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Monitoring enzyme expression of a branched respiratory chain of *corynebacterium glutamicum* using an EGFP reporter gene

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Abstract To investigate the expressional control of branched respiratory chain complexes of the amino-acid producing bacterium *Corynebacterium glutamicum* according to growth conditions, the expression indexes of the *ndh*, *sdh*, *qcrCAB*, *ctaCF*, *ctaD*, *ctaE*, and *cydAB* genes were estimated under aerobic and microaerobic, and carbon-rich and -poor conditions. The promoter region of each target gene was cloned upstream of the EGFP gene on expression vector pVK6, and the nine reporter constructs were transformed into *C. glutamicum* ssp. *lactofermentum*. The cytochrome content of cellular membranes obtained from each growth phase closely corresponded to the expression indexes based on EGFP fluorescence and cell density, indicating that this rapid and convenient method is suitable for analyzing the expression levels of respiratory chain complexes. Using this method, we demonstrated that a reciprocal change in the expression levels of cytochrome *bd*-type and aa_3-type oxidases occurs when *C. glutamicum* cells are held in stationary phase for extended periods.

Keywords *Corynebacterium glutamicum* · Promoter activity · Respiratory chain · Expressional control

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Abbreviations

EGFP	Enhanced green fluorescent protein
GFP	Green fluorescent protein
NDH	NADH dehydrogenase
NDH—II	NADH dehydrogenase type II
SDH	Succinate dehydrogenase
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis

Introduction

Biochemical and molecular biological studies, including whole genome sequence analyses, have revealed that enzymes in the respiratory chain of microorganisms are highly diverse, in contrast to those of mammalian mitochondria (Sone et al. 2004; Sakamoto and Sone 2004). Moreover, a single species typically possesses several types of electron transport pathways, which selectively operate in response to growth conditions. Although the major group of terminal oxidases is heme-copper oxidases, numerous bacteria and archaea also possess cytochrome *bd*-type oxidase, which contains hemes *b* and *d*, but not a Cu atom (Jünemann 1997). These two oxidase groups lack sequence similarity and common structural signatures, suggesting that they are unrelated with each other in terms of molecular evolution.

Heme-copper oxidases can be classified into three groups: type A (which is further subdivided into A1 and A2), type B, and type C (Castresana and Saraste 1995; Pereira et al. 2001). To date, biochemical and molecular biological researches on microbial respiratory chains have generally focused on Gram-negative bacteria such as *Escherichia coli*, *Rhodococcus* spp., and *Paracoccus* spp.

as model systems of mitochondrial-type pathways which are unbranched and compose of complex I, II, III, and IV; another, Gram-positive bacteria include many industrially and medically important species and also possess divergent respiratory enzymes of which the coordination and differential expression are unclear.

Corynebacterium glutamicum belongs to the phylum Actinobacteria, which is composed of Gram-positive bacteria with a high G + C ratio, and is utilized for the industrial production of amino acids. This bacterium also serves as a model organism for closely related pathogenetic bacteria, such as *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*. Recent studies by our and other groups have revealed that *C. glutamicum* possesses two electron transport pathways downstream of the quinone pool in the aerobic respiratory chain (Fig. 1; Sakamoto et al. 2001; Bott and Niebisch 2003). One pathway functions with cytochrome *bcc* complex-cytochrome aa_3-type cytochrome *c* oxidase as the terminal oxidase (Sakamoto et al. 2001), and the other utilizes cytochrome *bd*-type quinol oxidase (Kusumoto et al. 2000). Cytochrome *bcc* complex is a new type of complex III, which includes a hydrophobic diheme *c* subunit (Sone et al. 2001). Cytochrome aa_3-type cytochrome *c* oxidase is a conserved type-A cytochrome *c* oxidase, but contains a unique ampholytic domain rich in both acidic and basic amino-acid residues in the vicinity of the Cu_A binding site of subunit II which accepts electrons from the substrate cytochrome *c*.

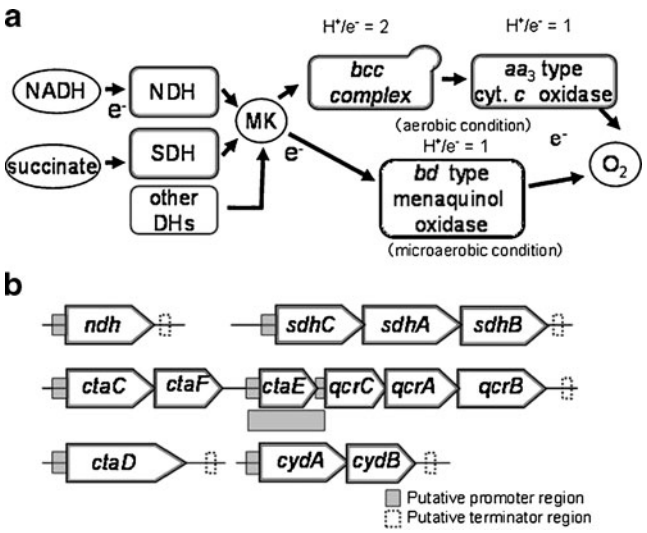

Fig. 1 Schematic representation of respiratory chain complexes and their associated genes in *C. glutamicum*. **a** Schematic representation of respiratory chain complexes of *C. glutamicum*. Square: enzyme of respiratory chain complex, circle: enzyme substrate, arrow: flow of electron transfer. NADH dehydrogenase (NDH), succinate dehydrogenase (SDH), and other dehydrogenases (DHs) transfer electrons to menaquinones (MKs), and the reduced menaquinols subsequently transfer electrons to the two branched respiratory pathways. The first utilizes cytochrome *bcc* complex–cytochrome aa_3 oxidase, which transports three protons per one electron, and the second pathway functions with cytochrome *bd* oxidase as the terminal oxidase, which transports one proton per one electron. **b** Five genomic loci for genes of respiratory chain enzymes. The genes encoding NDH (*ndh*), SDH (*sdhCAB*), subunits II and IV of cytochrome aa_3 oxidase (*ctaCF*), subunit I of cytochrome aa_3 oxidase (*ctaD*), subunit III of cytochrome aa_3 oxidase (*ctaE*), cytochrome *bcc* (*qcrCAB*), and subunit I and II of cytochrome *bd* oxidase (*cydAB*) are indicated by pentagons. Squares and dotted-line squares indicate putative promoter and terminator regions, respectively, with the exception of the *ctaE-qcrCAB* genes that include the putative promoter region and complete structural gene *of ctaE*

Upstream from the quinone pool, *C. glutamicum* utilizes non-proton pumping NADH dehydrogenase (type II or NDH-II, Matsushita et al. 2001) and succinate dehydrogenase, which is classified into five groups: types A through E (Lancaster 2003). *C. glutamicum* cells contain type-B dehydrogenase, which has been suggested to couple inward proton flow (Matsushita et al. 2001; Kurokawa and Sakamoto 2005). The other species in the phylum Actinobacteria, such as *Streptomyces griseus* and *Rhodococcus rhodochrous*, possess a similar branched respiratory chain and have industrial utility for the production of antibiotics and conversion of fine chemicals (Sone et al. 2003; Kobayashi et al. 1993, and Morii et al. 1998). Although the enzyme complexes in the actinobacterial respiratory chain have been characterized individually, as described above, the coordination and differential expression of these complexes are unclear.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a well-known method to simultaneously analyze expressional changes of numerous proteins; however, it cannot be used for continuous measurement in principle and has not been readily applicable to bacterial membrane proteins due to their hydrophobicity and structural complexity. Another method to estimate expressional changes of proteins is promoter activity assays that utilize enzymes, such as alkaline phosphatase and horseradish peroxidase, as reporters for the promoter activity of the target gene. However, these methods with alkaline phosphatase and horseradish peroxidase are also unsuitable for continual monitoring or detecting low-level expression of genes. The method to estimate quantification of the proteins including cytochromes in their molecules is a cytochrome spectra assay. However, this method is only for the proteins including cytochromes and has a low sensitivity. Green fluorescent protein (GFP) from *Aequorea victoria* has a molecular weight of 27 kDa and exhibits a stable β-barrel structure in which a chromophore moiety is embedded. GFP has many beneficial characteristics as a reporter enzyme, as it is resistant to protease cleavage and severe temperatures (−70 °C), has low cytotoxicity, and the chromophore matures spontaneously in numerous cell types. Enhanced GFP (EGFP) is a genetically engineered derivative of GFP, whose fluorescent intensity is 35-fold

higher than that of original GFP, and is widely used as a reporter for gene expression in cell and developmental biology.

Here, we utilized EGFP to monitor low-level expression of genes encoding the branched respiratory chain complexes of *C. glutamicum*. We constructed nine reporter plasmids, in which the EGFP gene was joined to the promoter region for the target respiratory enzyme complexes and inserted into expression vector pVK6. *C. glutamicum* was transformed with one of the plasmids and cultured under several divergent growth conditions, and the fluorescent intensities of cells were monitored at various growth stages.

Materials and methods

Bacterial strain and growth conditions

C. glutamicum subsp. *lactofermentum* (ATCC13869) was kindly provided by Ajinomoto Co., Inc., and was routinely cultured on a basal medium composed of 5 g polypeptone, 5 g $(NH_2)_2CO$, 1 g KH_2PO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4$, 0.01 g $MnCl_2 \cdot 4H_2O$, 5 mg nicotinamide, 0.2 mg thiamine, and 0.05 mg biotin per liter (pH 7.5). As a carbon source, the basal medium was supplemented with either 50 g (rich medium) or 5 g (poor medium) sucrose per liter. To prepare a low-biotin medium, the amount of biotin was decreased to 0.005 mg per liter.

Two aeration conditions were applied for the culture of bacteria. For aerobic conditions, bacteria were cultured in 50 ml medium in 500-ml baffled flasks shaken at 180 rpm, while microaerobic conditions were achieved using 100 ml medium in 500-ml baffled flasks shaken at 90 rpm. For both conditions, the cultures were incubated at 30 °C, and were inoculated with a 6-ml starter culture in basal medium. The optical density at 600 nm (OD_{600}) of cultures was measured using a UV-210A spectrophotometer (Shimazu) to monitor cell growth.

Measurement of fluorescent intensity

Growth media was collected at four time points, representing mid-exponential (I), late exponential (II), early stationary (III), and late stationary growth phases (IV), which corresponded to the times equaling 1/3,1/4, and 2/3 of the final culture turbidity in the stationary phase, and 3 h after the initiation of the stationary phase, respectively. In addition, culture medium was collected at 15 h (V) and 24 h (VI) after cells entered stationary phase. For the measurement of fluorescent intensity, 1 ml of collected medium was centrifuged at 9,100 × g for 2 min. The resultant pellet was washed in buffer containing 10 mM Na-phosphate (pH 7.4) and 0.5% (w/v) NaCl, and was resuspended in the identical buffer for sample measurements. Fluorescent intensity was measured using excitation and emission wavelengths of 484 and 510 nm, respectively, and a FP-6500 spectrophotofluorometer (Jasco). To normalize the fluorescence index with cell mass, the fluorescent intensity value was divided by the OD_{600} value, which was expected to be proportional to cellular mass. This normalized value is designated here as the expression index.

Membrane preparation and cytochrome content analyses

Collected cells in each growth phase were pelleted by centrifugation and then suspended in 1.0 ml membrane preparation buffer (10 mM Na-phosphate [pH 7.4], 0.5% [w/v] NaCl). Cells were disrupted by vigorous mixing with glass beads (diameter 0.18 mm) in a cell-disrupting mixer (Bead-Beater, Biospec), and unbroken cells were then removed by centrifugation at 5,000 × g for 10 min. The resultant supernatant was centrifuged at 100,000 × g for 20 min, and the precipitate was resuspended in 0.1–0.2 ml membrane preparation buffer. The concentration of cytochromes *a*, *b*, *c*, and *d* in each sample was determined by measurement of the $Na_2S_2O_4$-reduced minus oxidized difference spectrum using a DU-530 spectrometer (Beckman) at room temperature. Cytochrome contents were calculated using millimolar extinction coefficients of 19.1 at 552 nm, 21.0 at 562 nm, 10.5 at 600 nm, and 23.5 at 627 nm. Protein concentration was determined as described by Lowry et al. (1951).

DNA manipulations

Enzymes used for DNA digestion and ligation, plasmid pUC118, plasmid pEGFP-C1, and *Escherichia coli* JM109 were purchased from either Takara or New England Biolabs. Plasmid pVK6 was kindly provided by Ajinomoto Co., Inc. *C. glutamicum* genomic DNA was extracted using a gene extraction kit (Dr GenTLE® System; Takara). *E. coli* XL1-blue was used for the construction of plasmids and was grown at 37 °C in 2x TY medium (1.6% [w/v] polypeptone, 1.0% [w/v] yeast extract, and 0.5% [w/v] NaCl, pH 7.0) containing 50 μg/ml kanamycin. For the construction of pVK6-based plasmids, *E. coli* JM109 was used. Preparation of competent *E. coli* cells and genetic transformation were performed by the $CaCl_2$ method (Cohen et al. 1972).

Plasmid pVK6 functions as a shuttle vector between *E. coli* and *C. glutamicum*, and was previously constructed by combining plasmid pAM330 derived from *C. glutamicum* with the *E. coli* cloning vector pHSG299. The kanamycin-resistance cassette (Km^R) derived from pHSG299 was

utilized as a selection marker for pVK6 by supplementing culture medium with 25 μg/ml kanamycin.

In total, nine reporter plasmids were prepared (Fig. 1, Table 1). Approximately 100 or 400 bp of upstream DNA (putative promoter regions) from initiation codons of the following structural genes were amplified by PCR: (1) *qcrCAB* operon (cytochrome *bcc* complex); (2) *ctaCF* operon (subunits II and IV of cytochrome aa_3 oxidase); (3) *ctaD* (subunit I of cytochrome aa_3 oxidase); (4) *ctaE* (subunit III of cytochrome aa_3 oxidase); (5) *ctaE-qcrCAB* (6) *cydAB* (subunit I and II of cytochrome *bd* oxidase); (7) *ndh* (type-II NADH dehydrogenase); (8) *sdhCAB* (succinate dehydrogenase) (Patek et al. 2003; Melin et al. 1987). Since the upstream region of *qcrCAB* (73 bp) may have been too short to contain a functional gene promoter, the upstream region of the *ctaE-qcrCAB* genes (1,130 bp) was used as a promoter region of the cytochrome *bcc* complex.

For the ligation of PCR-amplified promoter regions into pUC118 and pVK6 plasmids, *Eco*RI sites were introduced in 5′-terminal sequences of forward primers. In addition, 3′-terminal sequences of reverse primers were partially replaced by *Nco*I sites for fusion with the EGFP gene. The EGFP gene was amplified by PCR using pEGFP-C1 plasmid as a template and suitable primers for the introduction of an *Eco*RI site at the 5′-terminus, and a *Sac*I site and stop codon at 3′-terminus. The amplified EGFP gene and pUC118 were digested with *Eco*RI and *Sac*I, and then ligated together to generate pUC118EGFP.

Plasmid pUC118EGFP was digested with *Eco*RI and *Sac*I to excise the EGFP gene, which was then ligated into *Eco*RI/*Sac*I-digested pVK6 to generate pVK6EGFP. Plasmid pVK6EGFP did not contain a promoter region upstream of the EGFP gene, and was used as a negative control.

The pVK6*ctaE*-EGFP, pVK6*ctaCF*-EGFP, pVK6*ctaD*-EGFP, pVK6*cyd*-EGFP, and pVK6 *qcrCAB*-EGFP reporter plasmids were constructed as follows. Putative promoter sequences upstream of the *ctaCF*, *ctaE, ctaD,* and *cydAB* genes were digested with *Eco*RI and *Nco*I, and then ligated into *Eco*RI/*Nco*I-digested pUC118EGP plasmid to generate pUC118*ctaCF*-EGFP, pUC118*ctaE*-EGFP, pUC118*ctaD*-EGFP, and pUC118*cyd*-EGFP, respectively. An upstream region of the *qcrCAB* operon, including entire the *ctaE* gene, was amplified by PCR with a *ctaE* forward primer and a *qcr* reverse primer. The amplified 1.8-kb PCR product was digested with *Eco*RI and *Nco*I and then ligated into *Eco*RI/*Nco*I-digested pUC118EGFP to generate pUC118*ctaE-qcrCAB*-EGFP. Plasmid pUC118*ctaE-qcrCAB*-EGFP was digested with *Hinc*II and *Sma*I and then dephosphorylated. A 0.9-kb DNA fragment including the 0.1-kb upstream region of the *qcrCAB* operon and the EGFP gene was ligated into *Sma*I-digested pUC118 to generate pUC118*qcrCAB*-EGFP. To express EGFP in *C. glutamicum*, promoter-EGFP gene fragments were excised from each pUC118-promoterEGFP construct with *Eco*RI and *Sac*I, and then ligated into *Eco*RI/*Sac*I-digested pVK6 to generate pVK6*ctaE*-EGFP, pVK6-*ctaCF*EGFP, pVK6-*ctaD*EGFP, and pVK6*cyd*-EGFP. Plasmid pUC118*qcrCAB*-EGFP was digested with *Eco*RI and *Sal*I, and the excised *qcrCAB*-EGFP fragment was ligated into *Eco*RI/*Sac*I-digested pVK6 digested.

Plasmids pVK6*ndh*-EGFP, pVK6*sdhCAB*-EGFP, and pVK6*ctE-qcrCAB*-EGFP were constructed as follows. Amplified *ndh* and *sdhCAB* putative promoter regions were

Table 1 The nine reporter plasmid constructs used in this study

No.	Sequence inserted into pVK6	Primers (5′-3′) Forward/reverse[a]	Promoter length (bp)
(1)	*qcrCAB*-EGFP	cagtag***gaattc***aggaatcctcaacgttgt tttag***ccatgg***tatttccctctggggt	73
(2)	*ctaCF*-EGFP	tacggc***gaattc***acatctgaggtgaaaagg cacgcttattttgctgtt***ccatgg***gtgttc	400
(3)	*ctaD*-EGFP	cgaga***gaattc***tctgaaagcgaaaagtggg gctcg***ccatgg***tatctagtatggctg	350
(4)	*ctaE*-EGFP	agaga***gaattc***tctgaaagcgaaaagtggg gctcg***ccatgg***tatctagtatggctg	400
(5)	*ctaE-qcrCAB*-EGFP	agaga***gaattc***tctgaaagcgaaaagtggg tttag***ccatgg***tatttccctctggggt	1,130
(6)	*cydAB*-EGFP	gcccg***gaattc***aaggagttgtgcaaccggt cgacat***ccatgg***taggtccccttcctcttc	400
(7)	*ndh*-EGFP	agtgg***gaattc***tgccaagaacgctc aactg***ccatgg***ttgtcaaagtctcc	400
(8)	*sdhCAB*-EGFP	ccgg***gaattc***tttaggcacctacgg taacag***ccatgg***cacctccagtgtc	365
(9)	EGFP		–

[a] underlined sequences are the area correspond to each promoter region, while the bold and italicized sequence indicate introduced restriction endonuclease sites

digested with *Eco*RI and *Nco*I, and ligated into *Eco*RI/*Nco*I-digested pVK6EGFP to generate pVK6*ndh*-EGFP and pVK6*sdhCAB*-EGFP, respectively. pUC118*ctE-qcrCAB*-EGFP and pVK6 were digested with *Eco*RI and *Sal*I, and the excised *ctE-qcrCAB*-EGFP fragment and pVK6 were ligated into *Eco*RI/*Sal*I-digested pVK6EGFP to generate pVK6*ctE-qcrCAB*-EGFP.

Each constructed pVK6 plasmid encoding a putative promoter region and the EGFP gene were transferred by electroporation (25 μF, 400 Ω, and 2.5 kV) into 100 μl competent *C. glutamicum* subsp. *lactofermentum* cells using 0.2-cm gapped cuvettes. Immediately after electroporation, 1 ml of L-medium (1.0% polypeptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose, pH 7.2) containing 10% (v/v) glycerol was added to the cells at room temperature, and the suspension was then transferred to a test tube with a lid. The test tube was incubated for 4 h at 30 °C with shaking at 120 rpm. Subsequently, recovered cells were then plated on selective L-medium agar plates containing 25 μg/ml kanamycin and incubated at 30 °C for 2–4 days to allow the selection of transformed cells.

Results

Construction of the GFP-promoter assay system and cellular cytochrome content under various growth conditions

Nine reporter plasmids were constructed to monitor the expression levels of several respiratory enzymes and subunits of *C. glutamicum* subsp. *lactofermentum*. In the constructs, the EGFP gene was fused in frame with the promoter regions of the target respiratory enzymes (Table 1 and Fig. 1). Cells of *C. glutamicum* subsp. *lactofermentum* were transformed with the reporter plasmids, in addition to a negative control construct containing a promoter-less EGFP gene. Growth curves of the transformed cells exhibited no significant differences from those of wild-type cells (data not shown). In addition, a clear fluorescent signal due to EGFP expression that was significantly higher than the background signal of cells containing the negative control construct was observed for each transformant (Fig. 2).

We next compared the fluorescence intensities resulting from reporter EGFP gene expression with cytochrome contents of the respiratory chain components in transformant cells cultured under four different growth conditions. The experimental conditions were alternative combinations of two aeration (highly aerobic vs. microaerobic) and carbon-rich and -poor (5% vs. 0.5% [w/v] sucrose, respectively] conditions (Fig. 3(a), see Materials and Methods). Compared to aerobic-rich conditions, the doubling times of cells grown under the microaerobic and aerobic-poor conditions were slightly longer (Table 2). In addition, the accumulated cell mass in the stationary phase was significantly lower under the three latter conditions than that observed under the aerobic-rich conditions (Table 2, Fig. 3(a)). To estimate cellular cytochrome content for each growth condition, cells were collected at four time points (I–IV), corresponding to mid-exponential, late exponential, early stationary, and late stationary growth phases, respectively, as indicated in Fig. 3(a), and redox difference spectra of the separated membrane fractions were measured (Fig. 4, Table 3). The levels of cytochromes *a* and *b* decreased with increasing culture time, although the levels inversely increased in the late stationary phase. The level of cytochrome *c* also decreased as the culture progressed, and became undetectable by the early stationary phase. In contrast, cytochrome *d* was undetectable in the middle growth phase, but had reached the highest level by the late stationary phase.

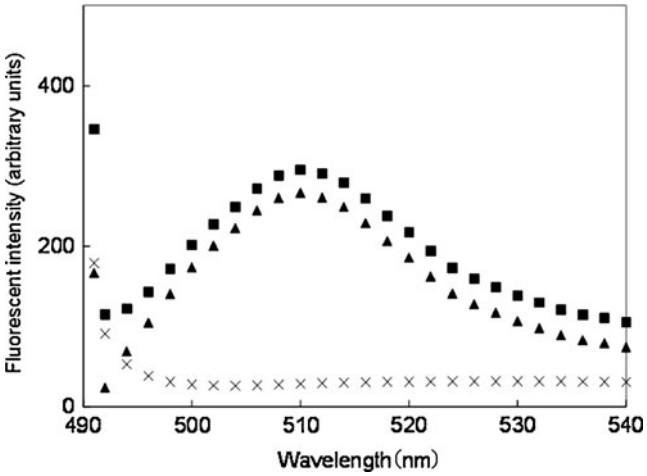

Fig. 2 Fluorescence spectra of cells transformed with the pVK6EGFP*cydAB* construct (*black square*), control cells (X), and transformed cells minus control cells (*black triangle*). Cells of each transformant were cultured until the stationary phase, collected, and washed. Fluorescent spectra were measured using an excitation wavelength of 484 nm

Promoter activity of respiratory chain enzyme genes under various growth conditions

To indirectly examine the expression of the target respiratory chain enzymes, cells transformed with the promoter-EGFP constructs were collected at the identical four time points (I–IV) as indicated in Fig. 3(a), washed with membrane preparation buffer, homogenized, and then subjected to spectrophotometry to quantify the fluorescence intensity of EGFP. To normalize fluorescence with cell mass, the value of fluorescence intensity was divided by the OD_{600} value, which was expected to be proportional to

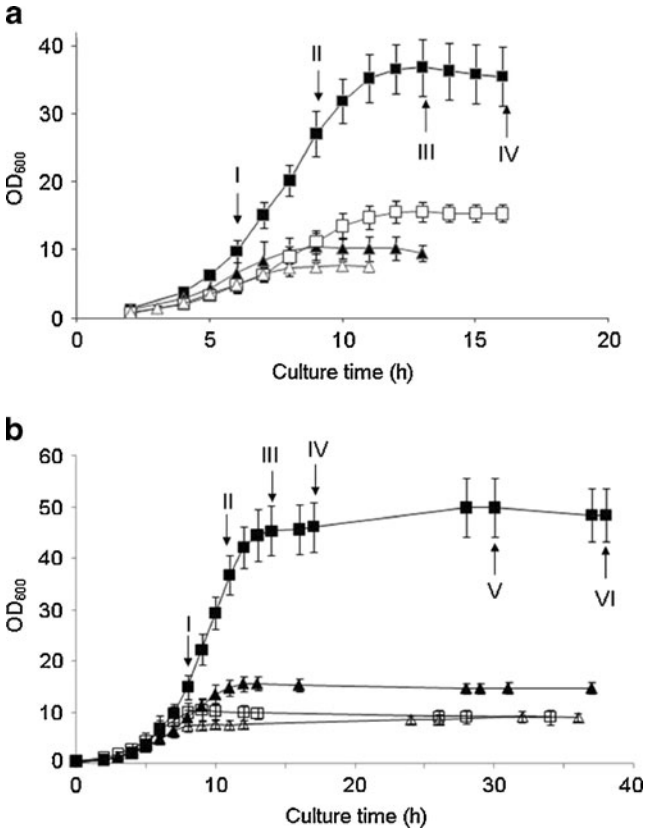

Fig. 3 Growth curves of *C. glutamicum* under various culture conditions. Cells were cultured under four conditions, consisting of either aerobic (50 ml medium in 500-ml baffled flasks, shaken at 180 rpm) or microaerobic (100 ml medium in 500-ml baffled flasks, shaken at 90 rpm) conditions, and either carbon-rich (5% sucrose) or carbon-poor (0.5% sucrose) conditions. Mean values and standard deviations of turbidity (OD_{600}) of cells of the nine transformants at each collection point are indicated as error bars. *black square*: aerobic + rich, *black triangle*: aerobic + poor, *white square*: microaerobic + rich, and *white triangle*: microaerobic + poor. Culture medium was initially collected at four time points during the first 20 h (**a**), representing mid-exponential (I), late exponential (II), early stationary (III), and late stationary growth phases (IV). In addition, culture medium was collected at 15 h (V) and 24 h (VI) after cells entered stationary phase (indicated by *arrows*) (**b**)

cellular mass. This normalized value was designated as the expression index. Examination of the calculated expression indexes for the nine transformants under the four growth conditions (Fig. 5), and comparison of the ratios of the indexes at the late stationary phase (IV) over those at the middle growth phase (I) (Table 4) revealed several trends in the expression of the respiratory chain genes. Under aerobic-rich conditions, the indexes of *ctaD* and *ctaE* increased, those of *ctaE-qcr*, *qcr*, and *ndh* decreased, while those of *cyd* and *sdh* slightly decreased. In contrast, all of the indexes, with the exception of *sdh*, decreased under aerobic-poor conditions. Under microaerobic-rich conditions, the indexes of *ctaC*, *ctaD* and *ctaE* were fairly constant, those of *ctaE-qcr*, *qcr*, and *ndh* decreased, while that of *cyd* increased slightly. Finally, under microaerobic-poor conditions, the index values of *cyd*, *ctaC*, *ctaD*, and *ctaE* increased, while those of *ctaE-qcr*, *qcr*, and *ndh* decreased. It was previously reported that cytochrome aa_3-type cytochrome *c* oxidase operates under aerobic conditions, whereas cytochrome *bd*–type quinol oxidase exhibits higher activity under microaerobic conditions (Kusumoto et al. 2000). These results were consistent with our findings that the expression indexes of the genes for cytochrome aa_3 oxidase decreased during the stationary phase, while those for cytochrome *bd* oxidase increased in the late stationary phase (IV) under microaerobic-poor conditions.

Since biotin is a key constituent of the growth medium for *C. glutamicum*, the effect of biotin on respiratory gene expression was also investigated. Lowering cellular biotin content induces glutamate production by slowing the citric acid cycle, which results in a reduction in energy conservation and cell growth, and likely also affects the expression levels of respiratory enzymes. Here, the biotin concentration was reduced 10-fold from 0.05% (w/v) in the basal medium to 0.005% (w/v), in both carbon-rich and -poor conditions. In low-biotin media, the OD_{600} value in the stationary phase was significantly lower (an average reduction of 0.25-fold) under the carbon-rich conditions

Table 2 Doubling time and OD_{600} at the stationary phase under various growth conditions

Culture duration	Aerobic condition	Doubling time (h)		OD_{600} at stationary phase	
		Rich	Poor	Rich	Poor
<20 h	Aerobic	1.34	1.69	35.57	9.35
		(1.00)[a]	(1.26)	(1.00)	(0.27)
	Microaerobic	1.80	1.77	15.36	7.72
		(1.35)	(1.32)	(0.43)	(0.22)
approx. 40 h	Aerobic	1.29	1.66	49.98	10.50
		(1.00)	(1.29)	(1.00)	(0.21)
	Microaerobic	1.55	1.70	15.63	9.19
		(1.20)	(1.32)	(0.31)	(0.18)

[a] Numbers in brackets indicate the doubling time and OD_{600} ratios of each condition with the aerobic-rich condition

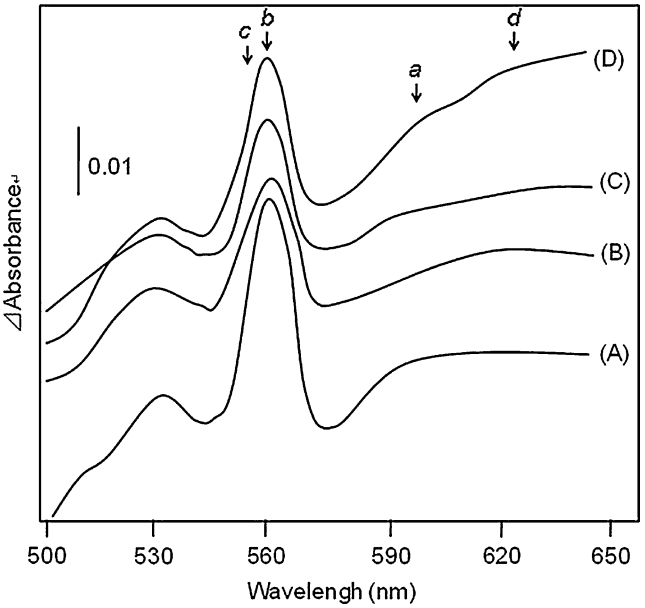

Fig. 4 $Na_2S_2O_4$-reduced minus oxidized difference spectra of membrane preparations at each growth phase. Membrane preparations of pVK6EGFP control cells collected at the middle (I) (**a**), late (II) (**b**), early stationary (III) (**c**), and late stationary growth phases (IV) (**d**) were analyzed. Arrows indicate the absorption wavelength of each cytochrome type; cytochrome *a*, 600 nm; cytochrome *b*, 562 nm; cytochrome *c*, 552 nm; cytochrome *d*, 627 nm

compared to that under carbon-poor conditions (data not shown). In contrast, the expression indexes of the respiratory oxidase subunit genes were higher under rich conditions, with mean increases of 1.07- and 1.45-fold for *cyd* and *ctaC*, respectively (data not shown). Moreover, the average expression indexes under rich conditions were also higher in low-biotin medium than those in high-biotin medium, with 2.25- and 1.80-fold higher values for *cyd* and *ctaC*, respectively. Differential expression was also detected in the different growth phases, as the expression indexes for *cyd* and *ctaC* increased 1.90- and 1.16-fold, respectively, from the early to the late stationary phase in carbon-rich medium, while decreases of 0.79- and 0.76-fold, respectively, were observed in carbon-poor medium. The average fluorescence intensities of *cyd* and *ctaC* were highest under the carbon-rich and low-biotin conditions.

Table 3 Cellular cytochrome content at each growth phase

Growth phase	Cytochrome content (nmol/mg)			
	a	*b*	*c*	*d*
Mid-exponential (I)	0.178	0.705	0.062	0.000
Late exponential (II)	0.033	0.396	0.016	0.024
Early stationary (III)	0.025	0.165	0.000	0.006
Late stationary (IV)	0.070	0.489	0.022	0.046

Cell growth and EGFP fluorescence intensity during extended culture

To investigate the influence of extended culture on the growth and promoter activities of *C. glutamicum*, cells were cultured for an additional 24 h after entering the stationary phase (Fig. 3(b)), against 3 h in other experiments (Fig. 3 (a)). The expression index ratios of *cyd* and *ctaC* at growth stages IV and V exhibited a reciprocal change (Fig. 6, Table 5). Further, the ratio of the expression levels of *cyd* to *ctaC* from growth stages IV to V increased during the extended stationary phase, and this increment was larger under microaerobic-poor conditions (4.56) than those observed under aerobic-rich (2.47) and aerobic-poor conditions (1.69) (Fig. 6, Table 5). In addition, the ratios of the expression levels of *cyd* to *ctaC* from growth stages V to VI were fairly constant, except under microaerobic-poor conditions, in which *ctaC* expression exhibited a relative increase.

The cell doubling times during the extended culture period and the corresponding ratios for each condition versus the doubling time under aerobic-rich conditions are presented in Table 2. Similar to the ratios obtained for growth phase III (stationary phase), the cell doubling times under the microaerobic and aerobic-poor conditions at growth stage IV were slightly longer compared to aerobic-rich conditions. However, the levels of cell mass under the four examined conditions at growth stage VI were fairly constant, compared to those at the initiation of stationary phase (Fig. 3).

Discussion

Six of the nine EGFP-reporter plasmids constructed here were used to monitor the expression levels of respiratory chain complexes of the two electron transport pathways in *C. glutamicum* subsp. *lactofermentum* cells, namely the cytochrome *bcc-aa*$_3$ and *bd* pathways (Fig. 1). Expression levels of cytochrome *bcc* complex genes, which were monitored using plasmids containing either the *qcr* or *ctaE-qcr* promoters fused to EGFP, decreased from mid-exponential to late stationary phases in all four examined growth conditions, although the extent of the decrease varied between 0.39 and 0.92-fold depending on the condition (Fig. 5, Table 4). In contrast, the expression level of cytochrome *bd* oxidase, monitored by the *cyd* reporter plasmid, increased 1.69-fold in the identical period under the microaerobic-poor conditions. These results are consistent with previous findings on the expression levels of these cytochrome complexes estimated by redox difference spectra, which demonstrated that the cytochrome *bcc-aa*$_3$ pathway operates mainly in earlier growth phases and is

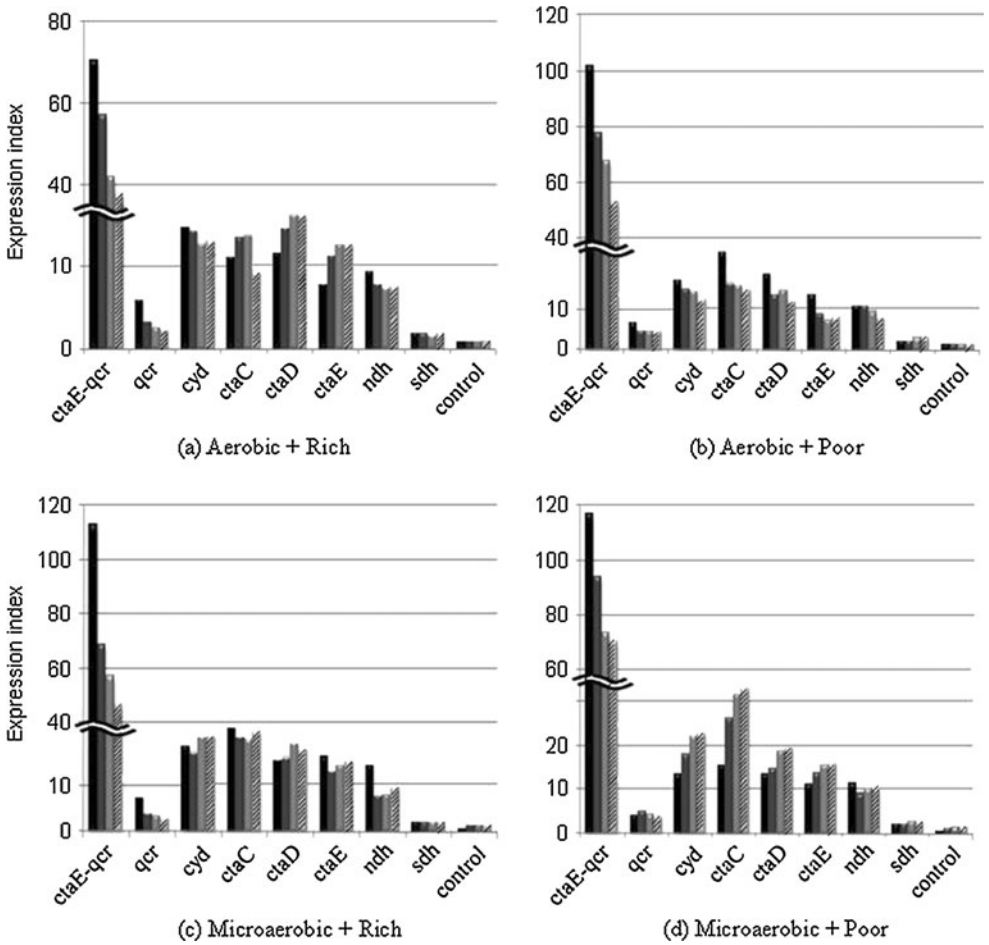

Fig. 5 Expression indexes under aerobic and microaerobic, and carbon-rich and -poor conditions. Each transformant was cultured until the middle (I) *black square*, late (II) ■, early stationary (III) ■, and late stationary growth phases (IV) ▨. The vertical axis represents the expression index, a normalized value of EGFP fluorescence intensity divided by the OD_{600} value at each phase; and the horizontal axis indicates the gene names of the cloned promoter regions

subsequently downregulated, while the cytochrome *bd* pathway operates mainly in later growth phases after a reciprocal increase in expression (Kusumoto et al. 2000). Notably, the expression of the *cyd* genes decreased under aerobic growth conditions with either sucrose-rich or -poor media, indicating that cytochrome *bd* oxidase genes are regulated not only by aeration, but also by the carbon source content of the growth medium. The expression indexes of *ctaC*, *ctaD*, and *ctaE*, which encode the subunits of cytochrome aa_3 oxidase, were higher at the late stationary phase than in the earlier phases, and also under microaerobic conditions than under aerobic conditions (Fig. 5, Table 4). The increased activity of *cta* promoters in later growth stages is not consistent with the previous report described above on the reciprocal behavior of the two electron transfer pathways.

Table 4 Ratio of expression indexes at growth stages IV/I under various growth conditions

Promoter	Ratio of promoter activities at stage IV/I[a]			
	Aerobic		Microaerobic	
	Rich	Poor	Rich	Poor
ctaE-qcr	0.54	0.54	0.63	0.60
qcr	0.39	0.68	0.60	0.92
cyd	0.89	0.75	1.05	1.69
ctaC	0.84	0.61	0.94	2.10
ctaD	1.39	0.66	1.08	1.44
ctaE	1.60	0.61	0.89	1.45
ndh	0.80	0.75	0.70	0.89
sdh	1.01	1.20	1.02	1.28

[a] Growth stages I and IV represent mid-exponential and late stationary growth phases, respectively

The analysis of the cytochrome content of cellular membranes under aerobic-rich conditions revealed that a decrease in cytochrome *c* levels in the late stationary phase corresponded to the observed expression index changes of *ctaE-qcr* and *qcr* (Fig. 5, Tables 3 and 4). The content of cytochrome *d* increased in the late stationary phase, even though the expression index of *cyd* decreased slightly. Moreover, the fluctuations in cytochrome *a* content estimated from membrane spectra corresponded to the behavior of the expression index changes of the *cta* operon (Fig. 5, Table 5).

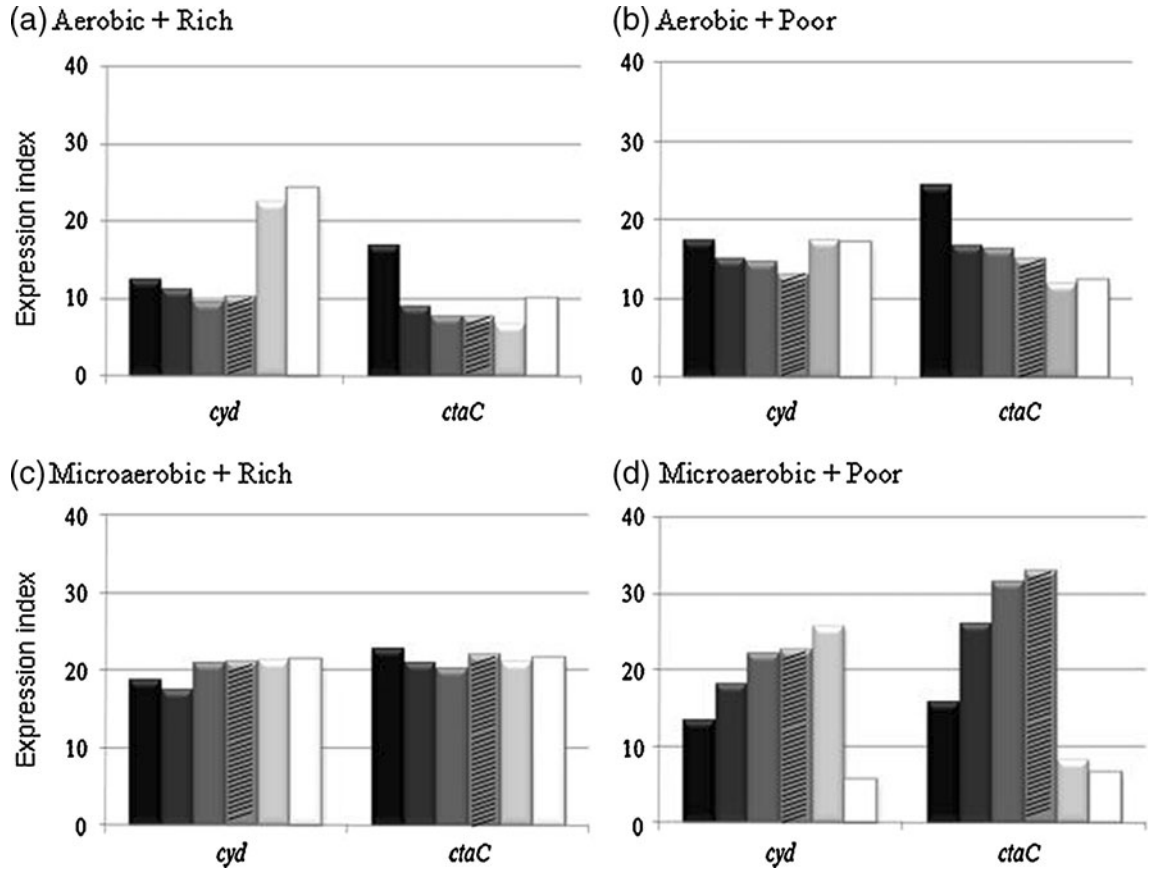

Fig. 6 Promoter activities of terminal oxidase genes (*cyd* and *ctaC*) during extended culture under four different growth conditions. *black square*: mid-exponential phase (I), ■: late exponential phase (II), ■: early stationary phase (III), ▨: 3 h after the initiation of stationary phase (IV), □: 15 h after the initiation of stationary phase (V), and *white square*: after 24 h after the initiation of stationary phase (VI)

To further investigate the expression profiles of the cytochrome *bcc-aa*$_3$ and *bd* pathways, we examined gene expression in cells cultured for extended periods of time. Following prolonged culture in the stationary phase, a clear enhancement in the expression of cytochrome *bd* oxidase was observed (Fig. 6, Table 5). Further, the ratio of the expression levels of *cyd* to *ctaC* increased during the extended stationary phase. Taken together, these results indicate that a reciprocal change in expression levels of cytochrome *bd* and *aa*$_3$ oxidases occurs when *C. glutamicum* cells remain in stationary phase for periods exceeding 15 h.

Table 5 Ratio of *cyd* and *ctaC* expression indexes at growth stages IV/V and VI/V during extended culture

Condition	Gene	Ratio of expression indexes			
		Stage IV/V[a]		Stage VI/V[a]	
		Rich	Poor	Rich	Poor
Aerobic	*cyd*	2.20	1.35	1.07	0.97
	ctaC	0.89	0.80	1.46	1.02
	cyd/ctaC	2.47	1.68	0.73	0.95
Microaerobic	*cyd*	1.01	1.14	0.99	0.21
	ctaC	0.96	0.25	1.01	0.75
	cyd/ctaC	1.05	4.56	0.98	0.28

[a] Growth stages IV, V, and VI represent the late stationary phase, and 15 and 24 h after entering the stationary phase, respectively

The expression level of NDH-II, as monitored by the *ndh* reporter plasmid, decreased under the four growth conditions with increasing age of the culture (Fig. 5, Table 3). This result is consistent with the expectation that the supply of NADH would continually decrease with depletion of the carbon source and reduced flux of the citric acid cycle with progressive growth stages. In contrast to NDH-II, the expression level of succinate dehydrogenase, as monitored by the *sdh* reporter plasmid, did not significantly differ by growth phase, and exhibited an extremely low level nearly equal to the negative control (Fig. 5). In addition, the expression of respiratory chain complexes in low-biotin medium which reduced 10-fold from 0.05% (w/v) in the basal medium were investigated, which revealed the ratio of expression indexes in low-biotin versus basal media increased (data not shown). This may indicate that the cellular metabolic activities are increased

for ATP reduction associated with the uptake of glutamate following the addition of biotin to culture medium. For subsequent investigations, the effect of glutamate concentration in the culture medium may need to be closely monitored.

In conclusion, conventional methods to detect and quantify respiratory enzymes have been dependent on analyzing individual redox difference spectra of cytochromes *a, b, c*, and *d*; however, this method lacks sensitivity, and cytochrome *d* in particular is relatively unstable and difficult to detect. Moreover, cytochrome spectra of closely related enzymes and polypeptides severely overlap each other, such as cytochromes *c* and c_1, and cytochrome *b* in succinate dehydrogenase and *bd*-type oxidase. We have demonstrated that the EGFP reporter system described here is highly sensitive and can be used to indirectly measure the expression of individual target polypeptides, even if the cytochrome types or redox spectra are indistinguishable.

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