was transferred by electroporation (37) into the *C. glutamicum* strains ATCC 13032 and MH20-22B and the transformation mixture was plated on LBHIS agar (37) containing 25 μ g kanamycin/ml. After selection for the first and second recombination events, genomic DNA of kanamycin-sensitive and sucrose-resistant clones was analyzed by PCR with the primers $\Delta cydAB$ -for and $\Delta cydAB$ -rev in order to distinguish between wild-type and $\Delta cydAB$ clones. For Southern blot analysis of selected $\Delta cydAB$ clones, the DIG-labeled 1.1-kb *EcoRI*/*PstI* insert of pK19mobsacB- $\Delta cydAB$ was used as a probe.

Overproduction of the cytochrome *bd* oxidase. In order to overproduce cytochrome *bd* oxidase, the *cydAB* genes or the *cydABDC* genes were cloned into the expression plasmid pKEEx2 (9), which contains an IPTG-inducible *tac* promoter. The *cydAB* genes including the *cydA* ribosome binding site were amplified by PCR using chromosomal *C. glutamicum* ATCC 13032 DNA as template and the primer pair *cydAB*-for/*cydAB*-rev. After digestion of the 2,587-bp PCR product

FIG. 2. Physical map of the *C. glutamicum* *cydABDC* gene cluster. The *cydA* and *cydB* genes encode subunit I and subunit II of cytochrome *bd* oxidase, respectively. The *cydC* and *cydD* genes encode an ABC transporter presumably required for the formation of active cytochrome *bd* oxidase. The sequence deleted in strains 13032 $\Delta cydAB$ and MH20-22B $\Delta cydAB$ is indicated. The gray bars indicate the regions amplified for construction of the plasmid pK19mobsacB- $\Delta cydAB$.

TABLE 1. Strains of *C. glutamicum* and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>C. glutamicum</i> strains		
ATCC 13032	Wild type, biotin auxotrophic	1
13032 Δ <i>cydAB</i>	ATCC 13032 derivative with a <i>cydAB</i> deletion	This work
13032/pEKEx2	Kan ^r ; ATCC 13032 with pEKEx2	This work
13032/pEKEx2- <i>cydABDC</i>	Kan ^r ; ATCC 13032 with pEKEx2- <i>cydABDC</i>	This work
MH20-22B	Lysine-producing derivative of ATCC 13032	32
MH20-22B Δ <i>cydAB</i>	MH20-22B derivative with a <i>cydAB</i> deletion	This work
MH20-22B/pEKEx2	Kan ^r ; MH20-22B with pEKEx2	This work
MH20-22B/pEKEx2- <i>cydAB</i>	Kan ^r ; MH20-22B with pEKEx2- <i>cydAB</i>	This work
MH20-22B/pEKEx2- <i>cydABDC</i>	Kan ^r ; MH20-22B with pEKEx2- <i>cydABDC</i>	This work
Plasmids		
pEKEx2	Kan ^r ; <i>E. coli</i> - <i>C. glutamicum</i> shuttle vector for regulated gene expression (P _{lac} <i>lacI</i> ^r pBL1 <i>oriV</i> _{C.g.} pUC18 <i>oriV</i> _{E.c.})	9
pEKEx2- <i>cydAB</i>	Kan ^r ; pEKEx2 derivative containing a 2,587-bp <i>SacI</i> - <i>EcoRI</i> fragment covering the <i>cydAB</i> genes	This work
pEKEx2- <i>cydABDC</i>	Kan ^r ; pEKEx2 derivative containing a 5,683-bp <i>SacI</i> - <i>EcoRI</i> fragment covering the <i>cydABDC</i> gene cluster	This work
pK19 <i>mobsacB</i>	Kan ^r ; vector for allelic exchange in <i>C. glutamicum</i> (pK18 <i>oriV</i> _{E.c.} <i>sacB lacZ</i> α)	30
pK19 <i>mobsacB</i> - Δ <i>cydAB</i>	Kan ^r ; pK19 <i>mobsacB</i> derivative containing a 1.1-kb crossover PCR product (<i>PstI</i> / <i>EcoRI</i>) which covers the flanking regions of the <i>C. glutamicum cydAB</i> genes	This work

with *SacI* and *EcoRI*, the fragment was ligated into the pEKEx2 vector cut with the same restriction enzymes, transferred into *E. coli* DH5 α , and plated on LB plates containing 50 μ g kanamycin/ml. Plasmids from kanamycin-resistant clones were isolated and restricted with *SacI* and *EcoRI*, and one of the plasmids containing the *cydAB* fragment (pEKEx2-*cydAB*) was transferred into *C. glutamicum* ATCC 13032 and MH20-22B by electroporation. The same strategy was used for the construction of a *cydABDC* expression plasmid. In this case, primers *cydAB*-for and *cydABDC*-rev were used to amplify the 5,683-bp *cydABDC* gene cluster. The pEKEx2-*cydABDC* plasmid was transferred into *C. glutamicum* strains ATCC 13032 and MH20-22B.

Determination of glucose and L-lysine. Glucose concentrations were determined enzymatically using hexokinase and glucose-6-phosphate dehydrogenase. Samples (1 ml) of the cultures were centrifuged for 10 min at 16,100 $\times$ g, and aliquots of the supernatant were directly used for glucose determination. NADH formation was measured spectrophotometrically at 340 nm in a microplate reader (ThermoMax; MWG, Ebersberg, Germany), and concentrations were calculated using an extinction coefficient (ϵ) of 6.3 mM⁻¹ cm⁻¹. Alternatively, glucose concentrations were determined with an enzyme electrode (EBIO Compact; Eppendorf, Hamburg, Germany). Quantitative determination of L-lysine in supernatants was carried out by reversed-phase high-pressure liquid chromatography after precolumn derivatization with a mixture of *o*-phthalaldehyde and β -mercaptoethanol (Pierce Biotechnology Inc., Rockford, IL) according to reference 19 using an Agilent 1100 LC system (Agilent Technologies, Waldbronn, Germany) equipped with an octyldecylsilane Hypersil 120- $\times$ 4-mm column (CS Chromatographie Service GmbH, Langerwehe, Germany) with a 5- μ m particle size and a guard cartridge (40 $\times$ 4 mm). Ornithine at a final concentration of 1 mM was used as an internal standard in each sample. Substances were eluted with a flow rate of 0.35 ml min⁻¹ within the first minute and 0.6 ml min⁻¹ for the

following 15 min at 40°C with a gradient of 0.1 M sodium acetate, pH 7.2, and methanol. Fluorescence of the amino acid isoindole derivatives was measured after excitation at 230 nm at an emission wavelength of 450 nm.

Determination of growth parameters. Growth was followed by measuring the OD₆₀₀ with an Ultrospec 500-Pro spectrophotometer (Amersham Biotech). The biomass concentration was calculated from OD₆₀₀ values using an experimentally determined correlation factor of 0.25 g (dry weight) of cells (cdw) liter⁻¹ for an OD₆₀₀ of 1.

Membrane isolation and determination of protein concentration. For the isolation of cell membranes, 5 g (wet weight) cells from stationary phase was suspended in 7 ml 100 mM Tris-HCl buffer, pH 7.5, containing 1 mM of phenylmethylsulfonyl fluoride and disrupted by five passages at 207 MPa through a French press cell (SLM Aminco). Cell debris was removed by centrifugation at 8,000 $\times$ g for 15 min, and the supernatant was ultracentrifuged at 150,000 $\times$ g for 90 min. The pellet containing the membrane fraction was resuspended in 7 ml 10 mM Tris-HCl (pH 7.5), ultracentrifuged once more for 90 min at 150,000 $\times$ g, and resuspended in 1 to 2 ml of the same buffer. For redox difference spectroscopy, suspensions containing 6 mg membrane protein ml⁻¹ were used. Protein concentrations were determined with the Bradford protein assay (5) using bovine serum albumin as a standard.

Redox difference spectroscopy. Dithionite-reduced-minus-ferricyanide-oxidized difference spectra of membranes and dithionite-reduced spectra of intact cells (oxidation of cytochromes in intact cells proved to be difficult) were recorded at room temperature using 5-mm-light-path cuvettes with a Jasco V560 spectrophotometer equipped with a silicon photodiode detector for turbid samples (7).

Oxygen consumption rates. Oxygen consumption rates were measured with intact cells from stationary phase in a thermostatically controlled, magnetically

TABLE 2. Oligonucleotides used in this work^a

Name	Sequence	Restriction enzyme
Δ <i>cydAB</i> -1	5'-GACGAATTCAAGCCCATCGCTGCGAATGCTG-3'	EcoRI
Δ <i>cydAB</i> -2	5'-CCCATCCACTAAACTTAAACAGATGTCGACGACATCCACGCTAG-3'	
Δ <i>cydAB</i> -3	5'-TGTTTAAGTTTAGTGATGGGTGGGTGTTCCGCAAACGACTTCAC-3'	
Δ <i>cydAB</i> -4	5'-GTCCTGCAGCCAACCCACCACCGCGAG-3'	
Δ <i>cydAB</i> -for	5'-TATCACAAGGCTGATGATGTATCC-3'	PstI
Δ <i>cydAB</i> -rev	5'-GGTCGCTGAGGCGTTGTTCCGG-3'	
<i>cydAB</i> -for	5'-GACTGAGCTCAATGGAAGAGGAAGTGACCTAGC-3'	SacI EcoRI
<i>cydAB</i> -rev	5'-GACTGAATTCCTCAACTTAAAGCAGACACTGGC-3'	
<i>cydABDC</i> -rev	5'-GACTGAATTCGCAAAATTACCTCACATGCCTC-3'	

^a Restriction sites are underlined, and complementary sequences of the primer pairs used for crossover PCR are shown in italic.

FIG. 3. Southern blot analysis of genomic DNA from *C. glutamicum* ATCC 13032 (lane 1), MH20-22B (lane 2), 13032 Δ *cydAB* (lane 3), and MH20-22 Δ *cydAB* (lane 4). DNA restricted with *Eco*RI and *Pst*I was separated by agarose gel electrophoresis, blotted onto a nylon membrane, denatured, and hybridized with the 1.1-kb DIG-labeled Δ *cydAB* insert from pK19*mobsacB*- Δ *cydAB*. St, DIG-labeled DNA standards II and III (Roche Diagnostics).

stirred 2-ml chamber with a Clarke-type oxygen electrode (Rank Brothers, Cambridge, United Kingdom) at 25°C. The chamber was filled with 940 μ l air-saturated 20 mM Tris-HCl buffer, pH 8.0, and 20 μ l air-saturated 222 mM glucose solution. Oxygen consumption was followed with a chart recorder after adding 40 μ l of a stock cell suspension in 20 mM Tris-HCl, pH 8.0, to give a final OD₆₀₀ of 5 in the chamber.

RESULTS

Influence of cytochrome *bd* oxidase absence on growth of *C. glutamicum* wild-type strain ATCC 13032. In order to test the relevance of cytochrome *bd* oxidase for growth of the *C. glutamicum* wild-type strain ATCC 13032, a mutant lacking the *cydAB* genes for subunits I and II of cytochrome *bd* was constructed as described in Materials and Methods. For the deletion, codons 7 to 507 of *cydA* and codons 1 to 319 of *cydB* were replaced by a 21-bp sequence tag. The genomic structure of the mutant was verified by PCR (data not shown) and Southern blot analysis (Fig. 3). In the parental strain, two hybridizing *Pst*I-*Eco*RI fragments of 1.7 kb and 4.6 kb were detected, whereas only a single hybridizing *Pst*I-*Eco*RI fragment of 3.8 kb was present in the *cydAB* deletion mutant. Figure 4A shows a growth comparison of the parental strain and the 13032 Δ *cydAB* mutant in CGXII minimal medium with 4% (wt/vol) glucose. The absence of cytochrome *bd* oxidase had no influence during the exponential growth phase, and the mutant reached the same maximal growth rate (0.35 h⁻¹) as the wild type did. However, growth after the exponential phase was strongly inhibited by the *cydAB* deletion and the mutant reached only ~60% of the maximal biomass of the wild type (10.6 versus 18.2 g cdw liter⁻¹). This result clearly indicates an important role for cytochrome *bd* oxidase under the prevailing culture conditions. Remarkably, a distinct cytochrome *d*-specific peak at 630 nm could be observed in the reduced spectrum of wild-type cells from stationary phase only after subtraction of the reduced spectrum of the Δ *cydAB* mutant (data not shown), indicating that the concentration of cytochrome *bd* oxidase in

FIG. 4. Influence of cytochrome *bd* oxidase absence (A) or overproduction (B) on growth of *C. glutamicum*. (A) Growth of *C. glutamicum* 13032 (filled squares) and 13032 Δ *cydAB* (open triangles) in CGXII medium with 4% (wt/vol) glucose. (B) Growth of *C. glutamicum* 13032/pEKEx2 (filled squares) and 13032/pEKEx2-*cydABDC* (open triangles) in CGXII medium with 4% (wt/vol) glucose, 25 μ g/ml kanamycin, and 1 mM IPTG. The inset shows dithionite-reduced spectra of intact stationary-phase cells from 13032/pEKEx2 (gray line) and 13032/pEKEx2-*cydABDC* (black line). The shoulder at 552 nm and the peaks at 562 nm, 603 nm, and 630 nm, which are characteristic for cytochromes of the *c*, *b*, *a*, and *d* types, respectively, are indicated. Each growth curve shows the mean values of three cultures including standard deviations.

the wild type is quite low. The observed growth defect of the Δ *cydAB* mutant could be complemented, at least partially, by transformation with plasmid pEKEx2-*cydAB*. The resulting strain, when cultivated in glucose minimal medium in the presence of 1 mM IPTG, showed a maximal growth rate in the exponential phase similar to those of the wild type and the Δ *cydAB*/pEKEx2 strain but reached a biomass concentration of 15.3 g cdw/liter after 28 h compared to 11.7 g cdw/liter of the Δ *cydAB*/pEKEx2 strain and 17.6 g cdw/liter of the wild type (mean values of two independent experiments).

Influence of cytochrome *bd* oxidase overproduction on growth of *C. glutamicum* wild-type strain ATCC 13032. In order

FIG. 5. Influence of cytochrome *bd* deletion on growth, glucose consumption, and lysine production by *C. glutamicum* MH20-22B. (A and B) Two independent growth experiments with MH20-22B (filled squares) and MH20-22 Δ *cydAB* (open triangles) in modified CGX minimal medium containing 10% glucose. (C and D) Glucose consumption and lysine formation (as lysine-HCl) of the four cultures shown in panels A and B, respectively.

to determine the effect of cytochrome *bd* oxidase overproduction on growth, the wild type was transformed with plasmid pEKEx2-*cydABDC*. As shown in the inset of Fig. 4B, the dithionite-reduced spectrum of stationary-phase cells of 13032/pEKEx2-*cydABDC* grown in the presence of IPTG revealed a clear cytochrome *d*-specific peak at 630 nm and an increased cytochrome *b*-specific peak at 560 nm compared to the reference strain 13032/pEKEx2, indicating that cytochrome *bd* was present. The respiration rate of suspensions of stationary-phase cells supplied with 4.4 mM glucose was 107 ± 11 nmol O_2 consumed min^{-1} mg protein $^{-1}$ for 13032/pEKEx2 and 140 ± 6 nmol O_2 consumed min^{-1} mg protein $^{-1}$ for 13032/pEKEx2-*cydABDC*, indicating that the overproduced cytochrome *bd* oxidase was catalytically active. As shown in Fig. 4B, growth of 13032/pEKEx2-*cydABDC* in glucose minimal medium differed significantly from that of the reference strain. The maximal growth rate ($0.19\ h^{-1}$) was reduced ~ 2 -fold and the maximal biomass ($11.2\ g\ liter^{-1}$) by $\sim 1/3$.

Influence of cytochrome *bd* oxidase absence on growth and lysine formation by *C. glutamicum* MH20-22B. In contrast to wild-type cells, a significant part of the carbon flux in strain MH20-22B is directed towards L-lysine rather than towards biomass production (32). Therefore, it was of interest to analyze the effect of a *cydAB* deletion on growth of and lysine formation by this producer strain. A *cydAB* deletion mutant of MH20-22B was constructed as described above for ATCC

13032, and the successful deletion was confirmed again by Southern blot analysis (Fig. 3). As shown in Fig. 5, the growth behavior of MH20-22 Δ *cydAB* did not differ significantly from that of MH20-22B. The *cydAB* mutant reached the same maximal growth rate ($0.29\ h^{-1}$) and a similar maximal biomass concentration ($10.8\ g\ cdw\ liter^{-1}$) as the parent strain did ($10.5\ g\ cdw\ liter^{-1}$). Redox difference spectroscopy of membranes revealed a small but distinct cytochrome *d*-specific peak at 630 nm in MH20-22B cells that was absent in the *cydAB* deletion mutant (inset of Fig. 5A). The presence of cytochrome *bd* oxidase in MH20-22B was also confirmed by the observation that respiration of stationary-phase cells (33 ± 1 nmol O_2 consumed min^{-1} mg protein $^{-1}$) was not inhibited by 10 mM cyanide, whereas that of MH20-22 Δ *cydAB* cells (27 ± 3 nmol O_2 consumed min^{-1} mg protein $^{-1}$) was rapidly and completely inhibited (data not shown). The increased level of cytochrome *bd* oxidase in MH20-22B compared to its parent ATCC 13032 is likely due to increased expression of the *cydAB* genes (33) and appears to result from the mutagenesis and screening procedures applied during the development of this strain (32).

Whereas the *cydAB* deletion in strain MH20-22B had no marked effect on growth and glucose consumption, it stimulated lysine synthesis. As shown in Fig. 5C and D, both the rate of lysine production and the lysine concentration were increased in MH20-22 Δ *cydAB*. In four independent experiments, the final lysine titer was increased on average by 12%,

TABLE 3. Influence of a *cydAB* deletion in *C. glutamicum* MH20-22B on lysine production parameters^a

Strain	Ratio of lysine-HCl (g) to:		Productivity (g liter ⁻¹ h ⁻¹)
	Glucose	Biomass	
MH20-22B	0.21 ± 0.02	2.18 ± 0.39	0.32 ± 0.03
MH20-22BΔ <i>cydAB</i>	0.24 ± 0.03	2.30 ± 0.29	0.35 ± 0.04

^a Numbers given are mean values and standard deviations of four independent experiments.

the glucose-specific lysine yield by 14%, the biomass-specific lysine yield by 6%, and the space-time yield by 9% (Table 3).

Influence of cytochrome *bd* oxidase overproduction on growth and lysine formation by *C. glutamicum* MH20-22B. As outlined in the Discussion, the increased lysine production by MH20-22BΔ*cydAB* might be a consequence of a bioenergetically more efficient respiratory chain. Therefore, it was of interest to test whether an increased level of cytochrome *bd* oxidase has an effect on lysine production opposite from that of the *cydAB* deletion. In a first attempt, only the structural genes for cytochrome *bd* oxidase, *cydA* and *cydB*, were cloned into the expression plasmid pEKEx2 and transferred to MH20-22B. The resulting strain, MH20-22B/pEKEx2-*cydAB*, was cultivated in CGXII minimal medium with 4% (wt/vol) glucose. Neither the size of the cytochrome *d* peak nor biomass formation nor lysine production showed any differences compared to the reference strain MH20-22B/pEKEx2 (data not shown). This indicated that overexpression of the *cydAB* genes alone is not sufficient for increased synthesis of an active cytochrome *bd* oxidase. As it is known that synthesis of functional cytochrome *bd* oxidase requires the products of *cydC* (11) and *cydD* (27), we cloned the entire *cydABDC* gene cluster into the pEKEx2 plasmid and transferred it into MH20-22B. Dithionite-reduced spectra of stationary-phase cells revealed significantly higher levels of *b*- and *d*-type cytochromes in MH20-22B/pEKEx2-*cydABDC* than in the reference strain MH20-22B/pEKEx2, confirming the overproduction of cytochrome *bd* (Fig. 6A, inset). The oxygen consumption rates of suspensions from stationary-phase cells were found to be 37 ± 6 nmol O₂ consumed min⁻¹ mg protein⁻¹ for MH20-22B/pEKEx2 and 63 ± 21 nmol O₂ consumed min⁻¹ mg protein⁻¹ for MH20-22B/pEKEx2-*cydABDC*. The 1.7-fold-increased respiration rate clearly showed that overexpression of *cydABDC* led to an active cytochrome *bd* oxidase. When strains were cultivated in CGXII minimal medium with 4% (wt/vol) glucose (Fig. 6A), the growth rate (0.22 h⁻¹) and the maximal biomass (9.9 g cdw liter⁻¹) of MH20-22B/pEKEx2-*cydABDC* were 12% and 16% lower, respectively, than those of MH20-22B/pEKEx2 (0.25 h⁻¹ and 11.8 g cdw liter⁻¹, respectively). The lysine titer of MH20-22B/pEKEx2-*cydABDC* (7.1 g liter⁻¹) was 14% lower than that of MH20-22B/pEKEx2 (8.3 g liter⁻¹). Thus, overproduction of cytochrome *bd* oxidase had a negative impact on growth of and lysine production by MH20-22B (Fig. 6).

DISCUSSION

The respiratory chain of *C. glutamicum* involves two branches for transfer of electrons to oxygen. One branch is formed by the cytochrome *bc*₁ complex and the terminal ox-

dase cytochrome *aa*₃ (23); the second branch is formed by cytochrome *bd* oxidase (18). We previously showed that mutants lacking either the *bc*₁ complex or cytochrome *aa*₃ had severe growth defects, suggesting that the *bc*₁-*aa*₃ supercomplex is of major importance for aerobic respiration (22, 23). In this study, we investigated the role of the second branch of the respiratory chain, cytochrome *bd* oxidase, by analyzing mutant strains which either lacked or overproduced this terminal oxidase. In the wild-type strain ATCC 13032, a *cydAB* deletion had no effect on growth in the exponential phase but inhibited growth thereafter, resulting in a strong reduction of the biomass formed (Fig. 4A). This shows that cytochrome *bd* oxidase is present and active in the wild type, although neither cells nor membranes of stationary-phase cells showed a distinct cytochrome *d*-specific peak in reduced or reduced-minus-oxidized spectra, respectively. The behavior of the 13032Δ*cydAB* mutant could be due to low dissolved oxygen concentrations at the

FIG. 6. Influence of cytochrome *bd* overproduction on growth (A) and lysine production (B) by *C. glutamicum* MH20-22B. (A) Growth of *C. glutamicum* MH20-22B/pEKEx2 (filled squares) and MH20-22B/pEKEx2-*cydABDC* (open triangles) in CGXII medium with 4% (wt/vol) glucose, 25 μg ml⁻¹ kanamycin, and 1 mM IPTG. The inset shows dithionite-reduced spectra of stationary-phase cells of MH20-22B/pEKEx2 (gray line) and MH20-22B/pEKEx2-*cydABDC* (black line). Peaks characteristic of *a*-, *b*-, *c*-, and *d*-type cytochromes are indicated. (B) L-Lysine formation by the cultures shown in panel A. Numbers given are mean values and standard deviations from three independent cultivations.

end of the exponential growth phase, which strongly restrict the activity of the low-affinity cytochrome *aa₃* oxidase but not that of the high-affinity cytochrome *bd* oxidase. In *Mycobacterium smegmatis*, like *C. glutamicum* a member of the suborder *Corynebacterineae* (36), cytochrome *bd* oxidase was recently shown to be important for growth under microaerobic conditions and to be induced two- to threefold upon reduction of the O₂ partial pressure in the growth medium from 21% to 0.5% air saturation (14).

Whereas the absence of cytochrome *bd* had no influence on exponential-phase growth of *C. glutamicum*, constitutive overproduction of this terminal oxidase by the expression plasmid pEKEx2-*cydABDC* caused a twofold reduction of the growth rate and a 35% reduction in biomass formation. Although inhibitory effects due to the overproduction of four membrane proteins cannot be excluded, the more likely reason for the reduced growth is the difference in bioenergetic efficiency between the cytochrome *bc₁-aa₃* supercomplex and cytochrome *bd* oxidase (6). As outlined previously (3), the former pathway should couple transport of two electrons from menaquinol to oxygen to the transfer of six protons from the cytoplasm to the outside, whereas the latter pathway can transfer only two protons to the outside per two electrons transported to oxygen (20, 28) (Fig. 1). This results in a threefold-lower P/O ratio of the *bd* branch than of the *bc₁-aa₃* branch. Consequently, a shift of the electron flow from the *bc₁-aa₃* branch to the *bd* branch, which is to be expected upon overproduction of an active cytochrome *bd* oxidase, might lead to a decreased rate of ATP synthesis by F₁F_o-ATP synthase, an increased amount of glucose required for catabolism and maintenance, and a decreased amount of glucose available for biomass formation. A rough calculation indicates that 13032/pEKEx2 uses 57% of the consumed glucose for energy generation and maintenance and 43% for biomass formation, whereas 13032/pEKEx2-*cydABDC* uses 72% of the glucose for catabolism and only 28% for anabolism. Assuming a Y_{ATP} value of 10 g (dry weight) cells/mol ATP for both strains, 13032/pEKEx2 forms 13.7 mol ATP/mol glucose, but 13032/pEKEx-*cydABDC* forms only 7 mol ATP/mol glucose.

Overall, the effects caused by the *cydAB* deletion and by *cydABDC* overexpression suggest that cytochrome *bd* functions as an emergency or rescue oxidase in *C. glutamicum* which permits respiration under conditions where cytochrome *aa₃* activity is insufficient due to low dissolved oxygen concentrations or other reasons. The low concentration of cytochrome *bd* oxidase indicated by the redox spectra appears to be sufficient to fulfill this function and ensures that, whenever possible, the major portion of electrons is transferred to oxygen via the energetically favorable cytochrome *bc₁-aa₃* supercomplex.

C. glutamicum MH20-22B uses a significant portion of the consumed glucose for lysine formation rather than for biomass formation. Deletion of *cydAB* in MH20-22B had only minor effects on growth rate, biomass formation, and glucose consumption, whereas the lysine synthesis rate and the final lysine titer were increased by 12%. Overproduction of cytochrome *bd* oxidase in MH20-22B led to reductions of the growth rate by ~10%, of biomass production by 16 to 19%, and of lysine synthesis by 14%. As discussed above, these effects might be explained by a reduced P/O ratio due to an increased electron flux through the inefficient *bd* branch. The positive effect of

cytochrome *bd* absence on lysine formation, on the other hand, can be explained by an increased P/O ratio in MH20-22BΔ*cydAB*, which results in a reduced glucose demand for energy generation and maintenance and an increased availability of glucose for lysine synthesis. A comparable stimulation of lysine production was also observed for a *cydAB* deletion mutant of *C. glutamicum* DM1730 (obtained from Degussa AG), a "minimal mutation strain" (*pyc*-P458S, *hom*-V59A, *lysC*-T311I, *zwf*-A243T) that was derived from ATCC 13032 by "genome breeding" (24) (data not shown). The results show that modulation of the respiratory chain composition in favor of an increased bioenergetic efficiency (P/O ratio) can be used as a metabolic engineering strategy to improve the performance of microbial producer strains. The conclusion derived here for lysine-producing strains of *C. glutamicum* was previously also suggested by Zamboni et al. (39), who showed that deletion of cytochrome *bd* oxidase improved riboflavin production by *Bacillus subtilis* (39).

ACKNOWLEDGMENT

Financial support by R&D Feed Additives of Degussa AG is gratefully acknowledged.

REFERENCES

1. Abe, S., K. Takayama, and S. Kinoshita. 1967. Taxonomical studies on glutamic acid producing bacteria. *J. Gen. Appl. Microbiol.* **13**:279–301.
2. Akashi, K., H. Shibai, and Y. Hirose. 1979. Effect of oxygen supply on L-lysine, L-threonine and L-isoleucine fermentations. *Agric. Biol. Chem.* **43**:2087–2092.
3. Bott, M., and A. Niebisch. 2003. The respiratory chain of *Corynebacterium glutamicum*. *J. Biotechnol.* **104**:129–153.
4. Bott, M., and A. Niebisch. 2005. Respiratory energy metabolism, p. 307–334. In L. Eggeling and M. Bott (ed.), *Handbook of Corynebacterium glutamicum*. CRC Press, Boca Raton, FL.
5. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
6. Calhoun, M. W., K. L. Oden, R. B. Gennis, M. J. Teixeira-de-Mattos, and O. M. Neijssel. 1993. Energetic efficiency of *Escherichia coli*: effects of mutations in components of the aerobic respiratory chain. *J. Bacteriol.* **175**:3020–3025.
7. Castiglioni, E., E. Grilli, and S. Sanguinetti. 1997. A new simple and low cost scattered transmission accessory for commercial double beam ultraviolet-visible spectrophotometers. *Rev. Sci. Instrum.* **68**:4288–4289.
8. Eggeling, L., and M. Bott, (ed.). 2005. *Handbook of Corynebacterium glutamicum*. CRC Press, Boca Raton, FL.
9. Eikmanns, B. J., E. Kleinertz, W. Liebl, and H. Sahm. 1991. A family of *Corynebacterium glutamicum*/*Escherichia coli* shuttle vectors for cloning, controlled gene expression, and promoter probing. *Gene* **102**:93–98.
10. Eikmanns, B. J., N. Thum-Schmitz, L. Eggeling, K. U. Luedtke, and H. Sahm. 1994. Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum* *gltA* gene encoding citrate synthase. *Microbiology* **140**:1817–1828.
11. Georgiou, C. D., H. Fang, and R. B. Gennis. 1987. Identification of the *cydC* locus required for expression of the functional form of the cytochrome *d* terminal oxidase complex in *Escherichia coli*. *J. Bacteriol.* **169**:2107–2112.
12. Hermann, T. 2003. Industrial production of amino acids by coryneform bacteria. *J. Biotechnol.* **104**:155–172.
13. Kalinowski, J., B. Bathe, D. Bartels, N. Bischoff, M. Bott, A. Burkovski, N. Dusch, L. Eggeling, B. J. Eikmanns, L. Gaigalat, A. Goesmann, M. Hartmann, K. Huthmacher, R. Krämer, B. Linke, A. C. McHardy, F. Meyer, B. Möckel, W. Pfefferle, A. Pühler, D. A. Rey, C. Rückert, O. Rupp, H. Sahm, V. F. Wendisch, I. Wiegand, and A. Tauch. 2003. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. *J. Biotechnol.* **104**:5–25.
14. Kana, B. D., E. A. Weinstein, D. Avarbock, S. S. Dawes, H. Rubin, and V. Mizrahi. 2001. Characterization of the *cydAB*-encoded cytochrome *bd* oxidase in *Mycobacterium smegmatis*. *J. Bacteriol.* **183**:7076–7086.
15. Keilhauer, C., L. Eggeling, and H. Sahm. 1993. Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. *J. Bacteriol.* **175**:5595–5603.
16. Kelle, L., T. Hermann, and B. Bathe. 2005. L-lysine production, p. 465–488.

- In L. Eggeling and M. Bott (ed.), Handbook of *Corynebacterium glutamicum*. CRC Press, Boca Raton, FL.
17. Kimura, E. 2005. L-glutamate production, p. 439–463. In L. Eggeling and M. Bott (ed.), Handbook of *Corynebacterium glutamicum*. CRC Press, Boca Raton, FL.
 18. Kusumoto, K., M. Sakiyama, J. Sakamoto, S. Noguchi, and N. Sone. 2000. Menaquinol oxidase activity and primary structure of cytochrome *bd* from the amino-acid fermenting bacterium *Corynebacterium glutamicum*. Arch. Microbiol. **173**:390–397.
 19. Lindroth, P., and K. Mopper. 1979. High-performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with *o*-phthalaldehyde. Anal. Chem. **51**:1667–1674.
 20. Miller, M. J., and R. B. Gennis. 1985. The cytochrome *d* complex is a coupling site in the aerobic respiratory chain of *Escherichia coli*. J. Biol. Chem. **260**:4003–4008.
 21. Möckel, B., A. Marx, and W. Pfefferle. July 2004. Nucleotide sequences coding for the *glbO* gene. U.S. patent 6,759,218.
 22. Niebisch, A., and M. Bott. 2001. Molecular analysis of the cytochrome *bc₁-aa₃* branch of the *Corynebacterium glutamicum* respiratory chain containing an unusual diheme cytochrome *c₁*. Arch. Microbiol. **175**:282–294.
 23. Niebisch, A., and M. Bott. 2003. Purification of a cytochrome *bc₁-aa₃* supercomplex with quinol oxidase activity from *Corynebacterium glutamicum*. Identification of a fourth subunit of cytochrome *aa₃* oxidase and mutational analysis of diheme cytochrome *c₁*. J. Biol. Chem. **278**:4339–4346.
 24. Ohnishi, J., S. Mitsuhashi, M. Hayashi, S. Ando, H. Yokoi, K. Ochiai, and M. Ikeda. 2002. A novel methodology employing *Corynebacterium glutamicum* genome information to generate a new L-lysine-producing mutant. Appl. Microbiol. Biotechnol. **58**:217–223.
 25. Pittman, M. S., H. Corker, G. Wu, M. B. Binet, A. J. Moir, and R. K. Poole. 2002. Cysteine is exported from the *Escherichia coli* cytoplasm by CydDC, an ATP-binding cassette-type transporter required for cytochrome assembly. J. Biol. Chem. **277**:49841–49849.
 26. Pittman, M. S., H. C. Robinson, and R. K. Poole. 2005. A bacterial glutathione transporter (*Escherichia coli* CydDC) exports reductant to the periplasm. J. Biol. Chem. **280**:32254–32261.
 27. Poole, R. K., H. D. Williams, J. A. Downie, and F. Gibson. 1989. Mutations affecting the cytochrome *d*-containing oxidase complex of *Escherichia coli* K12: identification and mapping of a fourth locus, *cydD*. J. Gen. Microbiol. **135**:1865–1874.
 28. Puustinen, A., M. Finel, T. Haltia, R. B. Gennis, and M. Wikstroem. 1991. Properties of the two terminal oxidases of *Escherichia coli*. Biochemistry **30**:3936–3942.
 29. Sassi, A. H., A. M. Deschamps, and J. M. Lebeault. 1996. Process analysis of L-lysine fermentation with *Corynebacterium glutamicum* under different oxygen and carbon dioxide supplies and redox potentials. Proc. Biochem. **31**:493–497.
 30. Schäfer, A., A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach, and A. Pühler. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145**:69–73.
 31. Schirawski, J., and G. Uden. 1998. Menaquinone-dependent succinate dehydrogenase of bacteria catalyzes reversed electron transport driven by the proton potential. Eur. J. Biochem. **257**:210–215.
 32. Schruppf, B., L. Eggeling, and H. Sahm. 1992. Isolation and prominent characteristics of an L-lysine hyperproducing strain of *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **37**:566–571.
 33. Sindelar, G. 2003. Ph.D. thesis. Heinrich-Heine-Universität, Düsseldorf, Germany.
 34. Sone, N., K. Nagata, H. Kojima, J. Tajima, Y. Kodera, T. Kanamaru, S. Noguchi, and J. Sakamoto. 2001. A novel hydrophobic diheme *c*-type cytochrome. Purification from *Corynebacterium glutamicum* and analysis of the *qcrCBA* operon encoding three subunit proteins of a putative cytochrome reductase complex. Biochim. Biophys. Acta **1503**:279–290.
 35. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
 36. Stackebrandt, E., F. A. Rainey, and N. L. Ward-Rainey. 1997. Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. Int. J. Syst. Bacteriol. **47**:479–491.
 37. van der Rest, M. E., C. Lange, and D. Molenaar. 1999. A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. Appl. Microbiol. Biotechnol. **52**:541–545.
 38. Yao, H. M., Y. C. Tian, M. O. Tade, and H. M. Ang. 2001. Variations and modelling of oxygen demand in amino acid production. Chem. Eng. Proc. **40**:401–409.
 39. Zamboni, N., N. Mouncey, H. P. Hohmann, and U. Sauer. 2003. Reducing maintenance metabolism by metabolic engineering of respiration improves riboflavin production by *Bacillus subtilis*. Metab. Eng. **5**:49–55.