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File ID 152379  
Filename Chapter 4: Respiration of Escherichia coli can be fully uncoupled via the non-electrogenic terminal cytochrome bd-II oxidase

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SOURCE (OR PART OF THE FOLLOWING SOURCE):

Type Dissertation  
Title Respiratory electron transfer in Escherichia coli : components, energetics and regulation  
Author M. Bekker  
Faculty Faculty of Science  
Year 2009  
Pages 150

FULL BIBLIOGRAPHIC DETAILS:

<http://dare.uva.nl/record/321143>

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## CHAPTER 4

Respiration of *Escherichia coli* can be fully uncoupled via the non-electrogenic terminal cytochrome *bd*-II oxidase.

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## Summary

The respiratory chain of *Escherichia coli* is usually seen as a device to conserve energy via the generation of a proton motive force, which subsequently may drive ATP synthesis by the ATP synthetase. It has been known that this system does not have a fixed amount of ATP synthesized per oxygen reduced (P/O ratio), due to alternative NADH dehydrogenases and terminal oxidases with different proton pumping stoichiometries.

Here we show that P/O ratios can vary much more than previously thought. First, we show that in wild type *E. coli* cytochrome *bo*, cytochrome *bd-I* and cytochrome *bd-II* are the major terminal oxidases: deletion of all the genes encoding these enzymes results in a fermentative phenotype in the presence of oxygen. Second, we provide evidence that the electron flux through cytochrome *bd-II* oxidase is significant but does not contribute to the generation of a proton motive force. Its kinetics support the view that it serves as an energy-independent system to endow the cell with metabolic flexibility by uncoupling catabolism from ATP synthesis under non-steady state conditions. The non-electrogenic nature of cytochrome *bd-II* oxidase implies that the respiratory chain can function in a fully uncoupled mode such that ATP synthesis is solely achieved by substrate level phosphorylation. As a consequence, the yield value on the carbon and energy source can vary 5- to 7-fold in dependence of the electron flux distribution in the respiratory chain. The full understanding and control of this distribution opens up new avenues for optimization of biotechnological processes.

## Introduction

The aerobic respiratory chain of *Escherichia coli* can function with a variety of different membrane-bound NADH dehydrogenases (NDH-1, NDH-2, WrbA (17, 133, 135, 141), YhdH and QOR (46, 179, 185)) at the electron input side, and three ubiquinol oxidases (cytochromes *bd-I*, *bd-II* and *bo*) (41, 42, 73, 102, 144) at the output side (Fig. 1). The stoichiometry of protons pumped per 2 electrons ( $H^+/2e^-$  ratio) transferred has unequivocally been determined for NDH-1 ( $H^+/2e^- = 4$ ) and NDH-2 ( $H^+/2e^- = 0$ ) (29, 110, 195). Although no specific data are available on WrbA, YhdH and QOR, it is generally assumed that these NADH:quinone oxidoreductases are not electrogenic because of the absence of (predicted) transmembrane alpha-helices (46, 179, 185). Similarly, the energy conserving efficiencies of the cytochrome *bd-I* oxidase and the cytochrome *bo* oxidase are different: the cytochrome *bd-I* complex does not actively pump protons, but due to the oxidation of the quinol on the periplasmic side of the membrane and subsequent uptake of protons from the

cytoplasmic side of the membrane, used in the formation of water, net electron transfer results in proton translocation with a stoichiometry of  $2\text{H}^+/2\text{e}^-$  (152). In contrast, the cytochrome *bo* complex actively pumps protons over the membrane, resulting in a stoichiometry of  $4\text{H}^+/2\text{e}^-$  (153, 197). The stoichiometry of proton translocation of the cytochrome *bd*-II complex is unknown.

Due to the differences in  $\text{H}^+/\text{e}^-$  ratio of the dehydrogenases involved, two-electron transfer from NADH to the quinone pool may be accompanied by the translocation of any number of protons between 0 and 4, and subsequent reoxidation of the quinol pool may contribute to proton translocation again with a stoichiometry that depends on the relative activities of the respective terminal oxidases. The loose coupling between energy conservation and electron flow in respiration has been interpreted as a physiological means for the cell to cope with sudden changes in the rate of electron influx into the respiratory chain and/or in the availability of terminal electron acceptors at its terminal side (29). The mere fact that this energetic efficiency can vary is of great interest, both for understanding of the physiological adaptive responses of the microbial cell and for biotechnological applications, *e.g.* synthesis of any oxidized compound with minimal biomass production. For this, it is important to quantify the flux distribution over and efficiencies of the components of the respiratory machinery in relation to environmental conditions.

Previous studies (29) have shown that NDH-1, NDH-2 and the two well-characterized cytochrome oxidases contribute significantly to the overall electron flux and furthermore that the distribution of fluxes over these components depends on environmental conditions such as the growth rate in glucose-limited chemostats (29). In addition, it has been suggested that the flux distribution over the terminal oxidases of *E. coli* is dependent on the culture pH (191). However, the cytochrome *bd*-II oxidase has not been taken into account in these former studies.

Here we present data that show that cytochrome *bd*-II oxidase participates significantly in oxygen reduction both during non-limited growth in batch cultures and in glucose-limited chemostat cultures. For further quantification of the contribution of the respiratory chain to oxidative phosphorylation, it is essential to assess the *in vivo*  $\text{H}^+/2\text{e}^-$  stoichiometry of the cytochrome *bd*-II oxidase (6, 178). Essentially the same approach has been followed as in the previous studies by (29): strains with respiratory chains that were modified such that their  $\text{H}^+/2\text{e}^-$  stoichiometry is fixed and known, were grown under identical, glucose-limited conditions in chemostat culture. A flux analysis with respect to

glucose catabolism and respiration allowed for the calculation of the rate of ATP synthesis for these strains. These data were then used as reference to the flux data for a strain that contained the cytochrome *bd-II* oxidase as the sole terminal oxidase. This strain showed a decreased yield value with respect to oxygen and glucose. In this way we demonstrate that electron flow through the cytochrome *bd-II* oxidase does not contribute to the generation of a proton motive force. These results are discussed in view of the biochemical characterization of the enzyme and its physiological importance with regard to adaptive responses by *E. coli* to an ever-changing environment.

## Material and Methods

### *Continuous cultures.*

*Escherichia coli* K-12, MG1655, and the various deletion strains were grown in an Applikon 2 L fermentor at a constant dilution rate (D) of  $0.15 \pm 0.01 \text{ h}^{-1}$ . A defined simple salts medium as described by Evans *et al.* (48) was used with nitrioloacetic acid (2 mM) rather than citrate as a chelator. Selenite (30  $\mu\text{g/l}$ ) and thiamine (15 mg/l) were added to the medium. Glucose was used as the sole carbon and energy source at 50 mM final concentration. The dilution rate was set by adjusting the medium supply rate. The pH value was maintained at  $7.0 \pm 0.1$  by titrating with sterile 4M NaOH and the temperature was controlled at 37°C at a stirring rate of 800 rpm. The air supply rate was set to 1 liter/min. In all cultures the steady state specific rates of fermentation product formation, and glucose and O<sub>2</sub> consumption were measured as described in Alexeeva *et al.* (5).

### *Batch cultures*

In batch cultures, the composition of the medium was similar to that described above, except that sodium phosphate (pH 7) was used at a concentration of 100 mM instead of 10 mM, to increase its buffer capacity. In selected experiments, glycerol or succinate was added as carbon- and energy source to 20 mM final concentration instead of glucose. High aeration of cultures during aerobic growth was accomplished by shaking 10 ml culture volumes in 100 ml flasks designed for aerobic cultivation. For anaerobic growth 30 ml culture volumes in sealed bottles (30 ml) were used. Cultures were inoculated from LB plates. The strains were maintained in vials in LB medium with 30% (w/v) glycerol at -70°C.

### *Construction of deletion mutants*

The single deletion strains were ordered from the KEIO collection (8, 43). In order to construct strains with multiple deletions, the kanamycin marker was first removed as described by Datsenko *et al.* (43). Double and triple deletions

were constructed by P1 phage transduction of the desired mutation (see Table 1). Mutants were checked by PCR.

#### *Quinone analysis.*

Culture samples (2 ml) were quenched with 6 ml of ice-cold methanol. Then 6 ml of petroleum ether (boiling point 40-60 °C) was added rapidly to the mixture and the mixture was vortexed for 1 minute. After centrifugation of the mixture (900 ×g, 2 minutes) the upper petroleum ether phase was removed and transferred to a test tube under a flow of nitrogen. Another 3 ml of petroleum ether was added to the lower phase, and the vortexing and centrifugation steps were repeated. The upper phases were combined. After evaporation under a flow of nitrogen to dryness extracts were stored. It was checked that extracts could be kept for at least 7 days under nitrogen at -20°C without changes in the quinone/quinol content. Immediately before use, the extracted quinone/quinol mixture was resuspended with a glass rod in 80 µl ethanol and fractionated by HPLC (Pharmacia LKB gradient pump 2249 system with a LKB 2151 Variable wavelength monitor) using a reversed phase Lichrosorb (Chrompack, Bergen op Zoom, The Netherlands) Reversed Phase 18 column (size 4.6 mm, internal diameter 250 mm). The column was equilibrated with ethanol:methanol (1:1, v/v) as the mobile phase. The flow rate was set at 1 ml/min at 20°C. Detection of the eluate was performed at 290 nm for ubiquinones and at 248 nm for menaquinones. The amount of each quinone species was calculated from the relevant peak area using ubiquinone-10 and menaquinone-4 as a standard, according to the method applied by Shestapov *et al.* (167). Methanol, ethanol, and petroleum ether were of analytical grade.

Peaks were identified by UV/Vis and MS/MS mass spectral analysis. A UV/Vis spectrum of DMK<sub>8</sub> was kindly provided by Dr Bogachev (Moscow University, Russia). For mass spectral analysis, fractions collected from the HPLC were evaporated under nitrogen and re-dissolved into 90% acetonitrile, 10% water, 1% formic acid (LC-Grade, Merck, Frankfurt Germany). The fractions were analysed by off-line electrospray-mass spectrometry using coated Picotips [Econo12] (New Objective, Woburn USA) on an ESI QTOF mass spectrometer (Micromass, Waters, Manchester UK). Ions selected for MSMS collided with Argon in the hexapole collision cell.

*Analysis of carbon fluxes*

Steady state bacterial dry weight was measured as described previously (3). Glucose, pyruvate, lactate, formate, acetate, succinate and ethanol were determined by HPLC (LKB) with a REZEX organic acid analysis column (Phenomenex) at a temperature of 45°C with 7.2 mM H<sub>2</sub>SO<sub>4</sub> as eluent, using a RI 1530 refractive index detector (Jasco) and AZUR chromatography software for data integration. All data present a carbon balance of 96 ± 4% as calculated from the glucose consumption and product formation rates.

*Determination of the cytochrome bd content*

The cytochrome *bd* contents were determined by UV/Vis spectroscopy in whole cells or purified membranes (20 mM KPi, pH 7) with an Olis upgraded DW2000 spectrophotometer using 1 mm cuvettes to reduce scatter. Reduced versus oxidized difference spectra were used for quantification of cytochrome *bd* content by use of the wavelength pair 629-652 nm. For membranes, an extinction coefficient of 24 mM<sup>-1</sup>cm<sup>-1</sup> (629-652 nm) was used for the reduced minus oxidized spectrum for cytochrome *bd*-I and *bd*-II oxidase (84). For whole cells, the reduced spectrum was used to calculate the cytochrome *bd* content after correction for scatter. The extinction coefficient for reduced cytochrome *bd* equals 17.2 mM<sup>-1</sup>cm<sup>-1</sup> for 631 minus 657 nm.

*Determination of enzymological parameters for cytochrome bd-I and bd-II.*

Ubiquinol oxidase activities (37 °C) were determined amperometrically using a Clark electrode and decyl-ubihydroquinone as substrate in membranes purified from strains MB30 (containing cytochrome *bd*-I only) and MB37 (cytochrome *bd*-II only). The buffer was 20 mM KPi, pH 7.0. The K<sub>M</sub> and V<sub>max</sub> values were determined by the integrated rate method using initial substrate concentrations between 100 - 250 μM; the traces showed no indication for product inhibition by decyl-ubiquinone. Values for turnover or V<sub>max</sub> (s<sup>-1</sup>) were calculated from the oxidase activities and cytochrome *bd* concentrations determined optically. The maximal solubility of decyl-ubihydroquinone is approximately 250 μM. Therefore, the K<sub>M</sub>-value determined and quoted for cytochrome *bd*-II of 250 ± 45 μM in conjunction with a V<sub>max</sub> of 1637 ± 150 s<sup>-1</sup> represents the K<sub>M</sub>/V<sub>max</sub> pair with the lowest K<sub>M</sub>-value compatible with the fit of the experimental traces.

*Calculation of specific ATP synthesis rates and H<sup>+</sup>/e<sup>-</sup> ratios.*

The rate of substrate level ATP synthesis (q<sub>ATP (SLPaerobic)</sub>) is stoichiometrically coupled to the rate of CO<sub>2</sub> production for complete oxidation of glucose according to (see Supplemental Fig. 1):



hence:  $q_{\text{ATP (SLPaerobic)}} = \frac{2}{3} \cdot q_{\text{CO}_2}$

For strains or conditions that involve acetate and/or ethanol production, the additional  $\text{CO}_2$  produced stoichiometrically per acetate and/or ethanol has been taken into account as it is assumed that under the conditions used in this study pyruvate formate lyase is not active (94). In addition, lactate production is accompanied by ATP synthesis with a stoichiometry of 1 :

$$q_{\text{ATP (SLPaerobic)}} = 2 \cdot q_{\text{Acet}} + q_{\text{EtOH}} + q_{\text{Lact}} + \left(\frac{2}{3} \cdot (q_{\text{CO}_2} - q_{\text{Acet}} - q_{\text{EtOH}})\right)$$

$$\text{or } q_{\text{ATP (SLPaerobic)}} = q_{\text{Lact}} + \frac{2}{3} \cdot q_{\text{CO}_2} + 1 \frac{1}{3} \cdot q_{\text{Acet}} + \frac{1}{3} \cdot q_{\text{EtOH}} \quad (1)$$

The rate of ATP synthesis by oxidative phosphorylation ( $q_{\text{ATP (ETP)}}$ ) is related to the rate of oxygen reduction, the number of protons translocated per electron transferred to oxygen and to the stoichiometry of ATP molecules synthesized per number of protons flowing back through the ATPase. Assuming that a stoichiometry of 4 for the latter *E. coli* (174) and taking into account that 4 electrons are needed to reduce molecular oxygen to water, it follows that

$$q_{\text{ATP (ETP)}} = 4 \cdot q_{\text{O}_2} \cdot (\text{H}^+/\text{e}^-) / 4 \quad (2)$$

Combining equations (1) and (2) and reducing yields

$$q_{\text{ATP (total)}} = (\text{H}^+/\text{e}^-) \cdot q_{\text{O}_2} + q_{\text{Lact}} + \frac{2}{3} \cdot q_{\text{CO}_2} + 1 \frac{1}{3} \cdot q_{\text{Acet}} + \frac{1}{3} \cdot q_{\text{EtOH}}$$

This formula was used for calculating the  $q_{\text{ATP}}$  of strains MB30 and MB34.

To determine the  $\text{H}^+/\text{e}^-$  of MB37 this formula was rewritten to:

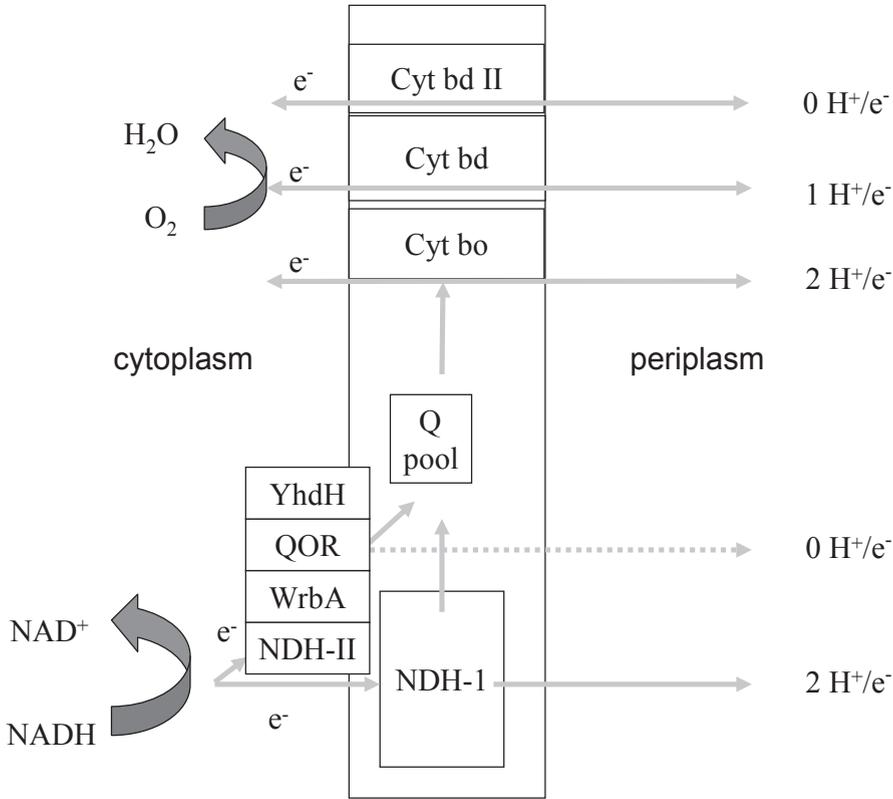
$$\text{H}^+/\text{e}^- = (q_{\text{ATP (total)}} - (q_{\text{Lact}} + \frac{2}{3} \cdot q_{\text{CO}_2} + 1 \frac{1}{3} \cdot q_{\text{Acet}} + \frac{1}{3} \cdot q_{\text{EtOH}})) / q_{\text{O}_2}$$

For  $q_{\text{ATP (total)}}$  the mean value for  $q_{\text{ATP (total)}}$  calculated of strains MB30 and MB34 was taken.

## Results

A comparative study was carried out with a number of isogenic *E. coli* strains that were modified with respect to their respiratory chain. Strain MB20 lacks the cytochrome *bo* oxidase, strain MB21 lacks cytochrome *bd-II* oxidase and strain MB28 lacks cytochrome *bd-I* oxidase. These strains were grown aerobically in batch cultures on a minimal medium with glucose as the sole energy and carbon source. Samples were taken at mid-log phase and assayed for the redox state and content of their ubiquinone pool (see Fig. 2A and 2B, and Material and Methods). From this figure it can be seen that deletion of *cyoB* (strain MB20) resulted in a significant increase in the reduction level of the ubiquinone-pool, whereas the total ubiquinone-pool content remained unchanged. In contrast, the *cydB* deletion (strain MB28) did not show a phenotype either with respect to the redox state or with respect to the cellular amount of total ubiquinone. This absence of a phenotype suggested that under these conditions cytochrome *bd-I* oxidase does not make a major contribution. Finally, absence of the cytochrome

Fig 1



**Fig. 1 Scheme of all NADH::quinone oxidoreductases and quinol::oxygen oxidoreductases in *E.coli* and their proton translocation properties.**

*bd*-II complex (strain MB21) caused a completely different effect on the ubiquinone pool: in this strain, the ubiquinone content was decreased almost threefold to 750 nmol/gram dry weight cells, but no change in the redox state of the ubiquinone pool, relative to the wild type, was observed.

Growth in steady state chemostat cultures allows for a quantitative analysis of specific oxygen consumption rates and NADH/NAD cycling (4, 5). To assess qualitatively the contribution of cytochrome *bd*-II to respiration in fully aerobic conditions, glucose-limited chemostats, set at a dilution rate of 0.2 h<sup>-1</sup> (corresponding to a doubling time of approximately 3.6 hours), were analyzed with respect to specific catabolic rates. Wild-type (MG1655) cells expressed

Strain	Genotype	Cytochrome(s) remaining	Reference
BW25113	K-12 <i>wild-type</i>	All cytochromes	(8)
MB20	JW0421 ( $\Delta cyoB$ ), kanamycin marker removed	Cytochrome <i>bd-I</i> , <i>bd II</i>	This work, (8)
MB21	JW0961 ( $\Delta appB$ ), kanamycin marker removed	Cytochrome <i>bd-I</i> , <i>bo</i>	This work, (8)
MB28	JW0723 ( $\Delta cydB$ ), kanamycin marker removed	Cytochrome <i>bo</i> , <i>bd II</i>	This work, (8)
MB30	BW25113, $\Delta cyoB$ , $\Delta appB$ , $\Delta nuoB$ , kanamycin marker removed	Cytochrome <i>bd-I</i>	This work
MB34	BW25113, $\Delta cydB$ , $\Delta appB$ , $\Delta nuoB$ , kanamycin marker removed	Cytochrome <i>bo</i>	This work
MB37	BW25113, $\Delta cyoB$ , $\Delta cydB$ , $\Delta nuoB$ , kanamycin marker removed	Cytochrome <i>bd II</i>	This work
MB44	BW25113, $\Delta cydB:kan$ , $\Delta cyoB$ , $\Delta appB$ , $\Delta nuoB$	None	This work
MG1655	K-12 <i>wild-type</i>	All cytochromes	
TBE016	K-12 <i>wild-type</i> $\Delta appB$	Cytochrome <i>bd-I</i> , <i>bo</i>	This work

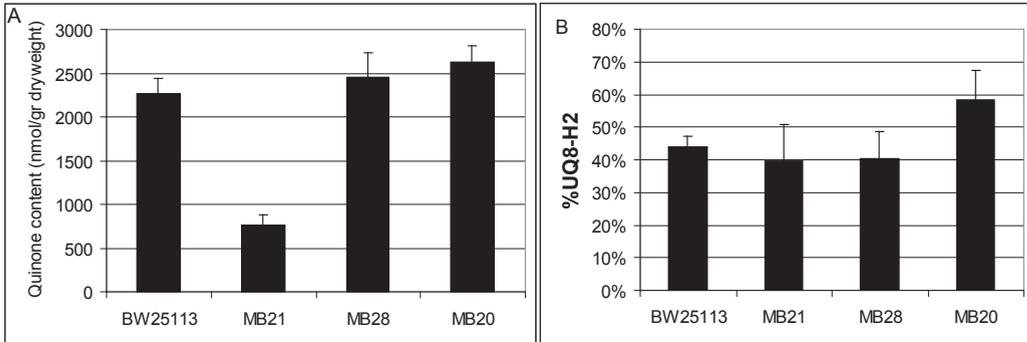
**Table 1** *E. coli* strains used in this study.

a specific respiration rate that was some 47% higher than the mutant strain (TBE016, similar to MB21 in a MG1655 background instead of BW25113) lacking cytochrome *bd-II* oxidase ( $8.7 \pm 0.7 \text{ mmol}(\text{g dw})^{-1}\text{h}^{-1}$  respectively  $5.9 \pm 0.4 \text{ mmol}(\text{g dw})^{-1}\text{h}^{-1}$ ). Also here (compare to Fig 2A) the total ubiquinone content was decreased threefold in the absence of cytochrome *bd-II* (TBE016) as compared to the wild-type. These changes in phenotype indicate that cytochrome *bd-II* oxidase under glucose-limited aerobic conditions is both present and active in the wild-type strain.

	qO <sub>2</sub> mmol (g [dw]) <sup>-1</sup> h <sup>-1</sup>	qGlucose mmol. (g [dw]) <sup>-1</sup> h <sup>-1</sup>	Y <sub>glucose</sub> g [dw] <sup>-1</sup> (g glc) <sup>-1</sup>	qAcetate mmol. (g [dw]) <sup>-1</sup> h <sup>-1</sup>	qATP mmol. (g [dw]) <sup>-1</sup> h <sup>-1</sup>	H <sup>+</sup> /2 e <sup>-</sup>
MB30 (cytochrome <i>bd-I</i> )	8.8 ± 1.3	3.2	0.26	1.5 ± 0.5	16.3 ± 2.2	2
MB37 (cytochrome <i>bd-II</i> )	11.1 ± 0.6	6.0	0.14	5.7 ± 0.7	16.6	0.2 ± 0.1
MB34 (cytochrome <i>bo</i> )	6.4 ± 0.4	2.3	0.36	0.4 ± 0.4	17.0 ± 0.4	4

**Table 2** Summary of properties of the various *E. coli* mutants that have a linear respiratory chain, grown aerobically in glucose-limited chemostat conditions at D = 0.15 h<sup>-1</sup>.

Fluxes are shown in mmol/gram dry weight/hour. q<sub>ATP</sub> values were calculated as described in Material and Methods with a H<sup>+</sup>/2e<sup>-</sup> stoichiometry of 2 for strain MB30 and of 4 for strain MB34, respectively. The averaged q<sub>ATP</sub> value was then used to calculate the H<sup>+</sup>/2e<sup>-</sup> stoichiometries for strain MB37. Other fluxes were determined as described in Material and Methods. Strain MB 37 produced lactate with qlactate = 0.8 mmol:(g dw)<sup>-1</sup>h<sup>-1</sup>. Carbon balances were 96±4 %, n=3.



**Fig. 2** Biomass-specific ubiquinone-8 content (A) and redox state (B) during exponential growth in minimal medium on glucose in a shake flask culture for different respiratory deletion mutants.

Content of the ubiquinone-pool during aerobic batch growth in minimal media supplied with 50 mM glucose of strains BW25113 (*wild-type*), MB21 ( $\Delta appB$ ), MB28 ( $\Delta cydB$ ) and MB20 ( $\Delta cyoB$ ). Error bars indicate standard deviation,  $n=3$ .

To further assess the functionality of the cytochrome *bd*-II complex as a quinol:oxygen oxidoreductase three deletion mutants were constructed that contained one single terminal oxidase only and further lacked the coupled NADH dehydrogenase (see Table 1). The three mutants, designated MB34 (containing cytochrome *bo* as sole terminal oxidase), MB30 (containing cytochrome *bd-I* as sole terminal oxidase) and MB37 (containing cytochrome *bd-II* as sole terminal oxidase) were tested for growth on the non-fermentable carbon sources succinate and glycerol.

All three strains were able to grow well on both glucose (with acetate as the sole overflow product) and the non-fermentable carbon-source glycerol as a carbon source. The same was found for MB34 and MB30 when succinate was supplied as the sole carbon source. However, MB37 could not be cultured on the latter substrate. Another control strain (MB44) that lacked all three above-mentioned quinol:oxygen oxidoreductases (see Table 1) could grow only on glucose and catabolized this energy source by full homolactic fermentation, *i.e.* all glucose consumed could be accounted for by lactate and biomass production. In accordance with the observed homolactic behaviour, this strain expressed no significant respiratory activity. When this control strain was grown under anaerobic conditions, homolactic fermentation was no longer observed: instead, the cells produced acetate and ethanol in amounts as usually found for wild type cells carrying out mixed fermentations (data not shown). In the absence of oxygen, apparently pyruvate formate lyase was functional. The inactivity of the pyruvate formate lyase under aerobic conditions (21) prevents

mixed acid fermentation and therefore strain MB44 ferments homolactic in aerobic conditions. It should be mentioned that the absence of respiration in a strain lacking the three terminal oxidases is in contrast to the findings reported in (149), where oxygen consumption remained after deletion of all three quinol oxidases.

Growth in steady state chemostat cultures allows for a quantitative analysis of specific oxygen consumption rates and NADH/NAD cycling (4, 5). Glucose-limited chemostats, set at a dilution rate of  $0.15 \text{ h}^{-1}$  (corresponding to a doubling time of approximately 4.6 hours) were analyzed with respect to specific catabolic rates. In contrast to the wild-type strain that oxidized glucose

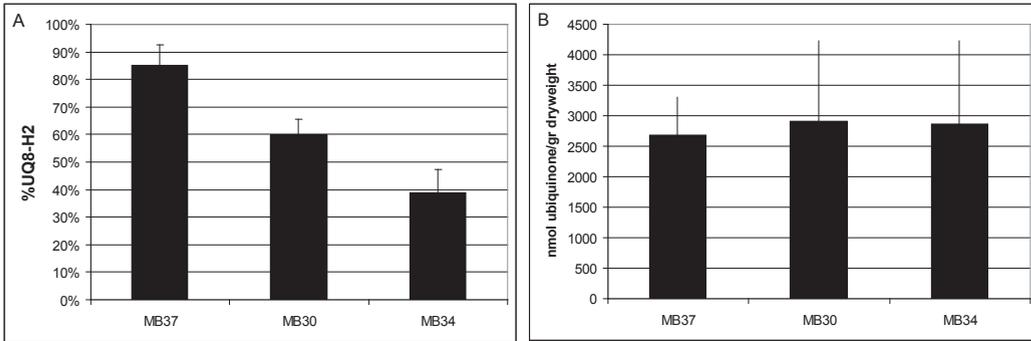
	$V_{\max}$ (mol O/mol bd/s)	$K_m \text{ O}_2$ ( $\mu\text{M}$ ) pH 7	$K_m \text{ UQ-H}_2$ ( $\mu\text{M}$ ) pH 7	cellular content (nmol/gr protein)	Spec. in vivo act. (mol $\text{O}_2$ /mol bd/s)
Cytochrome <i>bd</i> -I	218 $\pm$ 20	0.3 (122, 151)	85 $\pm$ 5	90 $\pm$ 29	58 $\pm$ 11
Cytochrome <i>bd</i> -II	818 $\pm$ 75	2.0 $\pm$ 0.3	250 $\pm$ 45	91 $\pm$ 32	70 $\pm$ 12
Cytochrome <i>bo</i>	225 (161)	6.0 (122)	47 (161)	ND	ND

**Table 3 Enzymatic characteristics of the two *bd* cytochromes from *E. coli*.**

In vitro  $V_{\max}$  and  $K_m$  were determined as described in Material and Methods. For the specific in vivo activity of the two quinol oxidases, the oxygen fluxes measured in continuous cultures (see Table 2) were divided by the measured cytochrome *bd* content/gram cells. n=3 (ND=not determined)

completely to carbon dioxide (4) the mutant strains that contain a respiratory chain with a fixed  $\text{H}^+/\text{e}^-$  stoichiometry, produced acetate to various extents as an overflow metabolite (see Table 2). This suggests that the overall capacity for NADH oxidation is insufficient to cope with the specific NADH production rate by glycolytic and tricarboxylic acid activity.

The highest acetate production, accompanied by a minor but significant lactate production, was observed for strain MB37 (see Table 2). All strains with a linear respiratory chain exhibited high oxygen consumption rates (see Table 2) and accordingly increased glycolytic fluxes as compared to the wild type. It should be emphasized that these strains, lacking the coupled NADH dehydrogenase ( $\Delta nuoB$ ) have a lowered efficiency of energy conservation, (*i.e.* a lowered overall P/O ratio; ATP synthesized per oxygen reduced) and therefore have to increase their catabolic rates to fulfill the energetic demands for growth and maintenance under the prevailing conditions. Apparently this is achieved partly by an increased activity of the respiratory chain and partly by increased substrate level phosphorylation. From Fig. 3A it can be seen that the ubiquinone pool of strain MB37 was much more reduced than that of the strains MB34 and MB30. No significant differences were observed in the ubiquinone



**Fig. 3 Analysis of the ubiquinone pool during continuous growth in glucose-limited chemostat conditions for different respiratory deletion mutants.**

Redox state (A) and content (B) of the ubiquinone-pool of MB30, MB37 and MB34 during aerobic glucose-limited chemostat growth in minimal media supplied with 50 mM glucose. Error bars indicate standard deviation,  $n=3$ .

content (see Fig. 3B) although it should be mentioned that very high standard deviations were found for unknown reasons. Throughout these experiments, the oxygen tension in the chemostats varied between 80 and 90% air saturation and therefore the observed differences in the redox state of the ubiquinone pool cannot be explained by changes in oxygen availability or ubiquinone content (13).

The results clearly show that the lack of two of the three terminal oxidases affects the balance between glycolytic and TCA fluxes on the one hand and the respiratory flux on the other. In order to determine whether this was due to the capacity of the oxidases or to differences in affinity for ubiquinol, the oxidase content of glucose-limited MB30 and MB37 cells was assayed as well as their  $V_{\max}$ ,  $K_{m, \text{QH}_2}$  and  $K_{m, \text{O}_2}$  values (see Supplementary Fig. 2). The data with respect to the kinetics of cytochrome *bd-II* oxidase and all kinetic parameters calculated are summarized in Table 3. Whereas the cellular content in the mutants is similar, the two *bd* type oxidases differ in  $V_{\max}$ ,  $K_{m, \text{O}_2}$  and  $K_{m, \text{QH}_2}$ . The cytochrome *bd-I* oxidase has a fourfold lower maximal turnover rate but a threefold higher affinity for ubiquinol and a 10-20 times higher affinity for oxygen. From the specific respiration rates and the cellular content, it is calculated that the specific in vivo activity of the oxidases is not significantly different under the conditions tested (see Table 3) and that they function at approximately 27 % (cytochrome *bd-I* oxidase) and 9 % (cytochrome *bd-II* oxidase) of the maximal attainable rate.

The quantitative results on the catabolic fluxes can be used to assess the *in vivo* contribution of the cytochrome *bd*-II oxidase to the build-up of a proton motive force. Previously we have used a similar method to determine  $H^+/2e^-$  stoichiometries *in vivo* by growth of *E. coli* cultures in glucose-limited chemostats (29), however without taking into account the alternative cytochrome *bd*-II oxidase, or the alternative NAD(P)H:quinone oxidoreductases WrbA, QOR and YhdH. Here we do take the presence of these enzymes into account. Essentially, the method takes the rate of ATP synthesis as calculated for strains with linear respiratory chains composed of mechanistically well-characterized components, as a reference value to calculate the rate in a mutant with the non-characterized cytochrome *bd*-II oxidase. It is intrinsically assumed that the ATP demand for growth and maintenance is identical in the three strains on the basis that the growth conditions (and hence the growth rate) are identical.

The equations for the calculations are presented in the Methods and Materials section. The validity of the method is shown by the fact that for the strains MB30 and MB34 (containing well-characterized oxidases with respect to  $H^+/2e^-$  stoichiometries, *i.e.* 0 for NDH-II, 2 for the cytochrome *bd*-I oxidase and 4 for the cytochrome *bo* oxidase (4, 29, 152, 153)) similar values for the specific ATP synthesis rates are calculated ( $16.3 \pm 2.4 \text{ mmol} \cdot (\text{g dw})^{-1} \cdot \text{h}^{-1}$  and  $17.0 \pm 0.4 \text{ mmol} \cdot (\text{g dw})^{-1} \cdot \text{h}^{-1}$  respectively). With an average value of  $16.6 \text{ mmol} \cdot (\text{g dw})^{-1} \cdot \text{h}^{-1}$  as reference it is then calculated that in MB37 all ATP is synthesized at the level of substrate phosphorylation and that consequently the cytochrome *bd*-II oxidase does not translocate protons electrogenically, *i.e.* a  $H^+/2e^-$  ratio as low as  $0.2 \pm 0.1$  was calculated. These results are summarized in Table 2.

## Discussion

Two of the three quinone:oxygen oxidoreductases present in *E. coli* have previously been shown to be active in batch cultures (39, 57) as well as during continuous glucose-limited growth (29). The role of the third quinone:oxygen oxidoreductase, cytochrome *bd*-II oxidase, in the actively growing and respiring cell has so far not been clarified *in vivo*.

We have now elucidated this role with respect to both the relative contribution in respiration and to energy conservation under glucose-excess and glucose-limited conditions. For these conditions, we firstly conclude that the cytochrome *bd*-II oxidase is the third and final significant catabolic oxygen-reducing system since strain MB44, that lacks all three quinol:oxygen oxidoreductases (See Table 1), is no longer able to reduce oxygen and behaves as a homolactic

fermentative organism. The reason that this mutant strain does not carry out a mixed fermentation must be sought in the absence of functional pyruvate formate lyase, as this enzyme is reversibly inactivated by oxygen (94, 205). This is consistent with the observation that under anaerobic conditions strain MB44 carries out a normal mixed-acid fermentation. Interestingly, it has recently been reported (149) that a similar mutant was able to respire. Whether this is due to differences in growth conditions or to differences in strains awaits further study.

Secondly, our data justify the conclusion that, in accordance with previous suggestions (6, 42, 178), cytochrome *bd*-II oxidase functions as a proper quinol:oxygen oxidoreductase. This conclusion is further supported by the observation that the expression of cytochrome *bd*-II oxidase as sole terminal oxidase allows growth on a highly reduced substrate like glycerol. The phenotype of a strain that lacks this oxidase (MB21) is a lowered ubiquinone-pool when grown under glucose-excess conditions. Furthermore such a strain shows a much decreased oxygen flux in glucose-limited chemostat conditions. These results strongly suggest that cytochrome *bd*-II oxidase contributes significantly to the overall respiratory electron flux under standard growth conditions.

Under the conditions tested, all strains that contain one single terminal oxidase only, lost the ability to oxidize glucose to completion. The shift to overflow and to fermentative catabolism was most clearly observed for strain MB37, *i.e.* when only cytochrome *bd*-II oxidase is functional. This seems to be compatible with the fact that the latter oxidase has the lowest affinity for ubiquinol (see Table 2) but its high  $V_{\max}$  value counteracts this. However, key to a proper kinetic analysis is reliable estimates on apparent substrate concentrations and since these cannot be made for processes taking place in the membrane, a straightforward kinetic analysis cannot be carried out. Therefore, we can only present a qualitative interpretation namely that the cytochrome *bd*-II oxidase is a low affinity system that, under the conditions tested, is active at approximately 10% of its activity as measured *in vitro*. As we have shown the cytochrome *bd*-II oxidase to be non-electrogenic, strain MB37 is energetically compromised and therefore must express a high catabolic flux, *i.e.* a high rate of NADH synthesis in order to maintain a sufficient ATP synthesis rate. Together with the impeded electron flux to oxygen this hampers complete oxidation of glucose and may give rise to an increased NADH pool: the resulting lowered TCA cycle and PDH complex activity will then lead to increased intracellular pyruvate levels, allowing lactate dehydrogenase, despite its relative low affinity for pyruvate, to compete successfully with the pyruvate dehydrogenase complex.

The conclusion that cytochrome *bd*-II oxidase is non-electrogenic is fully compatible with the observation that strain MB37 was unable to grow on succinate. It has been reported (178) that a strain with cytochrome *bd*-II oxidase as sole terminal oxidase can grow on succinate. However, in the study by Sturr *et al.* (178) the coupled NADH dehydrogenase (NDH 1) was present and apparently sufficient energy conservation could proceed via this complex. In MB37 NDH 1 is not active and therefore no proton motive force can be formed by respiration. Hence, ATP synthesis completely depends on substrate level phosphorylation: it should be noted that succinate catabolism yields only one ATP per succinate molecule and in addition that succinate import takes 2 protons per molecule (66, 115). Without oxidative phosphorylation, clearly the net ATP gain is too small to support growth on this substrate.

An attractive, but very speculative hypothesis as to why cytochrome *bd*-II does not build up a proton motive force may arise from the fact that the protons and electrons used for generation of water from oxygen are taken up from the periplasmic space whereas in cytochrome *bd*-I the protons are taken up from the cytoplasm (152). The di-heme center of cytochrome *bd*-I is supposedly located near the periplasm (14) and sequence comparison suggests this to be the case for *E. coli* cytochrome *bd*-I as well as cytochrome *bd*-II. One could assume that the overall transmembrane and heme topology of all *bd*-oxidases is essentially the same. However, changes of only a few amino acid residues in the di-heme region of the protein may lead to changes in the vectorial process.

Our observation of a non-coupled terminal oxidase in *E. coli* is in agreement with previous suggestions for the importance of an uncoupled respiratory chain component as a tool for the cell to respond appropriately to environmental changes such as a sudden influx of electrons at the starting point of catabolism (*e.g.* relief of an energy source limitation). We now may view the respiratory chain of *E. coli* as a device with several input and output channels for electrons (NADH dehydrogenases and terminal oxidases) that are or are not sensitive to back pressure from the proton motive force. Such a device leads to a greater metabolic (and regulatory) flexibility than highly 'rigid' but more efficient systems.

Glucose excess conditions presumably do not constitute energy-limited conditions and microbial species like *E. coli* are usually loosely coupled and do not tend to maximize energetic efficiency under these conditions. This is exemplified by the phenomenon of overflow metabolism. Cytochrome *bd*-II oxidase may have a role then in oxidation of ubiquinol at a high oxidative flux, not hampered by back pressure of the proton motive force (13). On the other hand, under steady state glucose-limited conditions, *i.e.* presumably

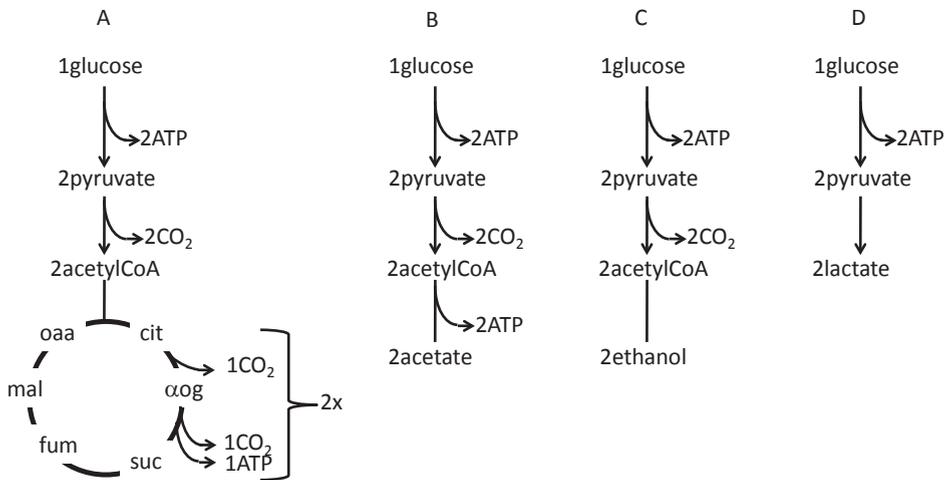
energy-restricted conditions, the contribution of the cytochrome *bd*-II oxidase is likely to be relatively low due to the more oxidized ubiquinone pool (6% (13)), coupled with the poor affinity of the oxidase for ubiquinol. However, upon relief of the limitation, increased NADH synthesis and hence increased ubiquinol pools can now be compensated for by the oxidase, thus preventing that the cell from becoming metabolically compromised. The low affinity characteristic of cytochrome *bd*-II oxidase is compatible with its role as an uncoupling device.

Our results expand the variability and flexibility of the respiratory chain of *E. coli* even further than has been assumed so far (29). Indeed, an electron flux distribution is feasible that results in the respiratory chain being virtually non-electrogenic (*i.e.* NDH-II  $\rightarrow$  Cytochrome *bd*-II) on the one extreme and or being fully coupled (*i.e.* NDH-1  $\rightarrow$  Cytochrome *bo*) on the other. Taking into account substrate level phosphorylation and redistribution of glucose over anabolism and catabolism, a five-fold difference in the yield on glucose between these extremes can be obtained. For apparent H<sup>+</sup>/ADP $\rightarrow$ ATP stoichiometries lower than the value 4 that has been assumed here (174), a seven-fold change would be obtainable. The implications and benefits for applications are obvious. Further insight in the regulation of the flux distribution will provide us with the tools to control the efficiency of carbon source conversion over a broad range. Thus, inducing a highly efficient respiratory activity during the growth phase, followed by a switch to a low efficiency activity during the phase in which the carbon source is to be converted to an end product, will contribute to optimization of a production process by minimalisation of ATP (and therefore biomass) synthesis in the production phase.

#### **Acknowledgements:**

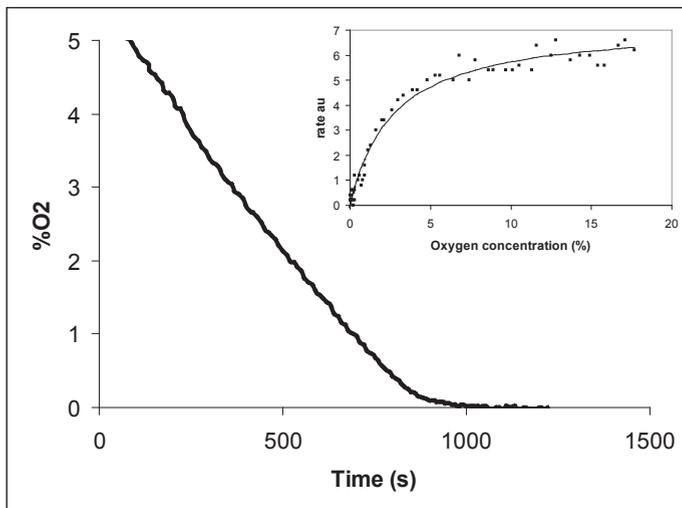
This work was supported by NWO-ALW project 812.05.004. A. Ter Beek contributed to this study as post doc for the SysMo-SUMO project supported by NWO-SysMo 826.06.002. We also thank Jorine Zandhuis and Marc J.F. Strampraad (TUD) for their contribution to this study.

Supplemental Fig 1



**Supplemental Fig. 1. Schematic metabolic pathways for the catabolism of glucose.**

(A) CO<sub>2</sub> by the TriCarboxylic Acid cycle, oaa=oxaloacetate, cit=citrate, αog = α-oxoglutarate, suc=succinate, fum=fumarate, mal=malate; (B) ethanol production; (C) acetate production; (D) lactate production. The stoichiometries for ATP/product are those used in this study for  $q_{ATP}$  calculations



**Supplemental Fig 2** Oxygen – time trace for cytochrome *bd-II* oxidase (see Material and Methods). Inset: Michaelis-Menten plot calculated from the trace