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Characterization of Two Terminal Oxidases in *Bacillus brevis* and Efficiency of Energy Conservation of the Respiratory Chain[1]

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The respiratory chain of *Bacillus brevis* was analyzed. Resting cells showed an H^+/O ratio of 4.8-5.2 (5.01±0.26), when measured using an oxygen pulse method with endogenous substrates. This value is intermediate between those of *Bacillus subtilis* (about 4), which predominantly expresses cytochrome aa_3-type quinol oxidase, and *Bacillus stearothermophilus* (about 6), which has quinol-cytochrome *c* reductase *plus* caa_3-type cytochrome *c* oxidase. Measurement of respiration with various substrates, and its inhibition by cyanide suggested that aa_3-type quinol oxidase and caa_3-type cytochrome *c* oxidase operate simultaneously in the respiratory chain of *B. brevis*. Both terminal oxidases were isolated by solubilizing *B. brevis* membranes with Triton X-100, and fractionating the extract using DEAE-Fractgel and gel-filtration columns. The quinol oxidase (aa_3) was composed of four subunits (57, 34, 23, and 15 kDa), like its counterpart of *B. subtilis*, while three subunits (52, 34, and 22 kDa) were identified in the cytochrome *c* oxidase (caa_3) preparation in *B. stearothermophilus*.

Key words: *Bacillus brevis*, cytochrome *c* oxidase, electron transfer chain, proton translocation, quinol oxidase.

Bacillus brevis, isolated from soil as a protein-hyperproducing bacterium (*1*), was found to show little extracellular protease activity, and has successfully been used as a host for high-level secretion of heterologous proteins (*2*) such as salivary α-amylase (*3*), epidermal growth factor (*4*), and growth hormone (*5*). Characterization of the respiratory chain of this aerobic bacterium is still incomplete, and may be important in relation to economical protein-production by this unique bacterium.

The respiratory chains of two groups of *Bacillus* have been characterized in some detail: thermophiles such as *B. stearothermophilus* and PS3, and probably also *B. caldolyticus*, have chains composed of dehydrogenases, quinol-cytochrome *c* reductase (*6-8*), and a caa_3-type cytochrome *c* oxidase (*9-11*), while mesophilic bacilli such as *B. subtilis* and *B. cereus*, when grown in regular rich media, mainly have aa_3-type quinol oxidase (*12-14*) encoded by *qoxABCD* (*15*). *B. subtilis* cells contain only small amounts of *c*-type cytochromes such as cytochrome *c*-550, whose role has not been elucidated (*16, 17*). Although *B. subtilis* has the genes (*ctaCDEF*) for a caa_3-type cytochrome *c* oxidase (*18*), which is expressed in an appreciable amount in succinate-grown cells and has been purified to homogeneity (*12, 14*), this enzyme is not the dominant terminal oxidase, but a minor one in vegetative *B. subtilis* cells grown under standard conditions. These differences in the composition of the respiratory chain in *Bacillus* species may explain the differences in the efficiency of H^+ translocation across the membrane measured using oxygen pulse methods, *i.e.* the ratio of proton ejected to oxygen atom consumed (H^+/O ratio) upon addition of a known amount of oxygen to resting cells under anaerobic conditions in the presence of an appropriate permeable ion (*19, 20*). The H^+/O ratio of thermophilic *Bacillus* was 6-7 (*20-22*), while those of mesophilic bacilli such as *B. subtilis* and *B. megaterium* were reported to be about 4 (*20, 23*). Jones *et al.* proposed that the apparent lack of cytochrome *c* in membrane preparations correlated with a lower H^+/O ratio of 4 (*20*). However, it seems more appropriate to suppose that the H^+/O of resting cells is the sum of the H^+ pumping activities of all respiratory complexes involved (*24*). The thermophilic bacilli use quinol-cytochrome *c* reductase (b_6c_1 complex) *plus* caa_3-type cytochrome *c* oxidase for menaquinol oxidation, while mesophilic bacilli mainly use aa_3-type quinol oxidase as the sole enzyme for the same reaction.

Here, we analyzed the respiratory chain of *B. brevis*, and found that its H^+/O ratio takes an intermediate value between those for the two groups of bacilli and that two types of terminal oxidases, caa_3-type cytochrome *c* oxidase and aa_3-type quinol oxidase, operate simultaneously.

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Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl *p*-phenylenediamine; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethane sulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; kDa, kilodalton; SDS, sodium dodecylsulfate; SDS-PAGE, polyacrylamide gel electrophoresis with SDS.

MATERIALS AND METHODS

Organism and Growth Conditions—*B. brevis* 47-5Q, kindly donated by Prof. S. Udaka of Tokyo University of Agriculture was cultured in Luria broth supplemented with 1% glucose at 37°C (*2*). A portion of an overnight starter culture (about 5 ml) was used to inoculate the pre-culture medium (200 ml in a 1-liter flask), and the content of the flask, after several hours of shaking culture, was added to a jar fermenter (Eyera MFB-801, Koshigaya) containing 6 liters of Luria broth with glucose. For vigorous aeration of the culture, the air-flow rate was set at 5.0 liters/min and the propeller was rotated at a high speed (500 rpm). The cells were harvested at late log phase when the optical density at 650 nm was 5-8 (it is about 15 at the stationary phase). Cells were washed once with 0.1 M NaCl containing 10 mM HEPES-NaOH buffer (pH 7.2), and suspended in the same medium. Cells were kept frozen until use.

Preparation of Membrane Fraction and Triton X-100 Extraction—Cells (16 g wet cells in 100 ml) were disrupted with a sonicator (Tomy UD-201) at an output of 10 for 2 min. The sonication was repeated 4 times with intervals of several minutes. The supernatant fraction of $4,000\times g$ was centrifuged at $104,000\times g$ for 60 min, and the resultant precipitate was used as the membrane fraction. Before extraction of integral membrane proteins, the membranes were treated with 2% sodium cholate in 30 mM Tris-Cl (pH 8.0) and 1 mM EDTA for 40 min with stirring and occasional sonication, and the mixture was centrifuged at $104,000\times g$ for 40 min. This procedure removed most peripheral membrane proteins. The pellet was suspended in the same medium and treated once more. The resultant pellet was then solubilized by incubation in 2% Triton X-100 containing 10 mM Tris-HCl, pH 8.0, for 60 min with stirring at 4°C. The amber-colored supernatant after centrifugation at $104,000\times g$ was used for the purification of the oxidases.

Measurement of Proton Movement in Resting Cells—The pH change induced by an oxygen pulse in resting cells under anaerobic conditions was measured in a closed vessel (3.0 ml) with a Beckman combination pH electrode (No. 39030). The solubility of O_2 in 0.14 M KCl was taken as 0.40 μg-atom/ml at 35°C.

Measurement of Respiration—Oxygen uptake of the membrane fraction was followed at 35°C using a Yellow Springs No. 4005 oxygen electrode in a semi-closed vessel (2.3 ml) containing 50 mM sodium phosphate buffer, pH 7.1, as the reaction medium.

Measurement of Quinol and TMPD Oxidase Activities—Quinol oxidase activity was measured spectrophotometrically with a dual-beam spectrophotometer (Hitachi 556) in a reaction mixture (0.5 ml) containing 20 mM sodium phosphate buffer, pH 6.7, with 1 mM EDTA at 35°C. The reaction was started by the addition of 50 μM quinol. Extinction coefficients of 19.5 $mM^{-1}\cdot cm^{-1}$ (264.5-283.5 nm), 2.2 (340-370 nm), and 9.0 (328-370 nm) were used for calculation of concentrations of duroquinone, menadione, and tetrachlorobenzoquinone. TMPD oxidase activity was measured spectrophotometrically at 562 nm with a single-beam spectrophotometer (Beckman DU-70) using 0.1 mM TMPD as described previously (*25*), unless stated otherwise.

Cytochrome *c* oxidase and TMPD oxidase activities were measured by following the pH change with ascorbate as a final electron donor according to the following equation (*26*):

$$\text{ascorbate H}\cdot\text{Na}+\text{H}^+ + 1/2\text{O}_2 = \text{dehydroascorbate} + \text{H}_2\text{O} + \text{Na}^+$$

The net alkali formation was back-titrated with aliquots of 5 mM HCl.

Spectrophotometric Measurement—Absorption spectra were measured on a Beckman DU-70 spectrophotometer. Contents of cytochromes aa_3 and caa_3 were determined from reduced *minus* oxidized difference spectra using millimolar extinction coefficient differences of 23.2 at 604 *minus* 630 nm (*9*).

Others—Peptide sequences were determined by the Edman degradation method using an Applied Biosystems model 473A gas-phase sequencer with polypeptide blotted onto polyvinylidene difluoride membrane after SDS-PAGE as the specimen. SDS-PAGE and protein determination were carried out as before (*25*). For heme analysis, *B. brevis* membrane preparation was vigorously mixed with acetone *plus* HCl, and hemes were extracted with ethyl acetate. After having been washed with water and supplemented with acetonitrile, the extract was applied to a reverse phase column Resource RPC (Pharmacia, Uppsala) and eluted with a gradient of 20-100% acetonitrile in water containing 0.05% trifluoroacetic acid. Gel-filtration glass columns G3000SW and G4000SW, as well as DEAE-Fractogel 650S, were purchased from Tosoh (Tokyo). Centricon-30 was obtained from Amicon (Beverly). Other chemicals, inhibitors, and detergents were obtained as described previously (*25*).

RESULTS

Proton Pumping Activity and Cytochrome Pattern of B. brevis—Figure 1 shows a typical trace of H^+ ejection in response to an oxygen pulse given to resting cells of *B. brevis* under anaerobic conditions. The H^+/O ratio extrapolated to time zero was 5.2 (trace A). In the presence of 0.1 mM KCN the H^+/O ratio was 4.0, and the initial velocity of H^+ ejection became slower than that in the previous trace without cyanide (trace B). Using three different preparations of *B. brevis* cells, the H^+/O ratios with and without cyanide were found to be 3.96 ± 0.19 ($n=8$) and 5.01 ± 0.26 ($n=10$), respectively. It is thus likely that a relatively cyanide-resistant alternative oxidase branch with a lower energy-yielding efficiency is operating in addition to a highly cyanide-sensitive one with a higher H^+/O ratio.

Figure 2 shows redox difference spectra of *B. brevis* membranes. In addition to the peaks at 602 nm and 558 nm indicating the presence of both *a*-type and *b*-type cytochromes, a clear shoulder at around 550 nm shows the presence of *c*-type cytochrome(s) in the $Na_2S_2O_4$-reduced *minus* air-oxidized difference spectrum (solid line). In the case of the thermophilic bacilli the peak due to *c*-type cytochromes is as high as or even higher than the peak due to *b*-type cytochrome(s) (*27*). *B. brevis* cells cultured in Luria broth containing succinate instead of glucose showed a similar H^+/O ratio and a similar cytochrome pattern to the glucose-grown cells. The ascorbate-reduced *minus* oxidized difference spectrum shows an α peak of *c*-type

cytochrome at 551 nm and no peak due to *b*-type cytochromes (broken line), indicating that almost all *b*-type cytochromes have lower redox potentials than ascorbate. Hemes were extracted from the membrane preparation and analyzed by reverse phase chromatography, showing that the extract contained hemes B and A, but no detectable amount of heme O (data not shown).

Oxidase Activity with Different Substrates—Table I summarizes rates of oxygen uptake of the membrane fraction of *B. brevis*. Both NADH and TMPD were oxidized rapidly. TMPD is an effective artificial electron donor for cytochrome *c* oxidase. Among organic acids tested, malate was oxidized most rapidly, suggesting the presence of an active membrane-bound malate dehydrogenase. Succinate and glycerophosphate were moderately oxidized. As expected by the rapid oxidation of TMPD, cytochrome *c* from *Saccharomyces cerevisiae* was oxidized. Duroquinol was also oxidized rather rapidly. This is in contrast to data obtained with the thermophilic *Bacillus* PS3, in which the rate of duroquinol oxidation was about 2% of the rate of TMPD oxidation (*28*), and to that with *B. subtilis*, whose TMPD oxidase activity was very low (*13*).

Figure 3 shows the effect of NaCN on TMPD and succinate oxidase activities. TMPD oxidation was inhibited by low concentrations of NaCN. A Dixon plot gave a K_i of 6.3 μM (not shown). In contrast, the cyanide inhibition of succinate oxidation was biphasic, suggesting that succinate was oxidized by two oxidases with different K_i values for cyanide. The three curves in the figure were drawn based on the assumption that oxidase activities with K_i values of 6.3 μM and 5.1 mM coexist in ratios of 40:60, 50:50, or 60:40. The latter K_i value is that obtained for cyanide inhibition of duroquinol oxidase activity by cytochrome aa_3 purified as described below. The data points best fitted the curve with the activity ratio of 50:50. Thus, two terminal oxidases,

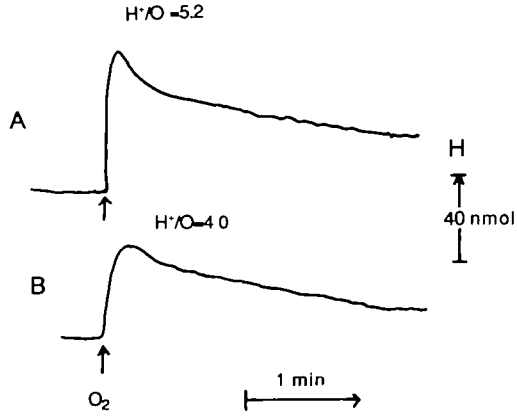

Fig. 1. **Change in pH of the medium containing *B. brevis* resting cells upon O_2 pulse.** The cells (5.5 mg dry wt.) were incubated at 35°C in 3.0 ml of 140 mM KCl containing 0.25 mM K-MOPS (pH 6.5-6.6). After incubation to anaerobiosis, 5 M KSCN (40 μl) and 0.5 mg/ml of valinomycin (4 μl) were added, and after about 15 min the reaction was started by adding air-saturated 0.14 M KCl. Cells were washed once with 0.1 M NaCl containing 10 mM HEPES-NaOH, pH 7.0, and then with 140 mM KCl containing 0.25 mM MOPS-KOH, pH 6.8. A, a control without KCN; B, in the presence of 0.1 mM KCN.

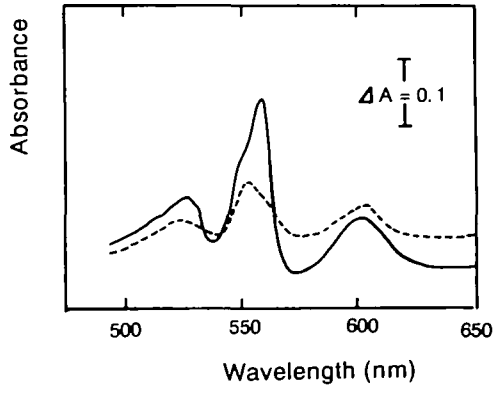

Fig. 2. **Redox difference spectra of membrane fractions showing cytochrome pattern.** The *B. brevis* membrane fraction was suspended in 50 mM Tris-HCl buffer (pH 8.0) containing 2% Triton X-100 and sonicated briefly. The reduced form was prepared by adding a few grains of $Na_2S_2O_4$ (–) or ascorbate in the presence of a very low concentration of TMPD (· ·).

TABLE I. **Oxygen uptake rates of *B. brevis* membrane fraction with various substrates.**[a]

Substrate	Conc. (mM)	Rate (ng-atom O/min/mg)
NADH	0.43	217
Malate	0.87	162
Glutamate	0.87	<1
Succinate	0.87	35
1-Glycerophosphate	0.87	22
Duroquinol	0.10	72
Yeast cytochrome *c*	0.002	25
TMPD	0.10	203

[a]The rate of oxygen uptake was followed polarographically at 35°C in 50 mM NaP_i buffer, pH 7.1. The protein concentrations used were 0.18-0.91 mg/ml.

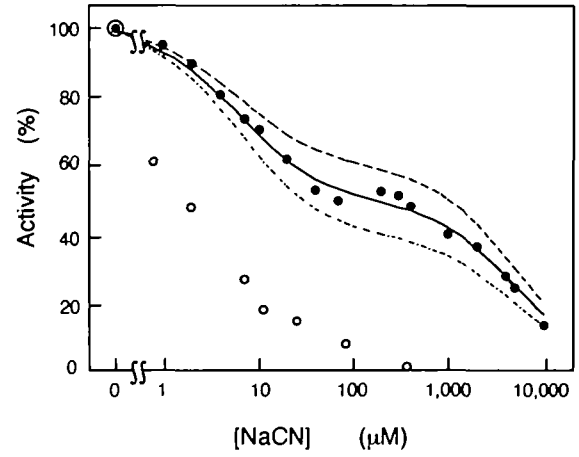

Fig. 3. **Effects of cyanide concentration on TMPD and succinate oxidation activity of *B. brevis* membrane fraction.** For TMPD oxidation (○), the reaction mixture contained membrane fraction (0.18 mg protein/ml), 0.1 mM TMPD, 50 mM NaP_i buffer (pH 7.1), and the indicated concentration of NaCN. Oxygen consumption was polarographically followed at 35°C. The K_i value was calculated to be 6.3 μM. For succinate oxidation (●), 2 mM sodium succinate and the membrane fraction at 0.91 mg protein/ml were used. The broken, solid, and chain curves show the calculated values based on the assumption that the sample contained two cyanide-sensitive pathways for succinate oxidation, one with a K_i value of 6.3 μM and the other with a K_i value of 5.1 mM, and that their ratio is 40:60, 50:50, or 60:40, respectively.

one highly sensitive to cyanide and the other moderately sensitive to it, are operating in the *B. brevis* respiratory chain.

Partial Purification of Two Terminal Oxidases—Figure 4 shows the elution pattern after DEAE-Fractogel chromatography of the Triton X-100-solubilized membrane fraction of *B. brevis*. TMPD oxidase activity was eluted at 50 mM NaCl, while quinol oxidase was mainly eluted at 100 mM. The 50 mM NaCl eluate contained *a*-, *b*-, and *c*-type cytochromes, while the 100 mM NaCl eluate fraction almost exclusively contained cytochrome *a*, suggesting that the quinol oxidase is of cytochrome aa_3-type. The 200 mM NaCl eluate included succinate dehydrogenase, which contains cytochrome *b*-558 and covalently bound flavin as in *B. subtilis* (*17*). There were minor peaks of duroquinol oxidation activity in the 10 and 200 mM NaCl eluates, although it is not known what entity manifests these activities. The 70 mM eluate contained *b*- and *c*-type cytochromes with α peaks at 550-562 nm, most likely being quinol-cytochrome *c* reductase, since SDS-PAGE showed 28, 23, 21, and 14 kDa bands as the main components, similar to *Bacillus* PS3 quinol-cytochrome *c* reductase (*17*). These 28 and 21 kDa bands had bound heme, revealed by peroxidase activity (not shown), as found with the PS3 enzyme (*28*). The material that was not absorbed on the column (flow through) also contained some *b*-type and *c*-type cytochromes. Most of the *c*-type cytochrome of this fraction was a small cytochrome *c* of about 13 kDa, which corresponds to *Bacillus* PS3 cytochrome *c*-551 (*29*) and *B. subtilis* cytochrome *c*-550 (*16*). These elution profiles of *B. brevis* cytochromes are very similar to those of the thermophilic *Bacillus* PS3, except for the presence of cytochrome aa_3 in the 100 mM NaCl fraction.

The 50 and 100 mM NaCl fractions were concentrated and then separately applied to two tandem-linked gel filtration columns (TSK G3000SW *plus* G4000SW) for further purification (*30*). Table II summarizes the overall purification of the TMPD and quinol oxidases. Yields of TMPD oxidase and quinol oxidase were 17.8 and 16.3%, respectively, and they were about 41- and 27-fold enriched during the purification. Heme A was distributed 1:9 between the cytochrome *c* oxidase and quinol oxidase fractions (Table II), suggesting that the latter enzyme is more abundant than the former, as expected from the low concentration of *c*-type cytochromes shown in the redox difference spectrum of the *B. brevis* membrane fraction (Fig. 2).

Properties of Cytochrome c Oxidase and Quinol Oxidase—Figure 5 shows redox difference spectra of cytochrome caa_3-type cytochrome *c* oxidase (trace A) and

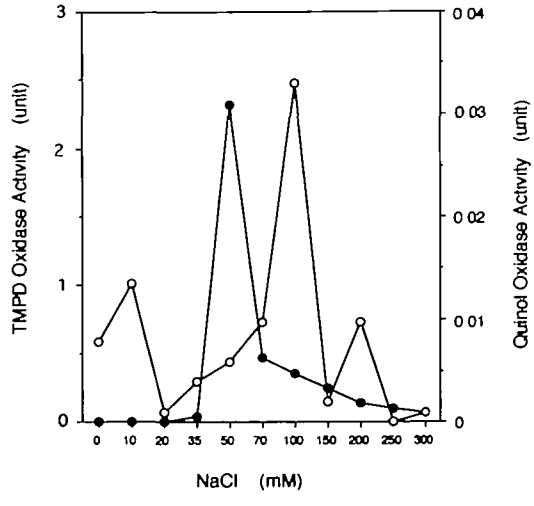

Fig. 4. **Elution profiles of *B. brevis* cytochrome components from a DEAE-Fractogel column.** *B. brevis* membranes (95.4 mg) treated with cholate and solubilized with 2% Triton X-100 were dialyzed against 10 mM Tris-HCl, pH 8.0, and applied to a DEAE-Fractogel column (2×5 cm) equilibrated with 0.5% Triton X-100 containing 10 mM Tris-HCl buffer, pH 8.0. Unabsorbed material flowed through the column before absorbed proteins were eluted by raising the concentration of NaCl in Tris buffer containing Triton X-100. Absorbance was monitored at 410 nm. TMPD (●) and quinol oxidase (○) activities of collected fractions were measured.

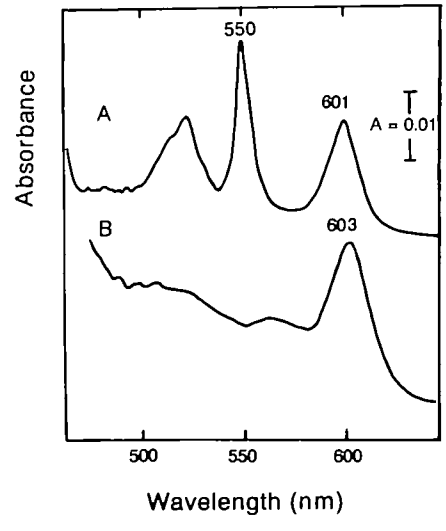

Fig. 5. **Reduced ($Na_2S_2O_4$) *minus* oxidized (as prepared) difference spectra of the cytochrome caa_3 (A) and cytochrome aa_3 (B).**

TABLE II. **Summary of purification of caa_3-cytochrome *c* oxidase and aa_3-quinol oxidase.**

	Protein (mg)	Cyt. aa_3 (nmol)	Activity (unit) TMPD-O_2	Activity (unit) QH_2-O_2	Cyt. aa_3 content (nmol/mg)	Specific activity[a] TMPD-O_2	Specific activity[a] QH_2-O_2
Triton extract	95.4	6.79	8.87	1.83	0.070	0.093	0.019
DEAE-eluate							
50 mM	3.1	0.58	–[b]	–	0.187	–	–
100 mM	8.4	5.92	–	–	0.70	–	–
Gel filtration							
caa_3	0.44	0.33	1.58	–	0.75	3.8	–
aa_3	0.58	2.41	–	0.30	4.16	–	0.52

[a]Unit/mg protein. [b]–, not determined.

cytochrome aa_3-type quinol oxidase (Trace B) preparations. There was no indication of the presence of *b*- or *d*-type cytochromes. Cytochrome *a* contents were 0.75 and 4.2 nmol/mg protein, respectively, and the former also contained cytochrome *c* (2.4 nmol/mg protein). Another preparation of cytochrome caa_3 showed better chromophore contents, as high as 1.8 nmol of cytochrome aa_3 and 2.4 nmol of cytochrome *c* per mg protein.

The preparations of the two terminal oxidases were analyzed by SDS-PAGE for purity and subunit composition. Figure 6 shows that the cytochrome aa_3 preparation contained mainly four polypeptides of 53, 34, 23, and 15 kDa (lane 2). The cytochrome caa_3 preparation contained a larger number of polypeptides (lane 3). However, the bands at 52, 37, and 22 kDa can be recognized as subunits I, II, and III of the oxidase, since these positions were similar to those in the cases of PS3 and *B. stearothermophilus* cytochrome caa_3 (*9, 31*). In particular, the 52-kDa band was broader than those of regular proteins, and the 37-kDa band retained covalently bound heme C as judged from its peroxidase activity (data not shown). These facts are consistent with the general observations that subunit I of cytochrome *c* oxidase shows a fuzzy appearance upon SDS-PAGE, and subunit II of the caa_3-type cytochrome *c* oxidase contains a *c*-type cytochrome moiety (*9, 31*). The bands other than these three are likely to be contaminants, since the amounts of the three bands were maximal in the peak fraction of the enzyme activity (30 min), and the other bands were mainly found in later fractions (31 and 32 min). The presence of subunit IV of cytochrome caa_3 was unclear. Subunits III and IV of the bacterial cytochrome *c* oxidases are often absent from purified preparations (*24, 31*). Cytochrome aa_3-type quinol oxidase from *B. subtilis* was once reported to have two subunits (*15*), and later revealed to contain four subunits in a pure preparation (*14*). The *qoxABCD* operon encodes subunits I through IV with molecular weights of 73.8, 33.6, 22.7, and 13.7 kDa, respectively (*14, 15*). The present *B. brevis* aa_3 preparation contained four subunits and appeared fairly pure (Fig. 6, lane 2), but its heme content was only about half of the expected value. This may be because the enzyme is one of the proteins whose amount would be overestimated by the assay method employed here. Similar deviation may also occur for cytochrome caa_3, although the main reason for the low value for the latter enzyme is the presence of contaminants.

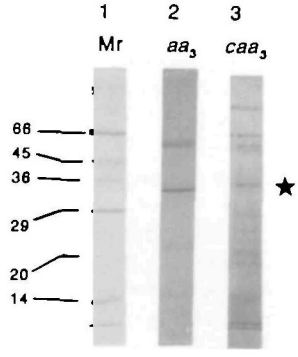

Fig. 6. **SDS-PAGE pattern of two *B. brevis* terminal oxidase preparations.** The preparations were carried out as described under "MATERIALS AND METHODS" (*2*). The polyacrylamide concentration of the gel was 13.5%, and the gel was stained with Coomassie Brilliant Blue R-250. Lane 1, molecular weight marker proteins of bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme (Biorad); lane 2, cytochrome aa_3 preparation (cytochrome aa_3 content of 4.2 nmol/mg protein); lane 3, cytochrome caa_3 preparation (cytochrome caa_3 content of 1.8 nmol/mg protein). The star indicates that the band has covalently bound heme.

In order to determine the N-terminal sequences of the subunit peptides of cytochrome aa_3, those separated by SDS-PAGE were transferred to a sheet of polyvinylidene difluoride membrane, and applied to a peptide sequencer. The second largest subunit (subunit II, 34 kDa) gave the sequence XGSEYLVLDPKGPVAEEQYNLI-. This sequence is very similar to the N-terminal sequence of mature cytochrome aa_3 quinol oxidase from *B. subtilis*, that was reported to be CSNASVLDPKGPVAEQQSDLI- (*14, 15*). The yields of N-terminal amino acids of the bands corresponding to subunits I and III were very low, suggesting that the N-terminus of each of these subunits is blocked. The blocking groups are unlikely to be formyl groups, because the sheet of polyvinylidene difluoride was pretreated with 0.6 N HCl (*32*) to cleave possible formyl groups.

Table III summarizes the substrate specificity of the two terminal oxidases from *B. brevis*. Cytochrome caa_3 oxidized cytochromes *c*. Yeast cytochrome *c* and PS3 cytochrome *c*-551 (with lower K_m) were better substrates than horse heart cytochrome *c*. Cytochrome caa_3 did not oxidize duroquinol. This substrate specificity is very similar to that of PS3 cytochrome caa_3 (*19*). On the other hand, the cytochrome aa_3 oxidized duroquinol, menadiol, and tetrachlorobenzoquinol at appreciable rates, but hardly oxidized menaquinol-3. The enzyme also did not rapidly oxidize ubiquinol-1, as cytochrome aa_3-600 of *B. subtilis* does not (*12*). The quinol-oxidation rates by the *B. brevis* enzyme were not linearly correlated to the redox potentials of those quinol/quinone couples. This finding suggests that the electron-transfer itself is not the rate-limiting step, but compatibility between the stereostructures of the substrate quinol and its binding site on the enzyme may be important in determining the overall reaction rate. The reason why cytochrome aa_3 did not oxidize menaquinol-3 may be that this quinol is too hydrophobic to be a substrate when

TABLE III. **Substrate specificity of caa_3 cytochrome *c* oxidase and aa_3 quinol oxidase.** Oxidation of cytochrome *c* and TMPD was measured polarographically as in Table I, and quinol oxidation was determined spectrophotometrically as described in "MATERIALS AND METHODS."

Substrate (conc, μM)	Activity (e^-/s)	
	Cytochrome caa_3	Cytochrome aa_3
TMPD (90)	141	–[a]
(180)	203	1.2 (<0.2*)
Yeast cyt. *c* (7)	49	–
(14)	104	0.6 (<0.2*)
Horse cyt. *c* (14)	28	–
PS3 cyt. *c*-551 (3.1)	75	–
Duroquinol (50)	<1	4.8
Ubiquinol-1 (200)	–	0.2
Menadiol (60)	–	19.8
Menaquinol-3 (100)	–	0.7
Tetrachlorobenzoquinol (100)	–	2.6
Tetrachlorobenzoquinol (100) *plus* menaquinone-3 (40)	–	9.4

[a]Not determined. *Inhibited by 0.1 mM KCN.

exogenously added, in spite of the fact that the endogenous quinone of *B. brevis* should be menaquinone-7, as in *B. subtilis* and *B. stearothermophilus* (*33, 34*). Another noteworthy observation was that tetrachlorobenzoquinol oxidation was accelerated by preincubating the cytochrome aa_3 with menaquinone-3. These findings suggest that besides the substrate binding site, there is another quinone binding site, which binds a hydrophobic quinone and then accelerates the oxidation of the substrate quinol. The present cytochrome aa_3 preparations showed low TMPD and yeast cytochrome *c* oxidase activities, but these activities seem to be due to contaminating cytochrome caa_3, since they were inhibited by the addition of 0.1 mM NaCN. In contrast, contamination of cytochrome aa_3 in cytochrome caa_3 was negligible, since the cytochrome caa_3 preparation did not oxidize duroquinol at all. The contamination is likely to be caused by the tendency of very hydrophobic membrane proteins to be eluted with tailing because of hydrophobic interactions, even when the salt concentration is dissociative. The inhibition by cyanide of the duroquinol oxidase activity of the cytochrome aa_3 preparation was measured. The results, analyzed by means of a Dixon plots, indicated a K_i of 5.1 mM (not shown). This value was used for the analysis of the succinate oxidation with the membrane fraction shown in Fig. 3.

DISCUSSION

The present data show two terminal oxidases in *B. brevis* vegetative cells. One is the aa_3-type quinol oxidase composed of four subunits, which is similar to cytochrome aa_3-600 of *B. subtilis* (*12-14*). The other is the caa_3-type cytochrome *c* oxidase containing at least three subunits. It is similar to that of *B. stearothermophilus*, for which three subunits have been identified (*31*), and that of thermophilic *Bacillus* PS3, which contains all four subunits encoded in the oxidase operon (*11, 35*). Cytochrome *bd*-type oxidase, found in *B. subtilis* at the stationary growth phase (*12, 16*), was not observed in our *B. brevis* cells harvested at late log phase. The α peak wavelengths in the absorption spectra were similar in the two reduced enzymes of *B. brevis* (Fig. 5); 603 nm in the quinol oxidase and 601 nm in the cytochrome *c* oxidase. Since in the case of *B. subtilis*, aa_3-type quinol oxidase and caa_3-type cytochrome *c* oxidase absorb at 600 and 605 nm, respectively (*16*), the order of peak wavelengths of two aa_3-type oxidases in an organism is not automatically determined by their substrate types. The cyanide-sensitivity of the two oxidases differed; the K_i of quinol oxidase was 5.1 mM, while that of cytochrome *c* oxidase was 6.3 μM. It would be of interest to know what kind of structural difference causes this different sensitivity to cyanide.

The substrate specificity of the two terminal oxidases of *B. brevis* may depend on structural differences in their subunit II. Subunit II of caa_3-type oxidase has a *c*-type cytochrome moiety and has a molecular mass of 37 kDa, while that of aa_3-type quinol oxidase is 34 kDa. Preincubation with menaquinone-3 accelerated the tetrachloroquinol-oxidation activity of the cytochrome aa_3 preparation. This finding suggests that the enzyme contains an intrinsic quinone-binding site besides the substrate binding site, as demonstrated for *E. coli bo*-type quinol oxidase (*36*), and that the bound quinone, which is necessary for high enzymatic activity and probably is menaquinone-7, was lost during the enzyme purification. Removal of intrinsic quinone may occur during chromatography in the presence of non-ionic detergent. Naphthoquinones are also known to be easily decomposed by UV light (*6*). We have also reported activation of PS3 quinol-cytochrome *c* reductase (cytochrome b_6c_1) by the addition of menaquinone 3-7 (*6*).

The activity of purified *B. brevis* quinol oxidase was not as high as that of the cytochrome *c* oxidase or that of *E. coli* cytochrome *bo*, which was reported to be about 500 s^{-1} with ubiquinol-1 (*37*), even after menaquinone-3 had been added. Judging from the result of the ion-exchange column chromatography (Fig. 4), the amount of cytochrome caa_3 was about one-tenth of that of aa_3. Based on this value, the molecular activities of TMPD oxidation and duroquinol oxidation of membrane preparations were calculated to be 400 and 10.2 s^{-1}, respectively. Both of these values are a little higher than those of the final preparations (Table III), indicating that the enzymes were inactivated in parallel during the course of the purification. The results of the experiment to evaluate cyanide inhibition of succinate-oxidizing activity (Fig. 3) suggests that both quinol oxidase and cytochrome *c* oxidase branches of the respiratory chain are working almost equally in *B. brevis* cells grown in the rich medium. Taking all these data, it seems likely that the lower molecular activity of the quinol oxidase than that of the cytochrome *c* oxidase is not mainly due to enzyme solubilization or isolation, and the low intrinsic activity is compensated by its abundance in the cells.

The intermediate H^+/O ratio of 5.01 with endogenous respiration (Fig. 1) also supports an approximately equal contribution of the two oxidase branches, since the ratio is 6-7 in the *Bacillus* cells which use the pathway composed of both b_6c_1-type quinol-cytochrome *c* reductase and caa_3-type cytochrome *c* oxidase (*20, 21*), while the ratio is 4 in cells which use aa_3-type quinol oxidase (*20*). Their coexistence is a characteristic feature of the *B. brevis* respiratory system. In the case of *B. subtilis*, aa_3-type quinol oxidase and *bd*-type oxidase are found, while caa_3-type cytochrome *c* oxidase is also found after growth in succinate medium (*12, 16*). In the case of *B. brevis*, change of the C-source from glucose to succinate did not cause a drastic change of the terminal oxidases. However, it would be interesting to examine the effects of other cultivation conditions on the amounts of respiratory cytochrome components in *B. brevis*. In addition, it would be interesting to analyze what happens in *B. brevis* cells if the quinol oxidase genes (*qoxABCD*) are deleted. Thermophilic bacilli are able to grow with a higher H^+/O ratio without aa_3-type quinol oxidase. Therefore, we can expect that *qox* gene-disruption may cause a higher growth yield of *B. brevis* and hence more economical protein production.

Figure 7 shows a tentative scheme for the respiratory chain of *B. brevis*. *B. brevis* contains menaquinone, as do in *B. subtilis* and *B. stearothermophilus*. Menaquinol in the membrane is oxidized with quinol-cytochrome *c* reductase *plus* caa_3 cytochrome *c* oxidase (cytochrome *c* branch) or with aa_3-type quinol oxidase (quinol oxidase branch). Electrons flow through both branches to nearly equal extents in *B. brevis* cells grown in glucose-polypepton-yeast extract medium. Other cytochrome components such as succinate dehydrogenase (cytochrome *b*-558) and a small cytochrome *c* were also present in *B. brevis*. Menaquinones

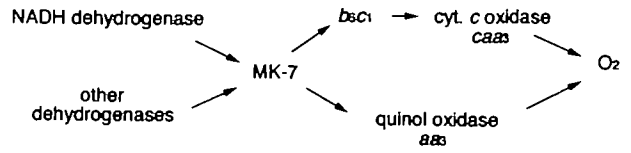

Fig. 7. **A tentative scheme for the respiratory chain of *B. brevis*.** Italic letters indicate the names of cytochromes, and "MK-7" means menaquinone-7.

are reduced by several primary dehydrogenases including NADH, malate, succinate, 1-glycerol phosphate, and lactate dehydrogenases. NADH dehydrogenase might not be a complex I-type, indicating that the enzyme does not pump protons. The H^+/O ratio of *B. brevis* with endogenous substrates was about 5, which is between 6 as found in *B. stearothermophilus* (*22*), and 4 as found in mesophilic *B. subtilis* and *B. megaterium* (*20, 23*). The value of 6 is consistent with the sum of the values of quinol-cytochrome *c* reductase and cytochrome *c* oxidase, while the value of 4 is consistent with the H^+/O ratios of ubiquinol-oxidizing *E. coli* cytochrome *bo* (*38*). We also obtained an H^+/O ratio of around 4 with resting cells of *B. brevis* in the presence of 0.1 mM KCN, which inhibited caa_3-type cytochrome *c* oxidase, but not aa_3-type quinol oxidase (Fig. 1).

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