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Correlation Between Proton Translocation and Growth: Genetic Analysis of the Respiratory Chain of *Corynebacterium glutamicum*

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Corynebacterium glutamicum **contains at least two terminal oxidases in the respiratory chain; cytochrome** ***aa*$_3$****-type cytochrome** ***c*** **oxidase and** ***bd*****-type menaquinol oxidase. Thus, the chain has two branches of electron flow. The** ***bcc-aa*$_3$** **branch translocates three protons per electron transferred, while the** ***bd*** **branch translocates only one. In this study, we constructed two mutant strains, lacking of either subunit I of the cytochrome** ***c*** **oxidase (Δ*ctaD*) or subunits I and II of the quinol oxidase (Δ*cydAB*), and also plasmids to complement the deficient genes to investigate their effects on energy conservation and cell growth. We measured H^+/O ratios of** ***C. glutamicum*** **wild-type and mutant cells grown aerobically. The H^+/O ratio of the wild-type cells grown in the semi-synthetic medium was 3.94 ± 0.30, while the value was 2.76 ± 0.25 for the Δ*ctaD* mutant. In contrast, the value was 5.23 ± 0.36 for the Δ*cydAB* mutant. The cells grown in the LB medium showed higher value compared to that of cells grown in the semi-synthetic medium. The Δ*ctaD* mutant grew less than the wild-type in LB medium, while they grew about equally in semi-synthetic medium. Correlation between bioenergetics and growth of** ***C. glutamicum*** **was significantly affected by the growth nutrients.**

Key words: ***Corynebacterium glutamicum*****, cytochrome** ***bd*****-type quinol oxidase, cytchrome** ***aa*$_3$****-type cytchrome** ***c*** **oxidase, Gram-positive bacteria, H^+/O ratio.**

Abbreviations: BN–PAGE, blue-native–polyacrylamide gel electrophoresis; Mops, 4-morpholinepropanesulfonic acid; TMPD, *N*,*N*,*N*′,*N*′-tetra methyl-*p*-phenylene diamine.

Corynebacterium glutamicum, which belongs to the group of Actinobacteria or high G + C gram-positive bacteria, is not only industrially important in amino-acid production but also useful as a model organism of pathogenic gram-positive bacteria, such as *Mycobacterium tuberculosis*. We have been using *C. glutamicum* as a model organism of this class of bacilli, which share conserved respiratory enzyme system. In order to improve the efficiency of cell growth and amino acid production, it is important to understand the aerobic energy metabolism. In our previous studies and those by others, four respiratory enzyme complexes were identified from this actinobacterium, succinate: menaquinone oxidoreductase, complex II (*1*), cytochrome *bcc*-type menaquinol:cytochrome *c* oxidoreductase, complex III (*2*, *3*), cytochrome *aa*$_3$-type cytochrome *c* oxidase, complex IV (*4*) and cytochrome *bd*-type menaquinol oxidase (*5*) (Fig. 1). Since cytochrome c_1 subunit of complex III contains two heme C, we call this protein cytochrome *cc* (*6*). The presence of cytochrome *cc* is a common feature of Actinobacteria. Additionally, this bacterium does not have type-I NADH dehydrogenase (NDH-I or complex I), which would translocate protons coupled with electron transfer, while it contains type-II NADH dehydrogenase instead, NDH-II (*7*), which is a peripheral membrane protein and does not translocate protons. The respiratory chain of this organism branches into two pathways; i.e. the electrons are passed from menaquinol either to the supercomplex composed of *bcc*-type complex and *aa*$_3$-type oxidase (*8*), or directly to *bd*-type quinol oxidase. This bacterium uses *bcc-aa*$_3$ supercomplex and *bd* branch alternatively or simultaneously depending on the growth conditions. *Mycobacterium smegmatis*, the model organism of the pathogens in the same genus, has similar branched respiratory chain as *C. glutamicum* (*9*), although it contains complex I or NDH-I, and much more amount of cytochrome *bd* oxidase expresses at 1% air saturation than at 20% (*10*). In addition to the aerobic respiration, *M. tuberculosis* also performs anaerobic nitrate respiration during infection of mouse lung, and therefore it uses several respiratory pathways depending on infection stages (*11*). Very recently, it was reported that *C. glutamicum* also could grow anaerobically using nitrate as a sole electron acceptor as *M. tuberculosis* does, and thus, this bacterium is a facultative anaerobe (*12*).

The respiratory electron transfer complexes translocate protons across the membrane and produce proton motive force for ATP synthesis. The stoichiometry of proton translocation per oxygen consumption (H^+/O ratios)

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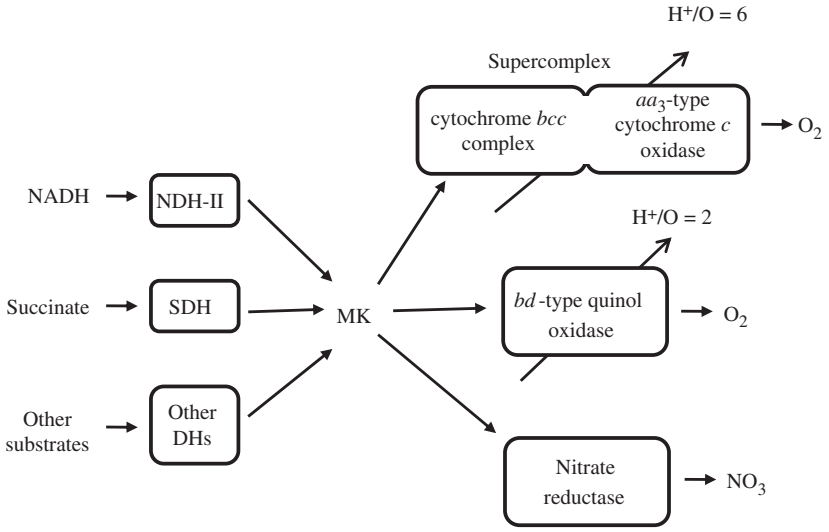

Fig. 1. **Schematic representation of a respiratory chain in *C. glutamicum*.** NDH-II, type-II NADH dehydorogenase; SDH, succinate dehydorogenase; MK, menaquinone; DHs, dehydrogenases.

is important for the bioenergy transduction by the enzyme molecules and the combined total value of the respiratory chain is crucial for the cell growth. To evaluate the efficiency of the respiratory chain, H^+/O ratio has been measured by the oxygen pulse method for wild-type and mutant cells deficient in a particular enzyme of a couple of bacterial species. The changes of H^+/O ratio are studied using the mutant strains of a firmicute or a low G+C Gram-positive bacterium *Geobacillus thermodenitrificans* K1041, which was formally named *Bacillus thermodenitrificans*, and a proteobacteria *Escherichia coli* in our studies. The wild-type strain of *G. thermodenitrificans*, mainly using the cytochrome b_6c_1 complex and cytochrome caa_3-type cytochrome *c* oxidase, showed the largest cell yield and the highest H^+/O ratio of 6–7, while strain K17, which is deficient in caa_3-type oxidase and instead cytochrome *bd* operates as the main terminal oxidase (*13*), and another strain K17q8 having the B-family cytochrome bo_3-type cytochrome *c* oxidase, showed lower growth yields and also lower H^+/O ratios (*14*). Besides, cytochrome *bd*- deletion mutant of *E. coli* showed the H^+/O ratio of ~5 and the highest cell yield, while cytochrome bo_3-deletion mutant showed both lower cell yield and H^+/O ratio than the former (*15*). In the case of *C. glutamicum*, several mutants have been constructed deficient in one of the oxidases (*2*, *16*, *17*), however, the H^+/O ratio has not been measured and therefore the relationship between the proton translocating activity and cell growth is unclear.

In this study, we constructed two *C. glutamicum* mutant strains, lacking either aa_3-type cytochrome *c* oxidase or *bd*-type quinol oxidase, and report here that the efficiency of energy metabolism of this bacterium is affected by the growth nutrients and the cell yield directly correlates with the H^+/O ratios of the wild-type cells, mutants, and transformants in which the oxidase deficiency is complemented by plasmids. These findings suggest that genetic engineering of the respiratory enzymes can result in improvement of the energy yield of the cells under aerobic conditions.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Growth Conditions—The bacterial strains and plasmids used in this study are shown in Table 1. *Corynebacterium glutamicum* subsp. *lactofermentum* (ATCC13869) was kindly provided by Ajinomoto Co., Inc. The bacteria were grown at 30°C in a semi-synthetic medium or LB medium (10 g polypepton, 5 g yeast extract, 10 g NaCl per litre at pH 7.5). The semi-synthetic medium contained 5 g urea, 1 g KH_2PO_4, 0.4 g $MgSO_4{\cdot}7H_2O$, 0.01 g $FeSO_4$, 0.01 g $MnCl_2{\cdot}4H_2O$, 0.5 g polypepton, 5 mg nicotinamide, 0.2 mg thiamine and 0.05 mg biotin per litre at pH 7.5, and 10 g glucose or 50 g sucrose as carbon sources. Fifty micrograms per millilitre kanamycin or 10 μg/ml chloramphenicol was added into the medium when needed. An overnight culture was inoculated into a 100-ml culture in a 500-ml baffled flask to an optical density of ~0.3 at 600 nm, and the cells were grown to the stationary phase under 180 rpm shaking. The absorbance was determined at 600 nm after dilution to monitor the cell growth. A 0.9% (w/v) of agar was added in solid mediums. *Escherichia coli* XL1-blue used for the construction of plasmids for gene deletion or expression was grown at 37°C in 2×TY medium (1.6% w/v polypepton, 1.0% w/v yeast extract, 0.5% w/v NaCl, pH 7.0), which contained 25 μg/ml chloramphenicol or 50 μg/ml kanamycin.

DNA Manipulations—Enzymes used for DNA digestion and ligation were purchased from TaKaRa or New England Biolabs. *Corynebacterium glutamicum* genomic DNA was extracted by gene extraction kit (Dr GenTLE® System; TaKaRa). Preparation of competent *E. coli* cells and genetic transformation were carried out by the $CaCl_2$ method (*18*). Electoro-competent cells of *C. glutamicum* were prepared as following. The *C. glutamicum* ssp. *lactofermentum* wild strain was grown at 30°C

Table 1. **Bacterial strains and plasmids used in this study.**

Strains	Relevant characteristic
ATCC13869	*C. glutamicum* subsp. *lactofermentum*, wild-type
Δ*ctaD*	Deletion of *ctaD* gene encoding the subunit I of cytochrome aa_3-type cytochrome *c* oxidase, Km^R
Δ*cydAB*	Deletion of *cydAB* genes encoding the subunit I and II of cytochrome *bd*-type quinol oxidase, Km^R
13 869/pPC4	ATCC13 869 with pPC4, Cm^R
Δ*ctaD*/pPC4	Δ*ctaD* with pPC4, Km^R and Cm^R
Δ*cydAB*/pPC4	Δ*cydAB* with pPC4, Km^R and Cm^R
Δ*ctaD*/pPC4-*ctaD*	Δ*ctaD* with pPC4-*ctaD*, Km^R and Cm^R
Δ*ctaD*/pPC4-*cydABDC*	Δ*ctaD* with pPC4-*cydABDC*, Km^R and Cm^R
Δ*cydAB*/pPC4-*cydAB*	Δ*cydAB* with pPC4-*cydAB*, Km^R and Cm^R
Δ*cydAB*/pPC4-*cydABDC*	Δ*cydAB* with pPC4-*cydABDC*, Km^R and Cm^R
Plasmids	
pHSG298	Cloning vector, Km^R
pPC4	*E. coli–C. glutamicum* shuttle vector for gene expression, Cm^R
pHSG4	*E. coli–C. glutamicum* shuttle vector used for the construction of deletion mutant, Cm^R, Temperature sensitive origin (TS ori)
pHSG4-*ctaD*::Km^R	pHSG4 derivative containing Km resistance cassette isolated from pHSG298 between the flanking regions of the *C. glutamicum ctaD* gene
pHSG4-*cydAB*::Km^R	pHSG4 derivative containing Km resistance cassette isolated from pHSG298 between the flanking regions of the *C. glutamicum cydAB* genes
pPC4-*ctaD*	pPC4 carrying a 2.3-kb fragment of the *C. glutamicum ctaD* gene
pPC4-*cydAB*	pPC4 carrying a 3.7-kb fragment of the *C. glutamicum cydAB* genes
pPC4-*cydABDC*	pPC4 carrying a 6.2-kb fragment of the *C. glutamicum cydABDC* genes

in a L-medium (1.0% w/v polypepton, 0.5% w/v yeast extract, 0.5% w/v NaCl, 0.1% w/v glucose, pH 7.0) until stationary growth phase. A 0.5-ml aliquot was inoculated into a 50-ml L-medium in a 200-ml flask under 150 rpm shaking until the optical density at 600 nm reached ~1.0.

Disruption of Cytochrome bd *or* aa_3 *Type Terminal Oxidase*—The mutants with disrupted *bd* type menaquinol oxidase or aa_3 type cytochrome *c* oxidase were constructed by homologous recombination. The gene encoding subunit I and II of *bd* type menaquinol oxidase or subunit I of aa_3 type cytochrome *c* oxidase were replaced with the kanamycin resistance cassette. The plasmid for disruption of *cydAB* gene, pHSG4-*cydAB*::Km^R was constructed as follow. A pUBD21 which include *cydAB* operon (*5*) was digested with *Msc* I and *Eag* I, these sites were located on *cydA* and *cydB* gene, respectively. The kanamycin resistance (Km^R) cassette isolated from pHSG298 digested with *Ava* II and *Sac* I was ligated between *cydA* and *cydB* gene. The DNA fragment of *cydAB* replaced by Km^R cassette was then ligated into pHSG4 vector which has temperature sensitivity origin. The plasmid for disruption of *ctaD* gene, pHSG4-*ctaD*::Km^R was constructed as follow. A pUAA31 which includes partial *nrdF* and complete *ctaD* gene (*4*) was digested with *Bsm* I and *Nco* I, these sites were located on *ctaD* gene. In the same way, the Km^R cassette isolated from pHSG298 was ligated between *Bsm* I and *Nco* I site of *ctaD* gene, and then the DNA fragment of *ctaD* gene replaced with Km^R cassette was ligated into pHSG4 vector.

Each of the two plasmids, pHSG4-*cydAB*::Km^R and pHSG4-*ctaD*::Km^R was transferred into the *C. glutamicum* subsp. *lactofermentum* by electoroporation in a 0.2 cm cuvette using 100 μl of competent cells with parameters set at 25 μF, 400 Ω, 2.0 kV. Immediately after electroporation, 1 ml of L-medium containing 10% (v/v) glycerol at room temperature was added and the cuvette and the suspension was transferred to a test tube. The test tube was incubated under shaking 80 rpm for 4 h at 30°C. Subsequently, they were plated on selective LB medium containing 50 μg/ml kanamycin and incubated at 25°C for 2 days. A plasmid pHSG4 is not replicated at 34°C because of its temperature sensitive origin, so the colony was not formed on the medium plates containing kanamycin at 34°C unless chromosomal insertion of kanamycin resistant cassette occur. After selection for the first and second recombination events, genomic DNA was extracted from clones which was kanamycin resistant and grew at 34°C. This genomic DNA was analysed by PCR with the primers that is located outside the mutated gene regions, Δ*ctaD*-for (5′-CGCCTGACGTAGC TTTCCCACGT-3′) and Δ*ctaD*-rev (5′-TTCGGGAACCAGA AGTAAACGCCTG-3′), or Δ*cydAB*-for (5′-AACTGGTCGG AATATTCGCG-3′) and Δ*cydAB*-rev (5′-TGCAACTACACC GATGACAG-3′) (Fig. 2A) in order to distinguish between wild-type and mutant strains. Agarose gel electrophoresis revealed that a PCR product of the subunit I of cytochrome aa_3 type terminal oxidase deletion mutant, called Δ*ctaD*, was about 0.75 kb larger than that of wild type, while that of the subunit I and II of cytochrome *bd* type terminal oxidase deletion mutant, called Δ*cydAB*, was ~0.46 kb smaller (Fig. 2B).

To complement the deleted genes, three expression plasmids were constructed. The entire *ctaD* gene was subcloned into pPC4 from pUAA21 and pUAA31 and this plasmid was designated as pPC4-*ctaD*. The *cydAB* genes were subcloned into pPC4 from pUBD21. The entire *cydABDC* genes including its authentic promoter region were amplified by PCR with the primers, *cydABDC*-for (5′-atatctagatcgatgcccggaaacaaaggagttg-3′)

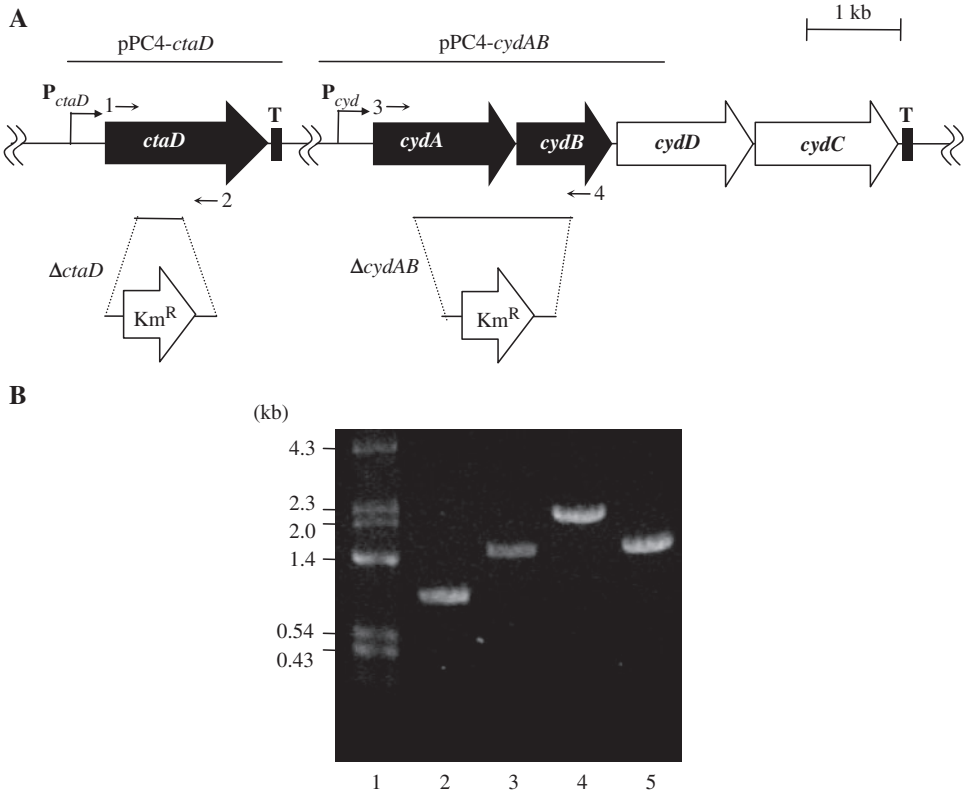

Fig. 2. **Verification of the insertional inactivation of the terminal oxidase.** (A) PCR products from *C. glutamicum* wild-type and mutant strain genomic DNA using primers located outside the mutated gene region. *Lane 1:* molecular mass markers (sizes indicated to the left); *lane 2:* 13 869 with primers Δ*ctaD*-for and Δ*ctaD*-rev; *lane 3:* Δ*ctaD* (cytchrome aa_3 deletion mutant) with primers Δ*ctaD*-for and Δ*ctaD*-rev; *lane 4:* 13 869 with primers Δ*cydAB*-for and Δ*cydAB*-rev; *lane 5:* Δ*cydAB* (cytochrome *bd* deletion mutant) with primers Δ*cydAB*-for and Δ*cydAB*-rev. (B) Physical map of *C.glutamicum ctaD* gene and *cydABDC* gene cluster. The *ctaD* gene encodes subunit I of cytochrome aa_3 oxidase. The *cydA* and *cydB* genes encode subunit I and II of cytochrome *bd* oxidase, respectively. The sequence deleted in strain 13 869 is indicated by *solid line*. These regions were replaced by kanamycin resistant gene isolated from pHSG298. P; putative promoter region, T; putative terminator region. Primer regions were indicated by the arrows; *1*, Δ*ctaD*-for; *2*, Δ*ctaD*-rev; *3*, Δ*cydAB*-for; *4*, Δ*cydAB*-rev.

and *cydABDC*-rev (5′-ataggtacctcgaatctgtggtggatat tcgc-3′). This 6.2-kb PCR product was digested with *Xba* I and *Kpn* I and ligated into pPC4. This plasmid was designated as pPC4-*cydABDC*. These plasmids were transferred into the *C. glutamicum* mutant strains, Δ*ctaD* and Δ*cydAB* by electoroporation in a 0.2 cm cuvette using 100 μl of competent cells with parameters set at 25 μF, 200 Ω, 2.0 kV.

Preparation of Membrane Proteins—The cells were suspended in 10 mM Na-phosphate buffer at pH 7.0 containing 0.5% (w/v) NaCl and disrupted by vigorous mixing with glass beads (diameter 0.18 mm) in a cell-disrupting mixer (Bead-Beater, Biospec). The unbroken cells were removed, following centrifugation at 5,000*g* for 10 min. The supernatant was then centrifuged at 100 000*g* for 30 min. The precipitate was resuspended in 10 mM Na-phosphate buffer at pH 7.0; this suspension was designated as the membrane preparation.

Electrophoretic Analysis—Blue-native–polyacrylamide gel electrophoresis (BN–PAGE) was performed according to the method of Schägger and co-workers (*19*). Non-denaturating electrophoresis was started at 100 V until the sample was within the stacking gel and was continued with a voltage and current limited to 350 V and 15 mA, respectively. After electrophoresis, the gel was incubated at 30°C in 20 mM sodium phosphate pH 6.0 buffer containing 2.5 mM TMPD.

Measurement of H+/O Ratio, Oxygen Uptake, Absorption Spectrum and Other Analysis—The pH change induced by an oxygen pulse to resting cells under anaerobic conditions was measured in a closed vessel (*20*). Cells were incubated at 30°C in 3.5 ml of 0.5 mM K-Mops (pH 6.8) containing 140 mM KCl and 50 mM KSCN. After onset anaerobiosis, 0.5 μg valinomycin was added, and after ~20 min the reaction was started by addition of 20 μl of air-saturated 140 mM KCl.

The rate of oxygen consumption was measured by using Clark-type oxygen electrode at 30°C. The reaction mixture (2.5 ml) contained 20 mM Na-phosphate buffer (pH 6.5) and 0.35 mg membrane preparation and then the reaction was started by addition of 1 mM NADH as a substrate.

Redox difference spectra were recorded at room temperature by using an Ultrospec 1100 pro spectrophotometer (GE Healthcare). The spectra of air-oxidized enzymes were obtained, and then, a few grains of solid

hydrosulfite was added to obtain their fully reduced forms. Heme contents were estimated from redox spectra using follow molar extinction coefficience: heme *a*, $\Delta\varepsilon_{600\,nm} = 11.6\,mM^{-1}\,cm^{-1}$ (*21*); heme *b*, $\Delta\varepsilon_{562\,nm} = 22\,mM^{-1}\,cm^{-1}$; heme *c*, $\Delta\varepsilon_{552\ nm} = 19.1\,mM^{-1}\,cm^{-1}$ (*22*); heme *d*, $\Delta\varepsilon_{627\ nm} = 27.9\,mM^{-1}\,cm^{-1}$ (*23*). Protein concentration was determined as described by Lowry and co-workers (*24*).

RESULTS

Knock Out of Cytochrome aa_3 or bd Type Terminal Oxidase in C. glutamicum—To investigate the relationship between the combination of respiratory enzymes and cellular energy efficiency in *C. glutamicum*, deletion mutants deficient in either cytochrome aa_3 or *bd* were constructed. Terminal oxidase disruption was performed by homologous recombination using a knockout vector carrying a *cydAB* or *ctaD* fragment having kanamycin resistance cassette in the centre, resulting in the plasmids named pHSG4-*ctaD*::Km^R and pHSG4-*cydAB*::Km^R.

Disruption of each terminal oxidases were confirmed by BN–PAGE and measuring redox difference spectra, dithionite reduced minus air oxidized form, of membranes from the cells grown aerobically in the semi-synthetic medium containing 1% (w/v) glucose (Fig. 3). Although *a*, *b* and *d*-type cytochrome peaks were observed in the redox difference spectra of the 13869 wild-type cells (*trace 1*), the peaks at either 600 or 627 nm, which is characteristic for *a*- and *d*-type cytochrome, were absent in the Δ*ctaD* (*trace 2*) or Δ*cydAB* (*trace 3*) mutant, respectively. In the cytochrome *bd* overexpression mutant, the peak at 627 nm was dominant instead of the cytochrome aa_3 peak at 600 nm (*trace 4*). The *c*-type heme did not give a clear peak or shoulder due to low expression level of cytochrome *c*, however, this type of cytochrome was obviously shown by heme stain after SDS–PAGE (data not shown). On activity staining of BN–PAGE analysis, the membrane preparation from wild type cells showed three bands presenting TMPD oxidase activity. Upper two bands showed strong TMPD oxidation activity, while the lowest one showed weak activity at 160 kDa (Fig. 4). Compared with wild-type cells, the Δ*ctaD* mutant lacked upper two bands which have strong activity, corresponding to the cytochrome *bcc*-aa_3 supercomplex and cytochrome aa_3, respectively, whereas the Δ*cydAB* mutant lacked a lowest band which has weak activity, corresponding to the cytochrome *bd* oxidase.

The Alteration of H^+/O Ratios—An H^+/O ratio was measured by the oxygen pulse method for *C. glutamicum* cells grown under the semi-synthetic and LB medium up to the exponential growth phase. Figure 5A shows typical trace of pH changes induced by an oxygen pulse with wild-type and mutant cells. The H^+/O ratio was 3.94 ± 0.30 ($n = 11$, Fig. 5B *closed bar 1*) for the 13 869 wild-type cells, while the value was 2.76 ± 0.25 ($n = 17$, *closed bar 2*) for the Δ*ctaD* mutant. In contrast, the value was as high as 5.23 ± 0.36 ($n = 12$, *closed bar 4*) for the Δ*cydAB* mutant. The drop in H^+/O ratios of the Δ*ctaD* mutant was complemented by the plasmid pPC4-*ctaD*. The value for the Δ*ctaD*/pPC4-*ctaD* mutant

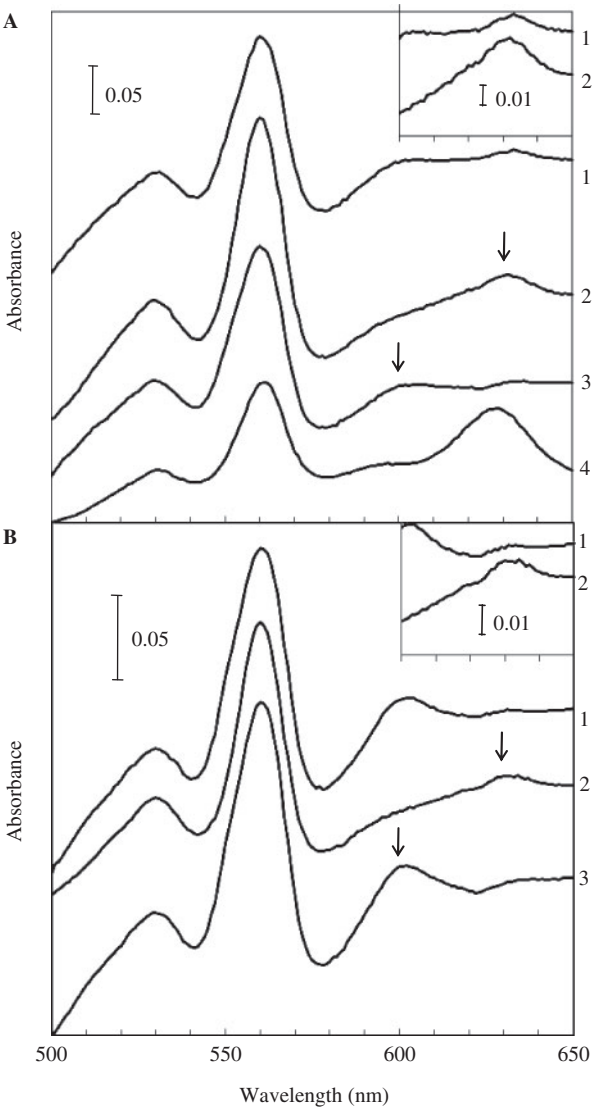

Fig. 3. **Redox difference spectra of membrane extracts from exponential-phase cells grown aerobically in the semi-synthetic medium containing 1% (w/v) glucose (A) and LB medium (B).** *Trace 1: C. glutamicum* 13 869 wild-type; *trace 2:* Δ*ctaD*; *trace 3:* Δ*cydAB*; *trace 4:* Δ*ctaD*/pPC4-*cydABDC*. Arrows indicate the peaks for the aa_3-type oxidase and *bd*-type oxidase. Spectra were measured at room temperature using 10 mg protein/ml in the presence of 5% (w/v) Triton X-100, 0.3 M NaCl and 10 mM sodium phosphate at pH 7.0. *Inset:* the data shows enlarged range of 600–650 nm.

was 4.12 ± 0.30 ($n = 16$, *closed bar 6*) compared to 4.04 ± 0.36 ($n = 16$, *closed bar 5*) for the 13869/pPC4 control cells. The H^+/O ratio was 4.80 ± 0.30 ($n = 16$, *closed bar 7*) for the Δ*cydAB*/pPC4-*cydAB* mutant, in which the deleted gene was complemented by the plasmid pPC4-*cydAB*, containing the *cydAB* gene with its authentic promoter region. This value is 19% higher than that of the control, but slightly less than the Δ*cydAB* mutant. The *cydAB* genes form operon with *cydDC* genes located downstream of the *cydAB* genes (Fig. 2A). The *cydDC* genes are needed for the maturation of cytochrome *bd*.

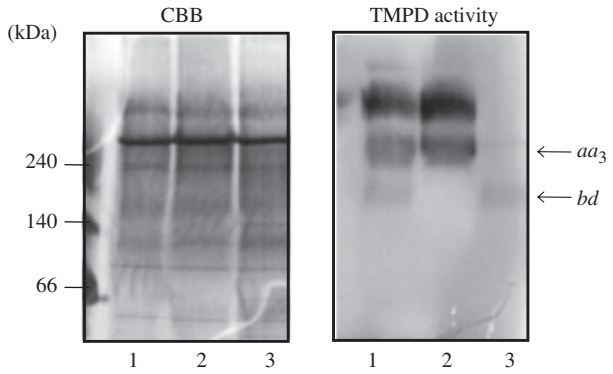

Fig. 4. **Blue-native–PAGE analysis of membrane protein from *C. glutamicum* wild-type and mutant strains.** The membrane protein was solubilized by 1% (w/v) *n*-dodecyl-β-D-maltoside containing 0.75 M aminocaproic acid and 50 mM bisTris–HCl pH 7.0. A 5–18% acrylamide gradient gel was used for native PAGE. The CBB stain and TMPD oxidase activity stain were performed after electrophoresis. *Lane 1:* 13 869 wild-type; *lane 2:* Δ*cydAB* mutant; *lane 3:* Δ*ctaD* mutant. Arrows indicate the bands for the aa_3- and *bd*-type oxidase which have TMPD oxidase activity.

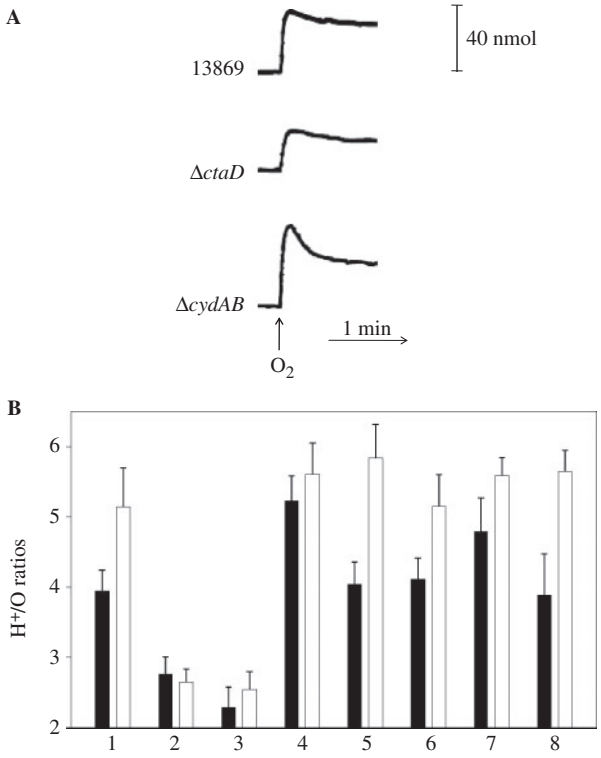

Fig. 5. **pH changes upon oxygen pulse of anaerobic cell suspensions of the resting *C. glutamicum* cells.** (A) The cells (50–100 mg wet wt.) were incubated at 30°C in 3.2 ml of 140 mM KCl containing 50 mM KSCN and 0.5 mM K-Mops (pH 6.8). After anaerobiosis, 0.5 μg valinomycin were added, and after ~20 min the reaction was started by adding air-saturated 140 mM KCl (20 μl, as indicated by the arrow). The buffer action of the medium was determined by the titration with a 4 μl aliquot of 10 mM HCl as indicated by the bar. (B) The means of eleven to 17 experiments on cells grown in the semi-synthetic medium (closed bar) or LB (open bar) were showed. *Lane 1:* 13 869 wild-type; *lane 2:* Δ*ctaD*; *lane 3:* Δ*ctaD*/pPC4-*cydABDC*; *lane 4:* Δ*cydAB*; *lane 5:* 13869/pPC4; *lane 6:* Δ*ctaD*/pPC4-*ctaD*; *lane 7:* Δ*cydAB*/pPC4-*cydAB*; *lane 8:* Δ*cydAB*/pPC4-*cydABDC*.

Although *cydDC* genes are conserved in the chromosome of the Δ*cydAB* mutant, the entire *cydABDC* operon might be necessary for the expression of this enzyme. Thus, we constructed the plasmid pPC4-*cydABDC* to complement the deleted genes completely, and the Δ*cydAB* mutant was transformed by this plasmid (the Δ*cydAB*/pPC4-*cydABDC* mutant). The H^+/O ratio was 3.89 ± 0.58 ($n = 11$, *closed bar 8*) for this mutant. In addition, the Δ*ctaD* mutant was transformed by the plasmid pPC4-*cydABDC* to overexpress cytochrome *bd* oxidase (the Δ*ctaD*/pPC4-*cydABDC* mutant) and investigate the effect of cytochrome *bd* pathway to the bioenergetics and cell growth in more detail. The overexpression of cytochrome *bd* in the Δ*ctaD* mutant caused further reduction of this value. The value was 2.29 ± 0.29 ($n = 16$, *closed bar 3*) for the Δ*ctaD*/pPC4-*cydABDC* mutant, which was 42% lower than that of wild-type cells.

On the other hand, cells grown in LB medium showed ~15–45% higher value compared to those grown in semi-synthetic medium except for the Δ*ctaD* mutant (Fig. 5B, *open bar*). For example, the wild-type cells had an H^+/O ratio of 5.14 ± 0.56 ($n = 11$, *open bar 1*), which is 30% higher than that of semi-synthetic medium. This kind of higher value was observed for all strains with exception of the Δ*ctaD* mutant, but the value of Δ*cydAB* mutant cells were almost similar to that of semi-synthetic medium.

Growth of the ΔcydAB and ΔctaD Mutants of C. glutamicum—Growth of the mutants was evaluated in cells aerobically grown in baffled-flasks as detailed in 'MATERIALS AND METHODS' section. We investigated the growth rate and cell yield of wild-type and mutant strains with different nutrients; semi-synthetic medium containing 1% (w/v) glucose and LB medium. Figure 6 shows the growth curves and colony formation on the plates of several strains of *C. glutamicum*. The cells showed exponential growth until they reached the stationary growth phase. Growth rate of LB grown cells was faster than that of semi-synthetic medium grown cells except for the Δ*ctaD* mutant. On the other hand, cell yields, which are evaluated by wet weight after cultivation, of the semi-synthetic medium showed 1.6–3.4-folds larger amount than that of LB medium. In the semi-synthetic medium culture, these three strains grew about equally (Fig. 6A). The doubling time of the Δ*cydAB* and Δ*ctaD* mutant was slightly longer than that of wild-type in the semi-synthetic medium. Moreover, the cell yield of two mutants at the stationary phase was slightly less compared to that of wild-type, 7 and 14% lower than that of wild-type, respectively (Table 2). In contrast, the Δ*cydAB* mutant showed severe growth defect under more aerobic conditions with higher content of carbon source [50-ml semi-synthetic culture in 500-ml baffled-flasks with 5% (w/v) sucrose, data not shown]. In the LB medium culture, the Δ*ctaD* mutant showed severe growth defect compared to

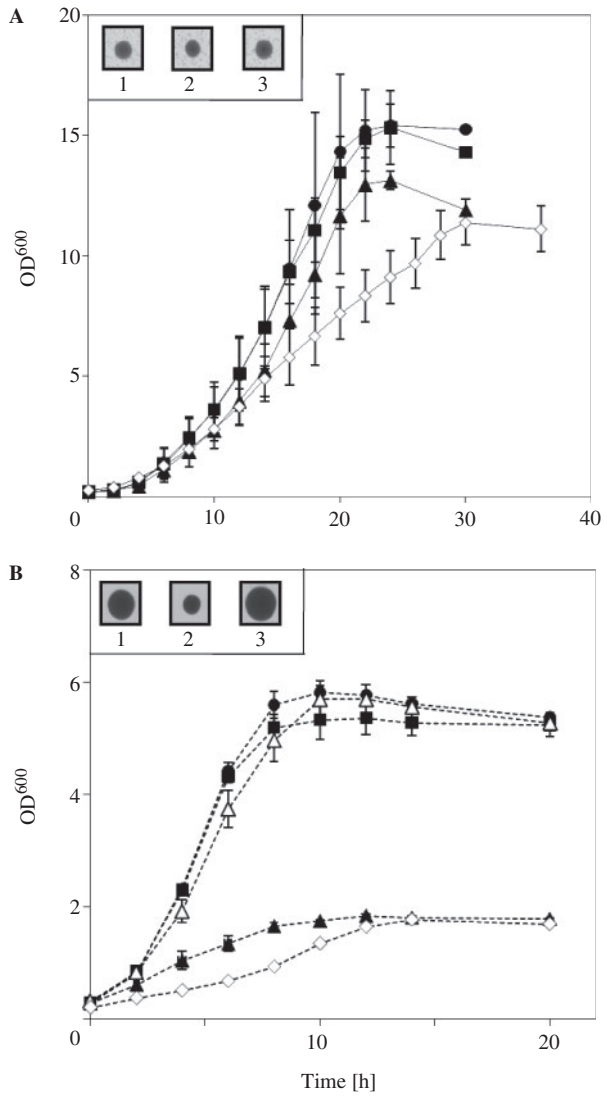

Fig. 6. **Effect of mutation in the terminal oxidase on the growth curves of *C. glutamicum* under aerobic conditions in liquid medium.** Strains were grown at 30°C in shaking flasks (180 rpm) in semi-synthetic medium (A) or LB (B), and growth was monitored by the absorbance at 600 nm. The semi-synthetic medium contained 1% (w/v) glucose as a carbon source. Symbols: *closed circle:* 13 869; *closed triangle:* Δ*ctaD*; *closed square:* Δ*cydAB*; *open triangle:* Δ*ctaD*/pPC4-*ctaD*; *open diamond:* Δ*ctaD*/pPC4-*cydABDC*. Each growth curve shows the mean values and standard division of three cultures. *Inset:* Growth of *C. glutamicum* strains on the plate medium. The colony grown on LB plate for 2–3 days was inoculated on new plates by a wooden pick. *Lane 1:* 13869/pPC4; *lane 2:* Δ*ctaD*/pPC4; *lane 3:* Δ*cydAB*/pPC4.

the 13 869 wild-type cells, while the Δ*cydAB* mutant showed growth largely similar to the wild-type (Fig. 6B). The doubling time of the Δ*ctaD* mutant was 1.6-fold longer than that of wild-type, while that of the Δ*cydAB* mutant was same as wild-type. The cell yield at the stationary phase as obtained by measuring wet weight of Δ*ctaD* mutant was 60% less than that of the wild-type, while that of Δ*cydAB* was 16% less (Table 2).

Table 2. **The cultural characteristic and cytochrome content of each strains.**

	Doubling time (h^{-1})	Cell yields (g wet-wt./l)	Cytochrome (nmol/mg)	
			a	*d*
Semi-synthetic				
13 869	1.7 ± 0.21	14.9 ± 1.1	0.19	0.02
Δ*ctaD*	2.0 ± 0.50	12.8 ± 0.72	ND	0.05
Δ*cydAB*	1.9 ± 0.41	13.8 ± 0.70	0.19	ND
LB				
13 869	1.3 ± 0.04	9.5 ± 0.61	0.26	ND
Δ*ctaD*	2.1 ± 0.04	3.8 ± 0.47	ND	0.03
Δ*cydAB*	1.3 ± 0.04	8.0 ± 0.23	0.33	ND

Cell yields are expressed as wet weights of the cells grown semi-synthetic medium containing 1% glucose as the carbon source or LB medium under aerobic condition. The weights were measured after harvesting at 30 h (semi-synthetic) or 20 h (LB) culture time. The means and standard deviation of three independent experiments are tabulated. ND, not detected.

These results are clear and comparable to those by the plate medium (Fig. 6, *inset*).

The drops in growth of Δ*ctaD* mutant in the LB and semi-synthetic medium were almost completely complemented by the plasmid pPC4-*ctaD*. The cell yield of the Δ*ctaD*/pPC4-*ctaD* mutant in the LB (8.3 ± 0.65 g wet-wt. l^{-1}) and semi-synthetic (15.2 ± 1.0 g wet-wt. l^{-1}) medium was 13% lower and 2% higher than that of wild-type, respectively. On the other hand, overexpression of cytochrome *bd* in the Δ*ctaD* mutant affected to growth rate in the both media. In the semi-synthetic medium, the Δ*ctaD*/pPC4-*cydABDC* mutant cells showed linear growth, and it took longer time until reaching stationary phase than other strains (Fig. 6A). Again, in the LB medium, this mutant showed similar behaviour to that in the semi-synthetic medium (Fig. 6B). However, overexpression of cytochrome *bd* in the Δ*ctaD* mutant did not affect to cell yield, the value was almost same as that of the Δ*ctaD* mutant (11.8 ± 0.48 g wet-wt. l^{-1} for semi-synthetic and 3.7 ± 0.20 g wet-wt. l^{-1} for LB). Interestingly, ~80% decrease of glutamate production was observed compared to the wild-type in the Δ*ctaD* mutant. In contrast, disruption of the cytochrome *bd* oxidase did not cause large change for glutamate production (data not shown).

Effect of Cyanide and p-*Benzoquinone on Respiration of Membrane Fraction*—The O_2 consumption rate of the membrane preparation was measured in the presence and absence of cyanide, a potent inhibitor of heme-copper oxidases. Figure 7A shows the effects of cyanide concentration on the respiration of the wild-type and mutant cells with NADH as the substrate. About 20% of the oxidation rate of the wild-type membranes was inhibited by cyanide biphasically at micromolar and millimolar concentration ranges. The two K_i values are 0.85 μM and 0.72 mM, and the extent of the first component is ~22%. That of the Δ*ctaD* mutant was relatively insensitive to cyanide and gradually inhibited at millli-molar concentrations range, while that of the Δ*cydAB* mutant was inhibited at rather lower concentration than that of the wild-type. In addition, that of the

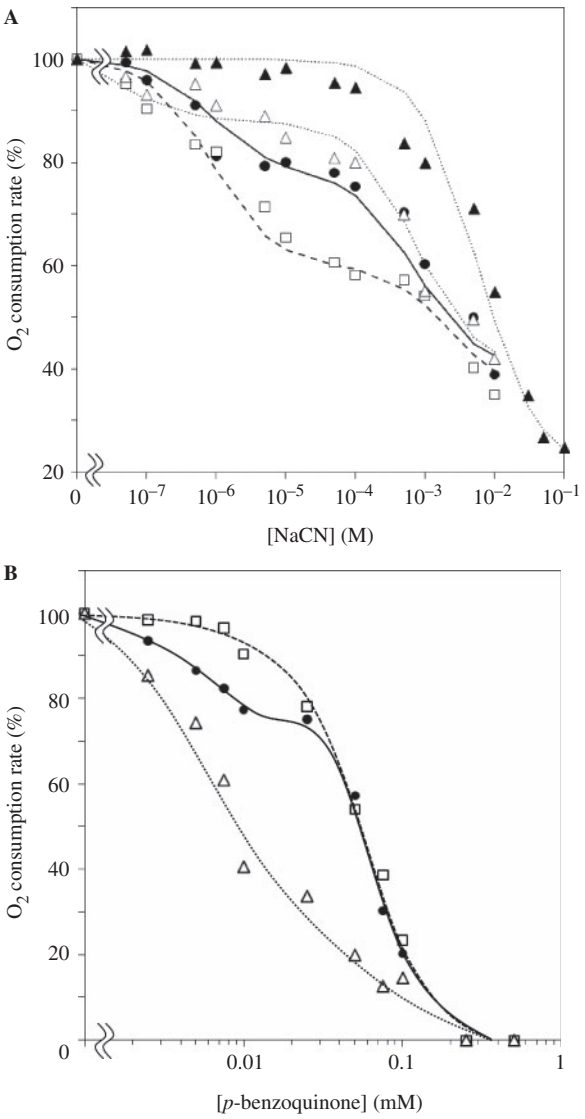

Fig. 7. **Titration by NaCN (A) or *p*-benzoquinone (B) on O_2 consumption of membrane fractions from cells grown in semi-synthetic medium containing 5% (w/v) sucrose as carbon source.** The reaction was carried out at 30°C in 20 mM Na-phosphate buffer, pH 6.5 with 1 mM NADH as substrate using 0.35 mg membranes. The oxidation rates of 100% were 822, 289, 441 and 657 ng-atom O min^{-1} mg-protein^{-1} for the 13 869, Δ*ctaD*, Δ*cydAB* and Δ*ctaD*/pPC4-*cydABDC* membranes, respectively. Symbols: *closed circle* and *solid line:* 13 869 wild-type; *open triangle* and *dotted line:* Δ*ctaD*; *open square* and *dashed line:* Δ*cydAB*; *closed triangle* and *dotted line:* Δ*ctaD*/pPC4-*cydABDC*.

Δ*ctaD*/pPC4-*cydABDC* mutant was the most insensitive to cyanide of all strains with single K_i value of 5.7 mM. About 30–40% of the oxidation rate of each membrane was not inhibited, increasing cyanide concentrations up to 10 mM. Interestingly, the membrane of the Δ*ctaD*/pPC4-*cydABDC* mutant was not inhibited completely up to 100 mM cyanide concentration, remaining ~25% activity.

p-Benzoquinone is known to be a potent inhibitor of *E. coli bd*-type quinol oxidase (*25*). The effect of *p*-benzoquinone on the respiration was also compared among the membranes from the three strains (Fig. 7B). Again, the wild-type membranes showed biphasic behaviour to *p*-benzoquinone. About 20% of the activity was inhibited with concentrations lower than 1 μM, while the rest of respiration was inhibited at those higher than 10 μM. The activity of the Δ*cydAB* mutant was inhibited only in the high *p*-benzoquinone concentration range with a single K_i value of 0.056 mM. The membranes of the Δ*ctaD* mutant were more sensitive against *p*-benzoquinone, and thus the respiration of the Δ*ctaD* membranes was inhibited at a lower concentration than other strains. The activity of the respiration of the Δ*ctaD* membranes decreased gradually from the concentration of 1 μM with a single K_i value of 0.011 mM. The respiration of every strain was fully inhibited at the concentration of 0.5 mM.

DISCUSSION

In this study, we constructed mutant strains of *C. glutamicum* by disrupting genes either for cytochrome aa_3-type cytochrome *c* oxidase or for *bd*-type quinol oxidase, called the Δ*ctaD* and Δ*cydAB* mutants, respectively. We constructed the plasmids to complement the deleted genes. Additionally, to investigate the cytochrome *bd* pathway in more detail, we constructed the Δ*ctaD*/pPC4-*cydABDC* mutant which overexpressed cytochrome *bd* in the cytochrome aa_3 deficient mutant. Then, we investigated the relationship between energy conservation and cell growth with semi-synthetic and LB medium. It was suggested that the bioenergetics of *C. glutamicum* was dramatically affected by the growth nutrients.

The 600 and 627 nm peaks, corresponding to cytochrome aa_3 and *bd*, respectively, were observed in the redox spectrum of the wild-type cells, while the former peak disappeared in the Δ*ctaD* mutant cells. Instead, complementary increase of cytochrome *bd* peak was observed in this mutant (Fig. 3 and Table 2). Additionally, the *d*-type heme peak at 627 nm became prominent in the Δ*ctaD*/pPC4-*cydABDC* mutant (Fig. 3A). Besides the absence of cytochrome aa_3 peak at 600 nm, the *b*-type cytochrome peak at 560 nm became to be sharper than that of wild-type cells. This similar observation, severe reduction of the *c*-type heme peaks at 552 nm with the deletion of the cytochrome aa_3 oxidase, has also been reported by Niebisch and Bott (*2*). On the other hand, the peaks at 627 nm, due to *d*-type cytochrome, disappeared in the Δ*cydAB* mutant cells. Complementary increase of cytochrome aa_3 peak was not observed in this mutant (Fig. 3A and Table 2). In the activity staining analysis for TMPD oxidase on BN–PAGE, the strong two bands shown in the wild-type, were absent in the Δ*ctaD* mutant, while the weak band remained (Fig. 4). In contrast, only the weak band disappeared in the Δ*cydAB* mutant. Additionally, cytochrome *bd* oxidase of this bacterium does not oxidize TMPD well, although that of *E. coli* oxidizes does well (*5*). Therefore, it is appropriate that lowest band of BN–PAGE has weaker activity than other bands. These results clearly indicate that cytochrome *bd* oxidase should have operated as the

main terminal oxidase instead of cytochrome aa_3 oxidase in the Δ*ctaD* mutant.

Cytochrome aa_3 has high affinity to cyanide, thus the respiration of this oxidase is inhibited at low concentration of cyanide. The NADH oxidation by the wild-type cell membranes, which contained both cytochrome aa_3 and *bd*, was inhibited biphasically at cyanide concentration with K_i values of 0.85 μM and 0.72 mM (Fig. 7A). The Δ*cydAB* mutant was more sensitive to cyanide than wild-type because of the sole presence of cytochrome aa_3. In contrast, the Δ*ctaD* mutant was more tolerant to cyanide than the wild because of the absence of cytochrome aa_3. Thus, in the case of the wild-type, the former decrease might be due to the inhibition of cytochrome aa_3 and the latter to the inhibition of cytochrome *bd*. Interestingly, the oxidase activity was not inhibited completely in all strains, increasing cyanide concentration up to 10 mM. In our previous study, this type of oxidase activity was insensitivity up to 30 mM cyanide (*5*). The purified cytochrome *bd* oxidase was inhibited at the milli-molar range. Thus, it is considered that cytochrome *bd* oxidase is not contributed to this cyanide insensitive component. Nantapong *et al*. (*26*) has reported that purified NADH dehydrogenase of *C. glutamicum* directly reacted with oxygen and thus NADH oxidase activity of the membranes showed high cyanide resistance, and this cyanide insensitive component of the membranes is due to this enzyme.

Again, the wild-type cell membranes showed biphasic behaviour to *p*-benzoquinone (Fig. 7B). The Δ*cydAB* mutant membranes were more resistant to *p*-benzoquinone than that of the wild, while that of the Δ*ctaD* mutant were more sensitive. Since the Δ*ctaD* mutant uses the cytochrome *bd* as the principal terminal oxidase, the effect of *p*-benzoquinone at very low concentrations is a result of inhibition of the cytochrome *bd* in the Δ*ctaD* mutant. Thus, in the case of the wild-type, the former decrease might be due to the inhibition of cytochrome *bd* and the latter to the inhibition of cytochrome aa_3. These results suggest that *C. glutamicum* cytochrome *bd* is inhibited strongly as well as *E. coli* cytochrome *bd*, although the type of Q-loop structure is different (*5*). The inhibition of the remaining respiration in the Δ*ctaD* membranes at high concentration of *p*-benzoquinone seems to be due to inhibition of quinol cytochrome *c* reductase (the *bcc* complex).

The H^+/O ratio was altered by disruption of a terminal oxidase in *C. glutamicum* (Fig. 5). The Δ*cydAB* mutant cells showed the ratio of 5.23, which is 33% higher than that for the wild-type cells (Fig. 5B). The H^+/O ratio of *bcc*-aa_3 branch is expected to be 6, based on the stoichiometry of 4 and 2 for *bcc* and aa_3, which has been obtain for the other organisms such as *G. thermodenitrificans* wild-type and mutant strains (*13*, *14*, *20*). The ratio of 5.23 is close to 6, the value expected when only cytochrome *bcc*-aa_3 supercomplex branch is used as main electron transfer pathway (Fig. 1). In contrast, the Δ*ctaD* mutant cells showed the H^+/O ratio of 2.76, which is 30% lower than that for the wild (Fig. 5B). The amount of cytochrome *bd* oxidase is very low even in the Δ*ctaD* mutant, since this oxidase operates alternatively. The Δ*ctaD*/pPC4-*cydABDC* mutant showed lower value than that of the Δ*ctaD* mutant. It may be suggested that another component other than cytochrome aa_3 and *bd* affected to the H^+/O ratio. The value was 2.29, which is 42% lower than that for the wild (Fig. 5B). The H^+/O ratio of cytochrome *bd* branch should be 2, because this type of oxidase has been shown to catalyse only electron transfer across membranes (*27*). The ratio of 2.29 is not far from 2, the value expected when only cytochrome *bd* branch is used as main pathway (Fig. 1). The deviation of the observed values from the calculated ones may be due to the heterogeneity of the endogenous substrates. The wild-type strain, containing both oxidase gene sets, showed an intermediate value of 3.94, which is reasonable when both electron transfer pathways are postulated to operate to comparable extent. The drop of H^+/O ratio in the Δ*ctaD* mutant was fully complemented by plasmid pPC4-*ctaD*, whereas the alteration of the ratio in the Δ*cydAB* mutant did not return to the value of the wild cells completely by plasmid pPC4-*cydAB* (Fig. 5B). The *cydAB* genes form operon with *cydDC* genes located downstream of the *cydAB* genes (Fig. 2A). The *cydDC* genes encoded an ABC transporter which is needed for the maturation of cytochrome *bd*-type quinol oxidase. Although *cydDC* genes are conserved in the chromosome of the Δ*cydAB* mutant, the entire *cydABDC* operon might be necessary for the expression of this enzyme. Thus, we constructed the plasmid pPC4-*cydABDC*, containing the entire *cyd* operon with its authentic promoter region. The Δ*cydAB* mutant was transformed by this plasmid and constructed the Δ*cydAB*/pPC4-*cydABDC* mutant. This mutant showed 3.89 of H^+/O ratio, and this value is close in value to that of control, 4.04 (Fig. 5B).

In this study, we also observed that the H^+/O ratio was affected by the growth nutrients. The cells grown in LB medium showed about 25% higher H^+/O ratios compared to those grown in semi-synthetic medium except for the Δ*ctaD* mutant cells (Fig. 5B). The former cells contained higher content of *a*-type cytochrome than the latter cells and did not contain *d*-type cytochrome (Table 2), suggesting that the cells grown in the LB medium use cytochrome aa_3 oxidase dominantly. Since much amount of cytochrome aa_3 oxidase expressed in the LB medium, it is considered that H^+/O ratios of cells grown in the LB medium are higher than that of semi-synthetic medium.

Then, we investigate the effect of the terminal oxidase disruption and concomitant alteration of H^+/O ratios to cell growth in the flak culture with different nutrients. While growth rate of LB grown cells was faster than that of the semi-synthetic medium, cell yields of LB grown cells were lower than those of the semi-synthetic medium. When the wild-type cells were grown in the totally synthetic medium, including 1% (w/v) glucose but excluding polypeptone, cell yields was less than one-sixth those of the LB grown cells (data not shown). Thus, contained polypepton might result in the larger amount of cell yields grown in the semi-synthetic medium than those in the LB medium. In the LB medium, the Δ*ctaD* mutant grew less than the wild-type, and this significant growth defect could be complemented by the plasmid pPC4-*ctaD* concomitantly with the recovery of the H^+/O ratio. It is suggested that the cells growth, especially growth rate, is directly correlated

to H^+/O ratio. However, there was no significant difference of cells growth in the semi-synthetic medium between wild-type and the Δ*ctaD* mutant cells despite the large difference of H^+/O ratios. Additionally, the growth rate of the Δ*ctaD*/pPC4-*cydABDC* mutant was slower than that of the Δ*ctaD* mutant in both media (Fig. 6 and Table 2). In contrast, cell yield of this mutant was equally to that of the Δ*ctaD* mutant. Cells grown in the LB medium showed higher H^+/O ratios than that of semi-synthetic medium as discussed above (Fig. 5B). It may be that the alteration of bioenergetics depending on growth nutrients affected to differential cell growth between LB and semi-synthetic medium grown cells. On the other hand, the simple enhancement of cell growth was expected by the deletion of cytochrome *bd*-type quinol oxidase, which really increased cellular H^+/O ratio, in other words, efficiency of energy metabolism as observed in our previous report on thermophilic bacilli and their mutant strains (*13*, *14*). However, the deletion of this enzyme did not cause any large change on cell growth in both medium (Fig. 6). In fact, the contrary result was previously reported. Kabus *et al*. (*17*) reported that the Δ*cydAB* mutant of *C. glutamicum* ATCC13032 strain showed growth defect in the glucose minimal medium. Also in our study, the cell yield of the Δ*cydAB* mutant was ~40% less than wild-type cells, when this mutant was grown under more aerobic condition, reducing medium volume to half (data not shown). Thus, this oxidase is also important for growth under aerobic conditions, although large amount of cytochrome *bd* expresses under microaerobic conditions. It may be that the absence of cytochrome *bd* results in severe stress on the cells as observed in our previous study (*15*). It is likely that deletion of cytochrome *bd* effective for enhancement of cell growth, since the H^+/O ratio of this mutant truly increased. However, growth conditions, such as kind of carbon source and aeration condition, are also important for enhancement of cell growth. We are currently attempting to find the conditions for this aim.

Recently, it was reported that cytochrome *bd* deletion on the lysine producing strain *C. glutamicum* MH20-22B caused ~12% increasing lysine production (*17*). The Δ*ctaD* mutant constructed in this work showed significant defect of glutamate production (data not shown). Disruption of respiratory oxidase affects other metabolic pathways in amino acid production. Although further work is necessary to confirm metabolic changes of *C. glutamicum* strains by the proteome analysis, the alteration of energy metabolism can be useful to improve materials production as shown in this study.

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CONFLICT OF INTEREST

None declared.

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