Title; The cytochrome *bcc*-*aa*$_3$-type respiratory chain of *Rhodococcus rhodochrous*

Running title; The cytochrome *bcc*-*aa*$_3$ pathway of *R. rhodochrous*

Jun-ichi Kishikawa,[1] Yoshiki Kabashima,[1] Tatsuki Kurokawa[2] and Junshi Sakamoto[1]*

[1]Department of Bioscience and Bioinformatics, Kyushu Institute of Technology, Kawazu 680-4, Iizuka, Fukuoka, 820-8502, Japan

[2]Department of Integrative Physiology, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka Suita, Osaka 565-0871, Japan

Keywords

Rhodococcus rhodochrous, respiratory chain, actinobacteria, H^+/O ratio, energy metabolism

Corresponding author;

Junshi Sakamoto Tel.: +81-948-29-7823; Fax: +81-948-29-7801; e-mail: sakamoto@bio.kyutech.ac.jp

Abstract

Rhodococcus rhodochrous is an active soil bacterium belonging to the *Nocardia* group of high GC Gram-positive bacteria. It is rich in various enzymes and thus important in the industrial production of chemicals and bioremediation. In this work, the respiratory chain of this aerobic organism was investigated and characterized. Grown under highly aerobic conditions, the membrane fraction of *R. rhodochrous* cells only contained *a*-, *b*- and *c*-type cytochromes, suggesting that it is the cytochrome *bcc*-aa_3-type pathway that mainly operates under these conditions. In contrast, the *d*-type cytochrome was also present under microaerobic conditions, indicating that the alternative pathway of the *bd*-type oxidase works in these circumstances. In addition, the results of H^+/O ratio measurements indicate that these two pathways have different energy efficiencies.

Introduction

Rhodococcus rhodochrous is a red soil saprophyte belonging to the *Nocardia* group of actinobacteria and actively produces lipases, esterases, hydroxylases and nitrilases. This organism is thus important in the industrial production of certain useful chemical compounds such as acrylamide (1). It is also useful in some bioremediation processes, such as the decomposition of polychlorinated biphenyl (PCB) (2) or phenol (3) and the emulsification of coal oil (4). The other subgroups of actinobacteria include both many useful bacteria such as *Corynebacterium glutamicum*, which is important in industry to produce amino acids, and many pathogenic ones such as *Mycobacterium tuberculosis*, which causes tuberculosis. The respiratory chains of these

actinobacteria, as well as the closely related species of the pathogenic bacteria, have been intensively investigated (5, 6, 7, 8, 9, 10).

In contrast to mammalian mitochondria, bacteria have extremely divergent respiratory chains and, in many cases, possess plural electron transfer pathways, which are used alternatively depending on the environment demands (11). Although the whole genome of *R. rhodochrous* has not been sequenced yet, the recent genome analyses of *Rhodococcus sp.* RHA1 (12) and the biochemical analyses of *R. rhodochrous* (13) have illustrated the constituents of the respiratory chain of the genus *Rhodococcus*. In the *Rhodococcus sp*. RHA1 genome, both type 1 and type 2 NADH dehydrogenases are coded. Type 1 NADH dehydrogenase (NDH-1) is an integral membrane protein complex that pumps protons and type 2 NADH dehydrogenase (NDH-2) is a peripheral membrane protein that does not conserve energy. Several other enzymes are also coded in the *Rhodococcus sp*. RHA1 genome, such as type A succinate dehydrogenase (SDH) (14, 15), the cytochrome aa_3-type cytochrome *c* oxidase which is classified into the type A heme-copper oxidase family(16) and the cytochrome *bd*-type menaquinol oxidase which is completely different from the heme-copper oxidase family (11, 17). In our previous research, the genes coding the complex III of *R. rhodochrous* have been cloned. According to the results, this enzyme had the diheme *c* subunit (13). Although the genus *Rhodococcus* shares some features of the respiratory chain with the other actinobacteria, there are still some differences in the types of NDH and SDH or the sequences of the enzymes identified by genome comparison of some actinobacteria. Theoretically, these differences might affect the energy transduction and the regulation of the alternative use of plural pathways. However, the entire picture of the respiratory chain and its activity on the

cellular level are virtually unknown.

In this study, we investigated the enzyme composition of the respiratory chain of *R. rhodochrous*, the differential use of the branched pathways under aerobic or microaerobic conditions and the energy transduction of the cells. The obtained values of the H^+/O ratio, which were lower than expected from the enzyme composition, and the results of the inhibitor assay suggest that NDH-1 has little or no activity in this organism.

Materials and Methods

Bacterial growth conditions

R. rhodochrous DMS 43202 cells were aerobically grown at 34°C in the medium containing 0.5% D-glucose, 0.5% polypeptone, 0.5% yeast extract, 0.1% NaCl and 0.1% Na_2CO_3 at pH 7.0. A 6-ml aliquot was inoculated into a main culture in a 1L baffled flask, and the culture was carried out until the cells reached the stationary phase by vigorous shaking. The cells were cultured under four conditions: condition I, 300 ml medium with 180 rpm rotation speed; condition II, 300 ml with 140 rpm rotation speed; condition III, 500 ml medium with 140 rpm rotation speed; and condition IV, 500 ml medium with 100 rpm rotation speed. The condition I and IV are the most and least aerobic condition, respectively. Although the O_2 concentrations of each medium were the same at the beginning of the culture, the differences of the O_2 concentrations occurred as the bacteria grew. $OD_{600,}$ as an indicator of the growth of the cells, was measured at a 10-fold dilution with water.

Enzyme preparation

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 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 20 min. The precipitate was resuspended in 10 mM Na-phosphate buffer at pH 7.0; this suspension was designated as the membrane fraction. The membranes were washed with the buffer containing 2.0% (w/v) sodium cholate, 0.1 M NaCl, 10 mM Na-phosphate at pH 7.0 and then rinsed with 10 mM Na-phosphate at pH 7.0. The membrane proteins were collected by centrifugation at 100,000 g for 20 min and solubilized at 10 mg protein/ml in 1% (w/v) n-dodecyl-β-D-maltoside (DDM) in the presence of 0.1 M NaCl and 10 mM Tris-HCl at pH 8.0 for the purification of the aa_3-type cytochrome *c* oxidase or 10 mM Na-phosphate at pH 6.0 for supercomplex analysis. The mixture was centrifuged at 100,000 g for 30 min, and the supernatant was applied to a DEAE-Toyopearl column. The absorbed proteins were eluted with 0.1% DDM solution containing increasing concentrations of NaCl at same pH as DDM extracts. The peak fractions of cytochrome aa_3 were applied to a Q-sepharose column. The proteins were eluted with a series of buffers containing 0.1% DDM at pH 8.0 and increasing concentrations of NaCl.

Electrophoretic analyses

Blue-native polyacrylamide gel electrophoresis (BN-PAGE) was performed according to the method of Schägger and co-workers (18). 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. The apparent molecular weight in native- PAGE was calculated

using molecular weight standard (240, 140, 66 kDa). For a two-dimensional analysis, a slice of blue-native PAGE gel was excised and soaked in 1% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol buffer for 1 h, which was then embedded in a separating gel containing 13.5% acrylamide. The two-dimensional analysis was performed at room temperature with the current limited to 20 mA. SDS-PAGE was performed according to the method of Laemmli (19). Stacking and separating gels contained 4% and 13.5% acrylamide, respectively. SDS-PAGE analysis was performed at room temperature with the current limited to 20 mA.

Enzyme activities

N,N,N',N'-Tetramethyl-*p*-phenylenediamine (TMPD) oxidation activity was assayed at 30°C in a final volume of 600 μl containing the suitable amount of enzyme, 9.2 mM NaCl, 4.6 mM Na-phosphate (pH 6.0) and 0.2 mM TMPD. The increase in the absorbance of TMPD was measured at 562 nm, and the activity was calculated using a millimolar absorption coefficient of 10.5 mM^{-1} cm^{-1} (20). Succinate dehydrogenase activity was assayed at 30°C by measuring the reduction rate of 2,6-dichloroindophenol sodium salt (DCIP) in a final volume of 600 μl containing 0.2 μg of enzyme, 50 mM sodium phosphate (pH 7.0), 0.1 mM DCIP, 0.4 mM phenazine methosulfate and 5 mM sodium succinate. The decrease in the absorbance of DCIP was measured at 600 nm, and the activity was calculated using a millimolar absorption coefficient of 21 mM^{-1} cm^{-1} (21). The activity of the cytochrome *bcc-aa*$_3$ supercomplex was measured by menaquinol oxidation activity. First, 1-5 μg enzyme, 2 μl 10 mM menaquinon-1 (MK-1) and 93 μl reactive buffer containing 20 mM Na-phosphate (pH 7.0), 1 mM EDTA, 0.1% DDM and 50 mM NaCl were

mixed and preincubated for 5 min on ice. The preincubation with menaquinone-1 enhanced and stabilized the enzyme activity. Afterward, 586 µl reactive buffer, 12 µl reduced MK-1 (menaquinol-1) and the preincubated mixture were mixed and the increase in the absorbance of MK-1 was measured at 275 nm at 30°C. The activity was calculated using a millimolar absorption coefficient of 8.13 mM^{-1} cm^{-1} (22). The rate of oxygen consumption was measured in a small closed reaction vessel at 30°C using a Clark-type oxygen electrode. The 2.5 ml reaction mixture contained 10 mM Na-phosphate and 0.05-0.4 mg of protein of the membrane preparation. The reaction was started by the injection of 1 mM NADH as the substrate at the final concentration. The rate was calculated by the amount of dissolved oxygen at 30°C in buffer of 435 ng-atom O ml^{-1} (23). The H^+/O ratio was measured through the changes of pH recorded by pH electrode. Cells harvested from steady-state cultures were washed with the buffer containing 10 mM Na-phosphate (pH 7.0) and 100 mM NaCl. The washed cells were resuspended in the reaction buffer containing 0.5 mM K-MOPS (pH 6.8), 140 mM KCl and 50 mM KSCN. A total of 3 ml of reaction buffer and cell suspension (2-4 mg dry weight) was transferred to a closed incubated (30°C) reaction chamber. After the addition of 0.5 µg valinomycin, a defined amount of oxygen was added to the suspension by the injection of 20 µl air-saturated 140 mM KCl (8.7 ng atom-O), which was equilibrated with the syringe at 30°C. H^+/O ratio was calculated from the pH changes and the known amount of atom-O (24).

Other analyses

Redox difference spectra were recorded at room temperature using a Beckman DU-70 spectrophotometer. The spectra of air-oxidized enzymes were obtained,

and then, a few grains of solid sodium dithionite were added to obtain their fully reduced forms. The presence of cytochrome was roughly estimated with difference spectra. In the case of the membrane preparations, proteins were extracted with 5% (w/v) Triton X-100 to minimize the turbidity before the spectra were measured. The contents of heme *b*, *c*, *a* and *d* were estimated from redox absorption spectra using a molar extinction coefficient of 22 mM^{-1} cm^{-1} at Δ560 nm, 19.1 $mM^{-1}cm^{-1}$ at Δ550 nm, 11.6 $mM^{-1}cm^{-1}$ at Δ603 nm and 27.9 $mM^{-1}cm^{-1}$ at Δ630 nm, respectively (25, 26). Protein concentration was determined as described (27) or using the bicinchoninic acid protein assay.

Results

Growth curves and difference spectra

The cells of *R. rhodochrous* were cultured under four different aeration conditions by varying the medium volume and the rotation speed (FIG. 1). When the absorption showed no increase, the cells were harvested and the wet weight was measured for calculating the growth yields. The doubling time of condition I (the most aerobic condition) was the shortest one and it became longer as O_2 levels decreased. The lower the oxygen supply was, the lower the cell yield was. The doubling times and the cell yields under the respective conditions were as follows: 1.27 h, 15.4 g/l under condition I; 1.29 h, 15.9 g/l under condition II; 1.36 h, 13.0 g/l under condition III; and 1.64 h, 10.0 g/l under condition IV (the least aerobic condition). The cells of each condition were harvested at the stationary phase and the redox difference spectra of the membranes were measured to estimate the expression level of the cytochromes *bcc*, aa_3 or *bd*. The results indicated that the aeration of the medium differentially influenced the expression of different respiratory enzymes.

Specifically, under condition I, the peaks of cytochrome *b*, *c* and *a* were clearly detected at 560 nm, 550 nm and 603 nm, respectively. As O_2 levels decreased, the peak of cytochrome *d* became relatively sharper at 630 nm compared to the other cytochromes (FIG. 2). The contents of these cytochromes were estimated from the redox difference spectra. The contents of the *b*-, *c*-, *a*- and *d*-type cytochrome in the membranes under each condition were summarized in TABLE 1. The contents of the *b*-type cytochrome in the membranes were highest under condition I and about the same under the other three conditions. The contents of the *c*- and *a*-type cytochrome varied in parallel with those of the *b*-type cytochrome. But, in panel d, the 560 nm peak was equivalent to the 550 nm one, and sharper than the peaks in panel a and b (FIG. 2). From this observation, it was inferable that the expression of the *b*-type cytochrome of SDH, which has a peak at 557 nm, was decreased as O_2 levels decreased. The *d*-type Cytochrome was observed only in condition III and IV, indicating that cytochrome *bd*-type quinol oxidase was only expressed under the highly limited oxygen supply conditions.

H^+/O ratio as the efficiency of respiration

Because the transmembrane movement of H^+ during respiration is principally important for oxidative phosphorylation, the H^+/O ratio was measured of the cells grown under the two extreme conditions among the four ones shown in FIG. 1, by the oxygen pulse method to evaluate the efficiency of the respiration chain. Figure 3 shows the typical pH changes induced by an oxygen pulse in the medium of the resting cells under condition I (FIG. 3A) and condition IV (FIG. 3B). The cells under condition I had an H^+/O ratio as high as 4.2 ± 0.26 (n=3), while the cells under condition IV had an H^+/O ratio of 3.1 ±

0.16 (n=3). In the respiratory chain, many enzymes couple the proton transport across the cell membrane with the electron transfer. The *bcc*-aa_3 and *bd* pathways translocate 6 and 2 protons, respectively, during the reduction of 1 oxygen atom in *C. glutamicum* (17). Combined with the result of cell growth (FIG. 1), both the H^+/O ratio and the cell yield were higher under the more aerobic conditions than the microaerobic ones, as previously shown in *Escherichia coli* (28).

Oxygen consumption and the effects of inhibitors

The oxygen consumptions of the membrane fractions were measured using a Clark-type oxygen electrode. NADH was used as the substrate. The membranes prepared from the cells under condition I showed the respiration rate of 113 ng-atom O/mg/min, while those from the cells under condition IV showed 220 ng-atom O/mg/min, about twice of the former rate. FIG. 4 shows the effects of cyanide and *p*-benzoquinone (PBQ) on the oxygen consumptions of the two membrane fractions at various concentrations. Cyanide, a potent inhibitor of the heme-copper terminal oxidases, also inhibits the *bd*-type quinol oxidases, but only at much higher concentrations (29). PBQ has been used as an inhibitor analogous to the natural substrate of the quinol oxidases (30). The membranes prepared from the cells under condition IV showed a much higher sensitivity to PBQ and a little lower sensitivity to cyanide than those of the membranes prepared from the cells under condition I (FIG. 4). The condition I membranes were virtually insensitive to PBQ. These results indicate that different respiratory enzymes are differentially expressed depending on the growth conditions and they have different sensitivities to the inhibitors. In addition, myxothiazol, antimycin and rotenon were also used in the inhibition

experiments. Myxothiazol and antimycin were used as the inhibitors of the bc_1 complex. Even though the *bcc* complex was observed on difference spectra (FIG.2), both agents, myxothiazol and antimycin, showed no inhibition at 1 μM. Rotenone, known as an inhibitor of NDH-1, showed no inhibition to the oxygen consumption neither when it was used at 1-5 μM.

Purification of the aa_3-type oxidase

Proteins in the aerobic membranes were solubilized with a detergent DDM, and the aa_3-type cytochrome *c* oxidase was purified from them by two consecutive column chromatography: DEAE-Toyopearl and Q-Sepharose. The redox difference spectrum of the aa_3-type cytochrome *c* oxidase showed a peak of cytochrome *a* at 600.5 nm of α band. The purified oxidase was electrophoresed in 2D-PAGE. There were two main bands, migrating at the apparent molecular weights of 240 kDa and 190 kDa, in the first dimension of the native-PAGE (FIG. 5). It was believed that these main bands were the aa_3-type cytochrome *c* oxidase monomers based on the calculated molecular weight. In the second dimension of SDS-PAGE, 4 polypeptide spots were split from each main band of the native-PAGE. Each spot can be attributed to subunit I, II, III and IV of the aa_3-type cytochrome *c* oxidase, respectively. Since the polypeptide composition seems the same for the two original bands, the difference in size of the two bands might come from the difference in the micelle composition of the detergent and lipids.

Analyses of the *b*-type cytochromes of aerobic membranes

To analyze the respiratory components in the aerobic membranes prepared from the condition I cells, the membranes were solubilized with DDM

and fractionated by DEAE-Toyopearl column, resulting in two elution peaks of the *b*-type cytochrome (data not shown). The first one eluted at a lower NaCl concentration had succinate dehydrogenase activity; therefore, the first *b*-type cytochrome peak is a SDH peak. The other one eluted together with cytochrome *a* and *c* at a higher NaCl concentration showed both menaquinol oxidase activity (19.3 unit) and TMPD oxidase activity (0.38 unit), indicating that this *b*-type cytochrome is the cytochrome *b* from the *bcc* complex and eluted with the aa_3-type cytochrome *c* oxidase. The redox difference spectra of the *b*-type cytochromes showed different peaks (FIG. 6): while SDH has the absorption peak at 557 nm, the *bcc* complex has the peak at 561 nm. From the fact that the latter fraction showed menaquinol oxidase activity, it is presumed that the *bcc* complex and aa_3-type cytochrome *c* oxidase compose a quinol-oxidase supercomplex, although they migrated separately in the native-PAGE.

Discussion

The current picture of the respiratory chain of *R. rhodochrous* is shown in Fig. 7. The cytochrome *bcc* complex and aa_3-type cytochrome *c* oxidase are suggested to compose a supercomplex by the chromatographic analysis and enzymatic activity measurements, however, their association might be weak, since the polypeptides of the aa_3 oxidase migrated separately from those of the *bcc* complex in the native-PAGE (FIG. 5). The cytochrome *bd*-type quinol oxidase was detected spectrometrically only under microaerobic conditions (conditions III and IV) but not under highly aerobic ones (conditions I and II, FIG. 2). This difference of the extent of cytochrome expression seems consistent with the concomitant alteration of H^+/O ratios. It has been

commonly reported in other bacterial species that the H^+/O ratios of the bc_1-aa_3 and *bd* pathways are about 6 and 2, respectively (11, 17). Thus, the lower H^+/O ratio of the cells under condition IV (3.1 ± 0.16) than that of the cells under condition I (4.2 ± 0.26) might be due to the larger amount of the cytochrome *bd* oxidase under the former condition. Furthermore, the effects of inhibitors on the enzyme activity were also consistent to the conclusions above. First, cyanide, a famous selective inhibitor of heme-copper oxidases, inhibited the oxygen consumption activity of the membranes from the cells under condition I more potently than those under condition IV. Secondly, PBQ, an inhibitor of *E. coli* cytochrome *bd* oxidase, potently inhibited the oxygen consumption activity under condition IV, whereas it did not inhibit that under condition I.

Since the *Rhodococcus* sp. RHA1, which is a close relative to *R. rhodochrous*, has the genes for NDH-1 and NDH-2 in its whole genome (12), it is reasonable to believe that the latter species also has the genes for these two enzymes. NDH-1, the rotenone-sensitive dehydrogenase, can translocate approximately 4 protons per 1 oxygen atom, while NDH-2, the rotenone-insensitive one, does not translocate any protons in general (33). The H^+/O ratios observed in this study are lower than expected for the case that NDH-1 is operating in addition to the *bcc*-aa_3 and/or *bd* pathways (FIG. 3). Combined with the finding that the oxygen consumptions were not inhibited by rotenone, it is possible that NDH-1 is not expressed or, at least, has very low activity in *R. rhodochrous*, and that instead, NDH-2 is operating, although the presence of NDH-2 has not been directly proved in this species.

Moreover, we investigated the relationship between the energy conservation of the respiratory chain and the total cell growth. The growth rates and the cell yields were the highest under conditions I and II, the lowest

under condition IV and the middle under condition III (FIG. 1). These findings are also consistent to the relative extents in the operation of the two respiratory pathways with different energy efficiencies (or H^+/O ratios), as discussed above. The oxygen consumption rate of the microaerobic membranes (condition IV) was higher than that of the aerobic ones (condition I). It seems that the lower H^+/O ratio of the respiratory chain is compensated by the higher reaction velocity to achieve the comparative proton motive force. In other words, the low energy-transduction efficiency of the cytochrome *bd* oxidase is redeemed by the high enzymatic activity that is enhanced in this organism. In conclusion, *R. rhodochrous* has a combination of the respiratory enzymes roughly similar to those of the other actinobacteria, such as *Corynebacterium* (6, 7), but it has some different features in the energy transduction system.

References

1. **Raj, J., Sharma, N. N., Prasad, S., and Bhalla, T. C.:** Acrylamide synthesis using agar entrapped cells of *Rhodococcus rhodochrous* PA-34 in a partitioned fed batch reactor. J. Ind. Microbiol. Biotechnol., **35,** 35-40 (2008).
2. **Taguchi, K., Motoyama, M., and Kudo, T.:** Multiplicity of 2,3-dihydroxybiphenyl dioxygenase genes in the Gram-positive polychlorinated biphenyl degrading bacterium *Rhodococcus rhodochrous* K37. Biosci. Biotechnol. Biochem., **68,** 787-795 (2007).
3. **Przybulewska, K., Wieczorek, A., Nowak, A., and Pochrzaszcz, M.:** The isolation of microorganisms capable of phenol degradation. Pol. J, Microbiol., **55,** 63-67 (2006).
4. **Sorkhoh, N. A., Ghannoum, M. A., Ibrahim, A. S., Stretton, R. J., and Radwan, S. S.:** Crude oil and hydrocarbon-degrading strains of *Rhodococcus rhodochrous* isolated from soil and marine environments in Kuwait. Environ. Pollut., **65,** 1-17 (1990).
5. **Sone, N., Nagata, K., Kojima, H., Tajima, J., Kodera, Y., Kanamaru, T., Noguchi, S., and Sakamoto, J.:** A novel hydrophobic diheme *c*-type cytochrome. Purification from *Corynebacterium glutamicum* and analysis of the *QcrCBA* operon encoding three subunit proteins of a putative cytochrome reductase complex. Biochim. Biophys. Acta, **1503,** 279-290 (2001).
6. **Sakamoto, J., Shibata, T., Mine, T., Miyahara, R., Torigoe, T., Noguchi, S., Matsushita, K., and Sone, N.:** Cytochrome *c* oxidase contains an extra charged amino acid cluster in a new type of respiratory chain in the amino-acid-producing Gram-positive bacterium *Corynebacterium glutamicum*. Microbiology, **147,** 2865-2871 (2001).
7. **Kusumoto, K., Sakiyama, M., Sakamoto, J., Noguchi, S., and Sone, N.:**

Menaquinol oxidase activity and primary structure of cytochrome *bd* from the amino-acid fermenting bacterium *Corynebacterium glutamicum*. Arch. Microbiol., **173,** 390-397 (2000).

8. **Megehee, J. A. and Lundrigan, M. D.:** Temporal expression of Mycobacterium smegmatis respiratory terminal oxidases. Can. J. Microbiol., **53,** 459-463 (2007).
9. **Megehee, J. A., Hosler, J. P., and Lundrigan, M. D.:** Evidence for a cytochrome *bcc-aa*$_3$ interaction in the respiratory chain of *Mycobacterium smegmatis*. Microbiology, **152,** 823-829 (2006).
10. **Shi, L., Sohaskey, C. D., Kana, B. D., Dawes, S., North, R. J., Mizrahi, V., and Gennaro, M. L.:** Changes in energy metabolism of *Mycobacterium tuberculosis* in mouse lung and under *in vitro* conditions affecting aerobic respiration. Proc. Natl. Acad. Sci. U. S. A., **102,** 15629-15634 (2005).
11. **Sakamoto, J. and Sone, N.:** Biochemical and Molecular Features of Terminal Oxidases, p. 87-113. Zannoni, D. (ed.), Advances in Photosynthesis and Respiration, vol. 15. Respiration in Archaea and Bacteria Diversity of Prokaryotic Electron Transport Carriers, 1st edn, Kluwer Academic Publishers, Netherlands (2004).
12. **McLeod, M. P., Warren, R. L., Hsiao, W. W., Araki. N., Myhre, M., Fernandes, C., Miyazawa, D., Wong, W., Lillquist, A. L., Wang, D.,and other 19 authers:** The complete genome of *Rhodococcus* sp. RHA1 provides insights into a catabolic powerhouse. Proc. Natl. Acad. Sci. U. S. A., **103,** 15582-15587 (2006).
13. **Sone, N., Fukuda, M., Katayama, S., Jyoudai, A., Syugyou, M., Noguchi, S., and Sakamoto, J.:** *QcrCAB* operon of a nocardia-form actinomycete *Rhodococcus rhodochrous* encodes cytochrome reductase complex with

diheme cytochrome *cc* subunit. Biochim. Biophys. Acta, **1557,** 125-131 (2003).

14. **Kurokawa, T. and Sakamoto, J.:** Purification and characterization of succinate:menaquinone oxidoreductase from *Corynebacterium glutamicum*. Arch. Microbiol., **183,** 317-324 (2005).
15. **Schirawski, J. and Unden, G.:** Menaquinone-dependent succinate dehydrogenase of bacteria catalyzes reversed electron transport driven by the proton potential. Eur. J. Biochem., **257,** 210-215 (1998).
16. **Pereira, M. M., Santana, M., and Teixeira, M.:** A novel scenario for the evolution of haem-copper oxygen reductases. Biochim. Biophys. Acta, **1505,** 185-208 (2001).
17. **Sone, N., Hägerhäll, C., and Sakamoto, J.:** Aerobic Respiration in the Gram-positive Bacteria, p. 35-62. Zannoni, D. (ed.), Advances in Photosynthesis and Respiration, vol. 16. Respiration in Archaea and Bacteria Diversity of Prokaryotic Electron Transport Carriers, 1st edn, Springer, Netherlands (2004).
18. **Schägger, H., Cramer, W. A., and von Jagow, G.:** Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. Anal. Biochem., **217,** 220-230 (1994).
19. **Laemmli, U. K.:** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227,** 390-397 (1970).
20. **Sakamoto, J., Matsumoto, A., Oobuchi, K., and Sone, N.:** Cytochrome *bd*-type quinol oxidase in a mutant of *Bacillus stearothermophilus* deficient in caa_3-type cytochrome *c* oxidase. FEMS Microbiol. Lett., **143,** 151-158

(1996).

21. **Steyn-Parve, E. T. and Beinert, H.:** On the mechanism of dehydrogenation of fatty acyl derivatives of coenzyme A. VI. Isolation and properties of stable enzyme-substrate complexes. J. Biol. Chem., **233,** 843-852 (1958).

22. **Sakamoto, J., Koga, E., Mizuta, T., Sato, C., Noguchi, S., and Sone, N.:** Gene structure and quinol oxidase activity of a cytochrome *bd*-type oxidase from Bacillus stearothermophilus. Biochim. Biophys. Acta, **1411,** 87-98 (1999).

23. **Sone, N.:** Cytochrome oxidase from thermophilic bacterium PS3. Methods. Enzymol., **126,** 145-152 (1986).

24. **Yaginuma, A., Tsukita, S., Sakamoto, J., and Sone, N.:** Characterization of Two Terminal Oxidases in *Bacillus brevis* and Efficiency of Energy Conservation of the Respiratory Chain. J. Biochem., **122,** 969-976 (1997).

25. **Niebisch, A. and Bott, M.:** Purification of a Cytochrome bc_1-aa_3 Supercomplex with Quinol Oxidase Activity from *Corynebacterium glutamicum*. J. Biol. Chem., **278,** 4339-4346 (2003).

26. **Castiglioni, E., Grilli, E. and Sanguinetti, S.:** A new simple and low cost scattered transmission accessory for commercial double beam ultraviolet-visible spectrophotometers. Rev. Sci Instrum., **68,** 4288-4289 (1997).

27. **Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.:** Protein measurement with the Folin phenol reagent. J. Biol. Chem., **193,** 265-275 (1951).

28. **Minohara, S., Sakamoto, J., and Sone, N.:** Improved H^+/O Ratio and Cell Yield of Escherichia coli with Genetically Altered Terminal Quinol Oxidases. J. Biosci. Bioeng., **93,**.464-469 (2002).

29. **Junemann, S.:** Cytochrome *bd* terminal oxidase. Biochim. Biophys. Acta, **1321,** 107-127 (1997).

30. **Sato-Watanabe, M., Mogi, T., Miyoshi, H., Iwamura, H., Matsushita, K. Adachi, O., and Anraku, Y.:** Structure-function studies on the ubiquinol oxidation site of the cytochrome *bo* complex from *Escherichia coli* using *p*-benzoquinones and substituted phenols. J. Biol. Chem., **269,** 28899-28907 (1994).

31. **Matsushita, K., Yamamoto, T., Toyama, H., and Adachi, O.:** NADPH oxidase system as a superoxide-generating cyanide-resistant pathway in the respiratory chain of *Corynebacterium glutamicum*. Biosci. Biotechnol. Biochem., **62,** 1986-1977 (1998).

32. **Bott, M. and Niebisch, A.:** The respiratory chain of *Corynebacterium glutamicum*. J. Biotechnol., **104,** 129-153 (2003).

33. **Yagi, T., Bernardo, S. D., Nakamura-Ogiso, E., Kao, M. C., Seo, B. B., and Matsuno-Yagi, A.:** NADH Dehydrogenase (NADH-Quinone Oxidoreductase), p.15-40. Zannoni, D. (ed.). Advances in Photosynthesis and Respiration vol. 15. Respiration in Archaea and Bacteria Diversity of Prokaryotic Electron Transport Carriers, 1st edn, Kluwer Academic Publishers. Netherlands (2004).

Figure legends

FIG. 1. Growth curves of *R. rhodochrous* under various aeration conditions. The cells were cultured in 1 L baffled flasks with different medium volumes and different rotation rates. Symbols: open circles: condition I (the most aerobic condition); open squares: condition II; closed squares: condition III; closed circles, condition IV (the most microaerobic condition). The details of each condition were listed in Materials and Methods. OD_{600} was measured at a 10-fold dilution with water.

FIG. 2. Redox difference spectra of isolated membranes of *R. rhodochrous* grown under various conditions. The black bar indicates absorbance scale of 0.01. The membranes prepared from the cells were solubilized in 5% Triton X-100. The dithionite-reduced minus oxidized spectra (as prepared) were measured. The conditions for cell growth were the same as for FIG. 1. Traces a, b, c and d correspond to condition I (the most aerobic conditions), II, III and IV (the least aerobic conditions), respectively.

FIG. 3. pH changes of the medium containing the resting *R. rhodochrous* cells upon O_2 pulse. The cells (2-4 mg dry weight) were incubated at 30°C in 3 ml of reaction buffer containing 0.5 mM K-MOPS (3-morpholinopropanesulfonic acid) (pH 6.8), 140 mM KCl and 50 mM KSCN. After the addition of 0.5 μg valinomycin, a defined amount of oxygen was added to the suspension by injecting 20 μl air-saturated 140 mM KCl (8.7 ng atom-O). A stands for condition I (the most aerobic cells), and B stands for condition IV (the least aerobic cells), respectively.

FIG. 4. Inhibition of the oxygen consumption rates of *R. rhodochrous* membranes by cyanide and PBQ. The oxygen consumption rates of the most aerobic (condition I) and least aerobic (condition IV) membranes were

measured at 30°C in the presence of 0.16 mg/ml protein of the membrane preparations, 1 mM NADH, 10 mM Na-phosphate buffer and various concentrations of NaCN (A) or PBQ (B).

FIG. 5. Two-dimensional SDS-PAGE analysis of *R. rhodochrous* aa_3-type cytochrome *c* oxidase. The purified enzyme was applied to blue-native PAGE in the first dimension. The complete lane was then resolved by the second dimension SDS-PAGE. The acrylamide concentration of the gel was 13.5%, and the gel was stained with Coomassie brilliant blue R-250. Lane 1: molecular mass standard; lane 2: the purified aa_3 type cytochrome *c* oxidase.

FIG. 6. Redox difference spectra of the *bcc* complex and succinate dehydrogenase (SDH) from *R. rhodochrous*. The spectrum of the *bcc* complex has two peaks: one at 551 nm for the *c*-type cytochrome and the other at 561 nm for the *b*-type one (solid line). The spectrum of SDH has a peak at 557 nm (broken line). The black bar indicates absorbance scale of 0.015.

FIG. 7. A current scheme for the respiratory chain of *R. rhodochrous*. "MK" stands for menaquinone. Allow lines indicate the electron flow. The electron pathway that is mainly used is shown in thick lines. At the downstream, the pathway used under aerobic conditions is shown in solid lines, and the pathway used under microaerobic conditions is shown in broken lines. The existence of NDH-1 and -2 are not confirmed (enclosed with broken lines).