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Studies on the respiratory system in alkaliphilic *Bacillus*; a proposed new respiratory mechanism

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Abstract Respiratory electron transfer systems in two alkaliphilic *Bacillus* species, YN-1 and YN-2000, were investigated. In the cyanide-sensitive pathway of the obligate alkaliphilic *Bacillus* YN-1, the terminal enzyme was a *caa*$_3$-type cytochrome *c* oxidase constituting up to just 10% of the total oxygen-reducing activity, while 90% of the respiratory activity was due to cyanide-insensitive, nonproteinaceous material with a molecular weight of 662. These results were consistent with the cyanide-tolerant growth of the bacterium. The molecular and catalytic properties of the nonproteinaceous material were not identical with those of menaquinones extracted from the bacterium. Furthermore, the nonproteinaceous material was also found in the facultative alkaliphilic *Bacillus* YN-2000, when that bacterium was cultivated in alkaline conditions. A new respiratory oxygen-reducing mechanism comprising a nonproteinaceous component and a catalase is proposed for these alkaliphilic *Bacillus* species.

Key words Alkaliphile · Cytochrome oxidase · Cyanide-insensitive respiratory system · Nonproteinaceous respiratory terminal component · H_2O_2

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Introduction

Many bacteria, yeasts, and fungi with the ability to grow in extremely alkaline conditions have been isolated from soils, and have been designated "alkaliphiles." Alkaliphilic microorganisms have a number of potential applications, since some of them produce alkaline-tolerant extracellular enzymes, such as cellulases and proteinases (Horikoshi and Akiba 1982). These alkaliphiles have also presented a number of intriguing problems in regard to bioenergetics. ATP synthesis in alkaliphiles, like that in neutralophiles, is driven by the proton motive force generated by a combination of proton pumping and respiration (Guffanti et al. 1981). On the other hand, the almost neutral cytoplasmic pH of alkaliphilic bacterial cells is reported to be maintained by the effect of Na^+/H^+-antiporters (Krulwich and Guffanti 1989a,b). Therefore, a simple chemiosmotic mechanism cannot explain the generation of a proton motive force across the cytoplasmic membrane of an alkaliphilic bacterium growing under high-pH conditions. Krulwich and co-workers have proposed a "localized gradient" hypothesis involving a particular localization for both the ATP synthase and the proton-pumping respiratory components in the membrane (Guffanti and Krulwich 1992). Recently, we purified some respiratory components, including two *c*-type cytochromes, an *aco*-type cytochrome *c* oxidase, and a succinate dehydrogenase, from the facultative alkaliphilic *Bacillus* YN-2000 (Yumoto et al. 1991; Qureshi et al. 1990, 1996), and found that *Bacillus* YN-2000 cultivated in alkaline conditions contained higher amounts of cytochromes than were present after cultivation under neutral conditions (Yumoto et al. 1990).

In the present study, we investigated the respiratory system of two alkaliphiles: an obligate alkaliphilic *Bacillus*, YN-1, and a facultative alkaliphilic *Bacillus*, YN-2000. These bacteria had branched respiratory electron-transfer systems consisting of a cyanide-sensitive cytochrome *c* oxidase and a cyanide-insensitive nonproteinaceous material. A new respiratory mechanism that utilizes a nonproteinaceous

component and a catalase enzyme for reducing the oxygen is discussed.

Materials and methods

Organisms

Obligate alkaliphilic *Bacillus* YN-1 and facultative alkaliphilic *Bacillus* YN-2000 were both kindly provided by Dr. Y. Nosoh and Dr. N. Koyama (Chiba University, Chiba, Japan). The organisms were aerobically grown in medium containing 10 g of polypeptone, 1.5 g yeast extract, 1 g glucose, 1 g K_2HPO_4, 0.1 g $MgCl_2 \cdot 6H_2O$, and 10 g Na_2CO_3 per liter according to the method of Qureshi et al. (1990). The pH of the medium was adjusted to 10.0 or to 8.0 by adding $NaHCO_3$ or NaH_2PO_4. The bacterial cells were harvested at early stationary growth phase and stored at −80°C until use.

Preparation of bacterial cytoplasmic membrane

About 100 g (wet weight) of the cultured *Bacillus* YN-1 or YN-2000 cells was suspended in 300 ml of 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM disodium ethylenediaminetetraacetate (EDTA) and 10 μM phenylmethylsulfonyl fluoride (PMSF). After addition of deoxyribonuclease (1 μg/ml), the suspension was compressed in a French pressure apparatus at 9800 N/cm^2 to disrupt the cells. The resulting suspension was centrifuged at 6000 × *g* for 15 min to remove unbroken cells. The supernatant thus obtained was centrifuged at 187 000 × *g* for 1 h. The resulting precipitate was homogenized with 100 mM Tris-HCl buffer, pH 8.0, containing 0.3 M KCl, 1 mM disodium EDTA, and 10 μM PMSF, and then centrifuged at 187 000 × *g* for 1 h to remove contaminating soluble proteins. The resulting pellet was suspended in 10 mM Tris-HCl buffer, pH 8.0, containing 0.1 mM disodium EDTA and 10 μM PMSF, and used as the membrane fraction.

Respiratory activities

Respiratory oxygen consumption was assayed polarographically using a Clark-type oxygen electrode (model MP-1000, Iijima Products, Tokyo, Japan). The experiments were performed at 25°C in 25 mM Tris-HCl buffer, pH 8.0. The reaction was started by addition of the respiratory substrates, either 1 mM NADH or 5 mM ascorbate *plus* 0.1 mM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), in a total volume of 2 ml. The oxygen concentration in an air-saturated solution was measured as 259 μM (Winkler 1914). Cytochrome *c* oxidizing activities of the cytoplasmic membranes or the purified components were measured by following the decrease in absorbance at the α-peak (550 nm) of ferrocytochrome *c* with a spectrophotometer (model MPS-2000, Shimadzu, Kyoto, Japan). The reaction mixture contained 25 mM sodium phosphate buffer, pH 6.5, and 6 μM horse or *Saccharomyces cerevisiae* ferrocytochrome *c* as substrate, in a total volume of 1 ml. TMPD-oxidizing activity was also measured spectrophotometrically by following the increase in absorbance at 606 nm (millimolar extinction coefficient = 11.6). The reaction mixture contained 25 mM Tris-HCl buffer, pH 8.0, and 1 mM TMPD in a total volume of 1 ml.

Physical and chemical measurements

Absorption spectra were recorded with a spectrophotometer (Shimadzu model MPS-2000) using cuvettes of 1-cm light path. Extraction of heme *a* was performed according to the method of Drabkin (1942). The content of hemes *a* and *c* in the purified enzyme was determined on the basis of the millimolar extinction coefficient at the α-peaks of the pyridine ferrohemochromes: 26 $mM^{-1}cm^{-1}$ (Morrison et al. 1960) and 29.1 $mM^{-1}cm^{-1}$ (Drabkin 1942) for hemochromes *a* and *c*, respectively. The concentration of menaquinone was determined on the basis of a millimolar extinction coefficient of 18.9 $mM^{-1}cm^{-1}$ at 248 nm for the oxidized form (Dunphy and Brodie 1971). The purity of the enzyme was confirmed with native polyacrylamide gel electropharesis (PAGE) in the presence of Triton X-100 by the method of Davis (1964). The subunit composition of the enzyme complex was determined by PAGE in the presence of sodium dodecyl sulfate (SDS) according the method of Kadenbach et al. (1983). Heme *c* in the gel was stained with heme-staining regents (Connelly et al. 1958), while oxidase activity in the gel was detected with Nadi-reagents (Keilin 1927). Protein concentration was determined by the bicinchoninic acid (BCA) protein assay protocol (Pierce, Rockford, IL, USA). Electron ionization mass spectrometry was performed using a JEOL JMS-AX505HA spectrometer (JEOL, Tokyo, Japan).

Reagents

Menaquinones were extracted from *Bacillus* YN-1 membranes by hexane, and partially purified by the method of Wan et al. (1975). Horse cytochrome *c* (type VI), *Saccharomyces cerevisiae* cytochrome *c*, bovine liver catalase, 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), and antimycin A were purchased from Sigma (St. Louis, MO, USA), and deoxyribonuclease from Takara (Kyoto, Japan). Ferrocytochrome *c* was prepared by addition of small amounts of $Na_2S_2O_4$. NADH, TMPD, sodium ascorbate, PMSF, $(NH_3)_2SO_4$, and disodium EDTA were purchased from Wako (Osaka, Japan) and potassium cyanide from Koso (Tokyo). Diethylaminoethyl (DEAE)-Toyopearl (Fractgel TSK DEAE-650M) and Sephacryl S-300 were purchased from Tosoh (Tokyo) and Pharmacia (Uppsala, Sweden), respectively. The Cosmosil $5C_{18}$ packed column was purchased from Nacalai Tesque (Kyoto, Japan), while the poly-hydroxyethyl aspartamide column for HPLC was a product of Poly LC (Columbia, SC, USA). All chemicals were of the highest grade commercially available.

Results

Effects of respiratory inhibitors on respiration and growth

The effects of the inhibitors, cyanide, antimycin A, and HQNO, upon respiratory activity in *Bacillus* YN-1 are summarized in Table 1. The cytochrome *c* oxidizing activity of the membrane was completely inhibited by a very low concentration of cyanide. On the other hand, the oxygen-consuming activity with an artificial electron donor system of ascorbate *plus* TMPD was not affected, even up to 0.5 mM cyanide. When NADH was used as a respiratory substrate, the oxygen-consuming activities were totally inhibited by antimycin A and HQNO, which are potent inhibitors for quinol-cytochrome *c* reductase (complex III). These results suggested that there were at least two respiratory terminal components, one of which was found to be a cyanide-sensitive cytochrome *c* oxidase, which may play only a very small role in the respiratory process in *Bacillus* YN-1. The other terminal was found to be cyanide-insensitive and probably plays a major role in the respiratory electron-transfer system of this bacterium. These results also suggested that two parallel pathways diverged at the point of complex III in the respiratory system of this bacterium. Furthermore, respiratory redox components that contained *a*-, *b*-, and *c*-type cytochromes were observed in the membrane as shown in the difference spectrum, $Na_2S_2O_4$-reduced *minus* ferricyanide-oxidized, while no absorption peaks at around 630 nm were observed in the difference spectrum, indicating absence of *d*-type cytochromes.

The growth of the bacterium was highly tolerant to cyanide. Figure 1A shows the growth curves of *Bacillus* YN-1 in the presence of several concentrations of cyanide. No effect was observed on bacterial growth in the presence of even up to 1 mM cyanide. In contrast, the growth of the facultative alkaliphilic *Bacillus* YN-2000 was decreased up to 63% by 100 μM cyanide in alkaline conditions at pH 10, while the same concentration of cyanide completely inhibited the growth in neutral conditions, as shown in Fig. 1b,c. These results indicated the biological significance of cyanide-insensitive respiration in the alkaliphilic *Bacillus* YN-1 and in *Bacillus* YN-2000 cultivated under alkaline conditions.

Fractionation of respiratory terminals of *Bacillus* YN-1 and YN-2000

Respiratory terminal components of *Bacillus* YN-1 were solubilized from the cytoplasmic membrane by detergent, then fractionated by anion-exchange chromatography. The bacterial membrane, prepared from 100 g (wet weight) of cells, was suspended in 420 ml of 10 mM Tris-HCl buffer, pH 8.0, containing 0.1 mM disodium EDTA and 10 μM PMSF (buffer A), and Triton X-100 up to 1.5% in total concentration was added. The suspension was stirred gently for 2 h and then centrifuged at 187 000 × *g* for 1 h. In this solubilization step, 96% of the total TMPD-dependent oxygen consuming activity was recovered in the supernatant. The reddish supernatant obtained was applied to chromatography on a DEAE-Toyopearl column (4.0 × 24.5 cm) that had been equilibrated with buffer A containing 1% Triton X-100 (buffer B). After the column was washed with buffer B containing 0.1 M NaCl, the adsorbed components were eluted with a linear gradient produced from 500 ml each of buffer B containing 0.1 M NaCl and buffer B containing 0.5 M NaCl.

Figure 2A shows the elution profile of the anion-exchange chromatography column. Three fractions with TMPD-oxidizing activity were obtained. The catalytic activities in the first and the second fractions were cyanide-insensitive, while that of the third fraction was highly sensitive to cyanide. In the second fraction, 55% of the total activity in the bacterial membrane was collected, while 14% and 21% were collected in the first and the third fractions, respectively. Thus, there was only a small loss of catalytic activity during the chromatography. Cytochrome *c* oxidiz-

Table 1. Effects of inhibitors on the respiratory activities of the membrane from *Bacillus* YN-1

Activity	Electron donor	Inhibitor concentration	Residual activity		
			Cyanide	Inhibitors Antimycin A	HQNO
Cytochrome *c* oxidase activity	Cytochrome *c*	0 μM	100%[a]	100%	100%
		50 μM	11%	100%	100%
		500 μM	5.5%	n.d.	100%
Oxygen uptake	Ascorbate-TMPD	50 μM	100%[b]	100%	100%
		500 μM	100%	100%	100%
	NADH	50 μM	100%[c]	76%	42%
		500 μM	100%	20%	26%

n.d., not determined; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide.
Cytochrome *c* oxidase activity was measured spectrophotometrically at room temperature by following the decrease in the absorbance at 550 nm.
[a] 1.46 nmol cytochrome *c*/mg protein per min.
[b] 26.5 nmol O_2/mg protein per min.
[c] 12.3 nmol O_2/mg protein per min.

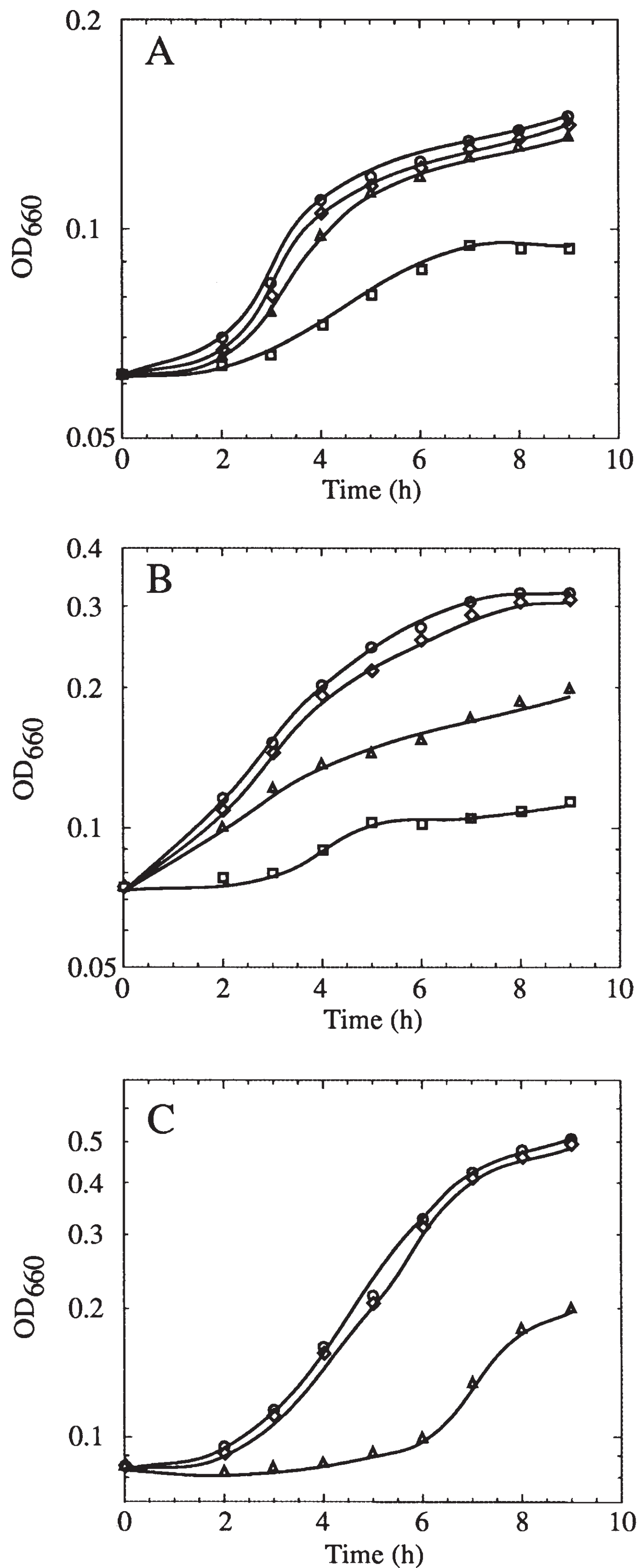


Fig. 1. Effects of cyanide on the growth of *Bacillus* YN-1 and *Bacillus* YN-2000. **A** *Bacillus* YN-1, **B** *Bacillus* YN-2000 cultivated at pH 10.0, **C** *Bacillus* YN-2000 cultivated at pH 8.0. *Circles*, 0 μM KCN; *diamonds*, in the presence of 10 μM KCN; *triangles*, in the presence of 100 μM KCN; *squares*, in the presence of 1 mM KCN. Growth was monitored by measuring optical density of the medium at 660 nm

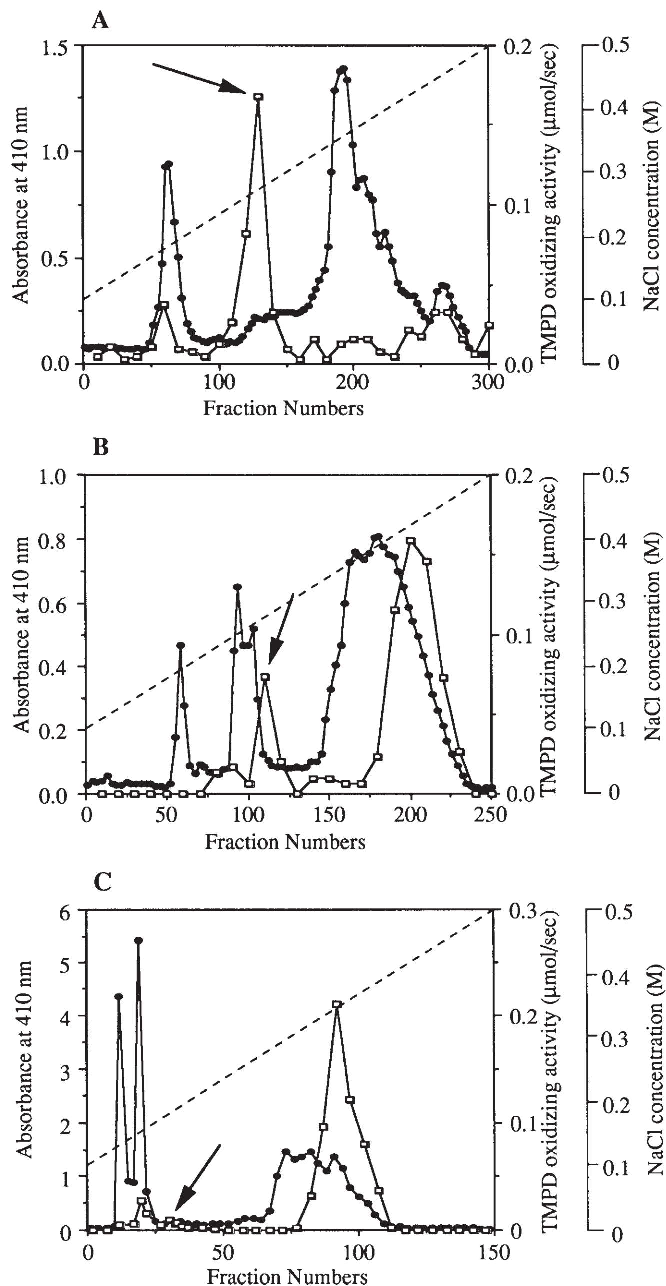


Fig. 2. Fractionation of respiratory terminal components by anion-exchange chromatography. **A** *Bacillus* YN-1, **B** *Bacillus* YN-2000 grown at pH 10.0, **C** *Bacillus* YN-2000 grown at pH 8.0. *Circles*, absorbance at 410 nm; *squares*, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) oxidizing activity; *dashed line*, NaCl concentration. The *arrows* indicate the cyanide-insensitive activity

ing activity was collected in only a single peak, corresponding well to the third TMPD-oxidizing fraction. The third fraction showed a visible spectrum which was typical of *a*-type cytochrome oxidase, whereas the second fraction, which exhibited TMPD-oxidizing activity accounting for more than half of the total activity, had no cytochrome *c* oxidizing activity. This fraction had a pale yellow color, and no hemoproteins could be detected from its visible spectrum. Surprisingly, the activity in this fraction was not af-

fected even by treatment with boiling water or with SDS, suggesting that this activity might be catalyzed by a stable, nonproteinaceous material. The activity in the first fraction was considered to be caused by a protoheme molecule that was released from the denatured *b*-type cytochromes.

Fractionation of the respiratory terminal components of *Bacillus* YN-2000, cultivated at pH 8 or 10, by chromatography was also performed. In the case of cells cultured at pH 10, two TMPD-oxidizing fractions appeared on the chromatography profile, as shown in Fig. 2B. The fraction eluted from the column at around 0.4 M salt concentration was cyanide-sensitive, and was identical to the *aco*-type cytochrome *c* oxidase reported previously (Qureshi et al. 1990). Additionally, a cyanide-insensitive and heat-stable TMPD-oxidizing fraction also appeared in the profile, as indicated by the arrow (Fig. 2b). The cyanide-insensitive activity occupied only a small part of the total activity in *Bacillus* YN-2000. Figure 2C shows the chromatography profile of the respiratory components extracted from *Bacillus* YN-2000 cultivated at pH 8. There was a low concentration of the cyanide-insensitive component in the profile.

From the results of inhibitory and chromatographic experiments, the cyanide-insensitive component was considered to correspond to a respiratory terminal component in the cyanide-insensitive pathway of the bacteria, suggesting the biological significance of cyanide-insensitive respiration for bacterial growth under alkaline conditions.

Purification and characterization of cyanide-sensitive respiratory terminal oxidase

The third fraction obtained from the DEAE-Toyopearl column (4.0 × 24.5 cm) chromatography was dialyzed against 2 l of buffer B for 12 h, and the resulting solution was fractionated with $(NH_4)_2SO_4$ from 30% to 70% saturation. The precipitated enzyme was dissolved in buffer B and subjected to gel filtration with a Sephacryl S-300 column (2.5 × 148 cm) that had been equilibrated with buffer B containing 0.25 M NaCl. The active fraction was collected, concentrated with a small-size DEAE-Toyopearl column, and used as the purified cyanide-sensitive respiratory terminal oxidase of the bacterium. The representative purification steps are summarized in Table 2.

As shown in Fig. 3A, the enzyme was purified to an electrophoretically homogeneous state. The subunit structure was also analyzed by SDS-PAGE (Fig. 3B). The enzyme consisted of three kinds of subunits, of molecular masses 47.5 kDa, 43 kDa, and 21 kDa, respectively. The 43-kDa subunit was stained with heme-staining reagents, suggesting that heme *c* was covalently bound to the 43-kDa subunit.

Figure 4 shows the absorption spectra of the purified cyanide-sensitive respiratory terminal oxidase. In the oxidized form, the oxidase showed a sharp absorption peak at 410 nm and small peaks at 527 and 595 nm, while in the reduced form it showed peaks at 416, 441, 522, 550, and 597 nm. These spectral properties suggest the presence of heme *c* and heme *a* molecules in the enzyme. Furthermore, the ratio of heme *a* to heme *c* in the enzyme was estimated at about 2 : 1 on the basis of the pyridine ferrohemochrome spectrum. On the other hand, the difference spectrum, CO-complex *minus* reduced, of the enzyme showed absorption peaks at 587, 430, and 413 nm and troughs at 550, 442, and

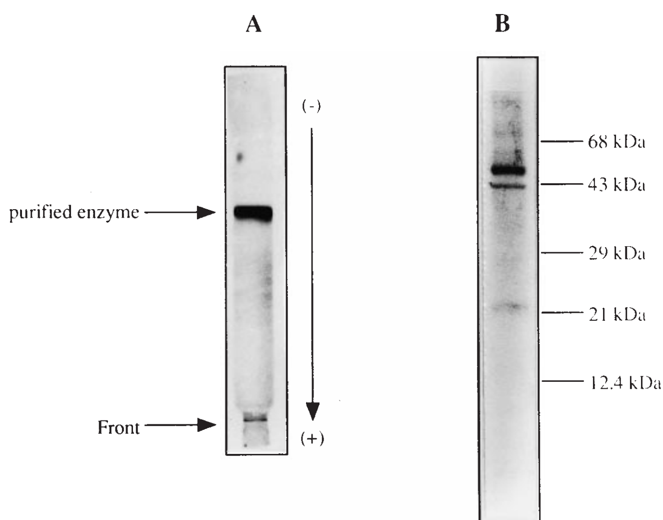


Fig. 3. Polyacrylamide gel electrophoresis of *caa*$_3$-type cytochrome *c* oxidase purified from alkaliphilic *Bacillus* YN-1. **A** The purified enzyme (30 μg) was electrophoresed on a 7% polyacrylamide gel in the absence of sodium dodecyl sulfate (SDS). **B** The purified enzyme (6 μg) was electrophoresed on a 14% polyacrylamide gel in the presence of SDS. The gels were stained with Coomassie Brilliant Blue

Table 2. Purification of cyanide-sensitive respiratory terminal oxidase from *Bacillus* YN-1

Purification step	Total protein (mg)	Total heme *a* (nmol)	Heme *a*/protein (nmol/mg)
Membrane fraction	121	33.9 (100%)	0.279
Solubilized fraction	102	48.4	0.474
DEAE ion-exchange chromatography	8.28	26.6	3.21
$(NH_4)_2SO_4$ fractionation	5.44	17.2	3.16
Sephacryl S-300 gel filtration	0.774	5.46 (16%)	7.05

DEAE, diethylaminoethyl.

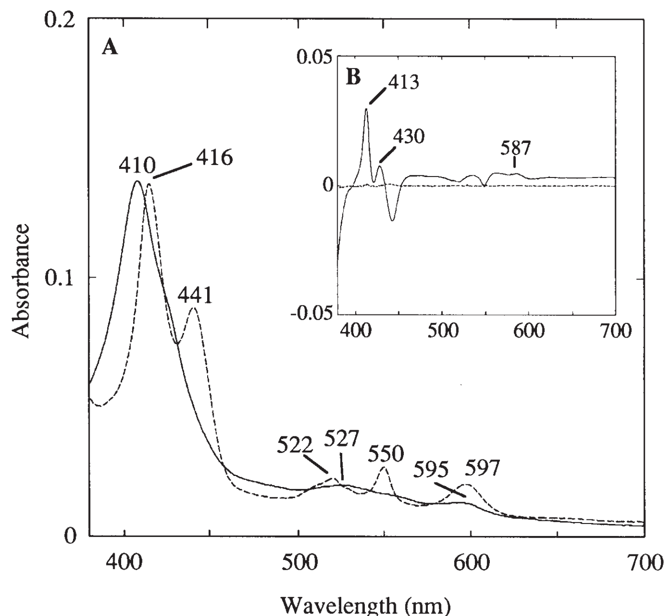


Fig. 4. Absorption spectra of *Bacillus* YN-1 cytochrome *caa*$_3$. The enzyme was dissolved in 10 mM Tris-HCl buffer, pH 8.0, containing 1% Triton X-100. **A** *Solid curve*, enzyme (air-oxidized); *dashed curve*, dithionite-reduced. **B** *Solid curve*, difference spectrum: (reduced + CO) *minus* reduced; *dashed curve*, base line

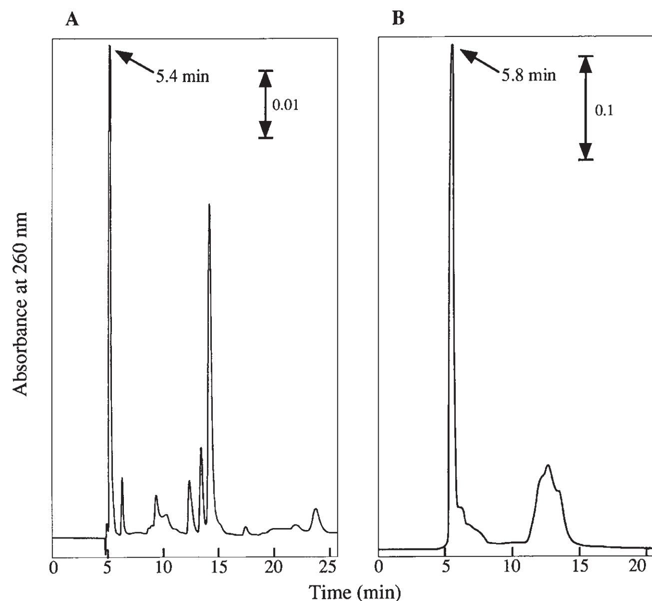


Fig. 5. Purification of cyanide-insensitive respiratory terminal component by HPLC. **A** The elution profile from reverse-phase HPLC. The elution was monitored by the absorbance at 260 nm, the flow rate was 0.5 ml/min. The activity was eluted at 5.4 min. **B** The elution profile from gel filtration HPLC. The activity was eluted at 5.8 min

421 nm. We therefore conclude that the cyanide-sensitive terminal of the alkaliphilic *Bacillus* YN-1 is a *caa*$_3$-type cytochrome *c* oxidase, which has already been reported for both neutralophilic bacteria (Hon-nami and Oshima 1984; Sone and Yanagita 1982) and the alkaliphile *Bacillus firmus* OF4 (Quirk et al. 1993).

The cytochrome *c* oxidase activity was measured spectrophotometrically with horse heart cytochrome *c* or *S. cerevisiae* cytochrome *c* as electron donor. The optimum pH of the enzymatic activity was found to be 6.5. The K_m value for horse heart cytochrome *c* was 5.32 μM, while the value for *S. cerevisiae* cytochrome *c* was 8.42 μM. The V_{max} values for horse heart and *S. cerevisiae* cytochromes *c* were 0.484 and 3.61 s^{-1}, respectively. Potassium cyanide inhibited the enzymatic activity, and its K_i was 3.3 μM. It should be noted that the cytochrome *c* oxidizing activity of the bacterial membrane was also strongly inhibited by cyanide.

Purification and characterization of cyanide-insensitive respiratory terminal component

The catalytic activity of the cyanide-insensitive respiratory terminal component was not inhibited by heat-treatment (100°C for 5 min) in the presence of 1% SDS, suggesting that the component was a stable, nonproteinaceous material. After the anion-exchange chromatography, the component was further purified using a reverse-phase HPLC column that had been equilibrated with H_2O containing 1% HCl, as shown in Fig. 5A. The active fraction was not adsorbed on the column, suggesting that the material was hydrophilic. The active fraction was collected, concentrated by evaporation, and subjected to a second HPLC, on a polyhydroxyethyl aspartamide gel filtration column (Fig. 5B). The active fraction thus obtained was lyophilized and used as a purified sample. The average content of this component in *Bacillus* YN-1 cells was estimated to be 2.0 mg per g of wet cells by three individual experiments.

Molecular and catalytic properties of the cyanide-insensitive respiratory component and menaquinone

Figure 6A shows the effects of cyanide and catalase on the oxygen-consuming reaction catalyzed by the purified cyanide-insensitive respiratory terminal component. When using ascorbate plus TMPD as an electron-donating system, the oxygen consumption rate was not affected by 5 mM cyanide. Addition of catalase into the reaction mixture, however, reduced the oxygen consumption by one-half, as shown in Fig. 6A. Figure 6B shows the oxygen consumption of the membrane fractions in the presence of 1 mM NADH and 5 mM cyanide. This activity was also halved by the addition of catalase. These phenomena suggested the likelihood that hydrogen peroxide was produced by the catalytic reaction of cyanide-insensitive respiratory terminal component.

Bacillus species are known to contain menaquinones as respiratory electron carriers that mediate electron-transfer from NADH-quinone reductase (complex I) to quinol-cytochrome *c* reductase (complex III). These quinones also have an oxygen-reducing activity that produces hydrogen

peroxide (Molina Portela et al. 1996). In this study, however, cyanide-insensitive respiratory terminal component showed molecular and catalytic characteristics distinct from those of menaquinones. The TMPD-oxidizing activity of the purified nonproteinaceous material was calculated to be 0.182 mol $O_2 \cdot mol^{-1} \cdot min^{-1}$, while that of menaquinone isolated from the same bacterial cells was 0.0435 mol $O_2 \cdot mol^{-1} \cdot min^{-1}$, as shown in Table 3. Although the nonproteinaceous material was extracted from the bacterial membrane by detergent, the purified sample would not dissolve into organic solvents such as methanol, ethylacetate, or xylene. This hydrophilic character was completely different from the highly hydrophobicnature of menaquinone and its biosynthetic precursors. The absorption spectrum in the near-ultraviolet region of the purified nonproteinaceous material is shown in Fig. 7A. The spectrum revealed a symmetrical absorption curve with a peak at 261.5 nm in the oxidized form, and a peak at 258.5 nm in the reduced form prepared by addition of 2.5 mg/ml of sodium borohydride. The millimolar extinction coefficient of the nonproteinaceous material was estimated as 0.88. The absorption spectrum was considerably different from that of menaquinone purified from the bacterium, as shown in Fig. 7B. Finally, the molecular mass of the nonproteinaceous material was analyzed by electron ionization mass spectrometry. Mass signals of the fragments were observed mainly at the positions corresponding to *m/z* values of 662, 647, and 316. No signal was observed in the large mass/charge (*m/z*) region from 800 to 1000. Therefore, the molecular mass of the nonproteinaceous material was determined to be 662.0. The signal observed at the *m/z* of 647 might derive from a degraded fragment resulting from the removal of a methyl group from the component. No menaquinones of molecular weight 662, or their chemical derivatives, have yet been reported.

Discussion

Aerobic organisms utilize oxygen as a final electron acceptor to drive respiratory electron transport systems. Heme-copper type cytochrome oxidase, which is characterized by the presence of a heme-copper binuclear cluster in its reaction center, functions as a terminal enzyme of respiration in most eukaryotes and aerobic bacteria. The enzyme catalyzes 4-electron reduction of oxygen to produce water molecules through the coordination of complicated redox reactions of hemes and copper atoms in the enzyme molecule. Although several types of heme-copper cytochrome oxidases have been found in bacteria, the structural diversity of these enzymes is relatively small, and can be consid-

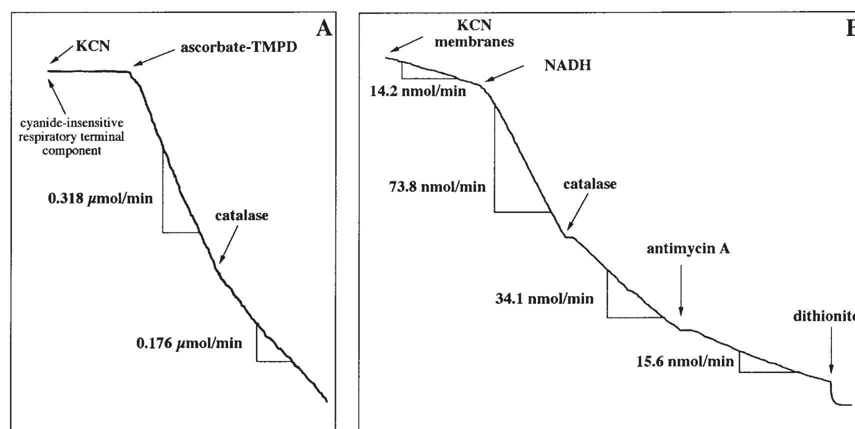


Fig. 6. Effect of catalase on the oxygen-consuming activity. **A** Oxygen-consuming activity of the purified cyanide-insensitive respirtory terminal component. Before starting the reaction, 1.1 mg of the purified component was incubated with 5 mM potassium cyanide in a reaction mixture containing 25 mM Tris-HCl buffer, pH 8.0. The reaction was started by mixing 5 mM ascorbate and 0.1 mM TMPD; finally, 0.2 mg/ml of catalase was added into the solution. **B** Oxygen-consuming activity of the membranes prepared from *Bacillus* YN-1. The membrane fraction containing 4.31 mg protein per ml was incubated with 5 mM potassium cyanide for 15 min at 25°C in 25 mM Tris-HCl buffer, pH 8.0. The reaction was started by addition of 1 mM NADH, then the effects of catalase (0.2 mg/ml) and antimycin A (0.2 mM) were observed

Table 3. Comparison of characteristics of the cyanide-insensitive respiratory terminal component and menaquinone of *Bacillus* YN-1

	Cyanide-insensitive respiratory terminal component	Menaquinone
Solubility in water	Soluble	Insoluble
O_2 consumption ($mol\ O_2 \cdot mol^{-1} \cdot min^{-1}$)	0.182	0.0453
Spectral properties	Oxidized 261.5 nm Reduced 258.5 nm	Oxidized 269, 261, 248 nm Reduced 272 nm
Molecular weight	662	MK-6: 580.9 MK-7: 649.0 MK-8: 717.1

MK, menaquinone.

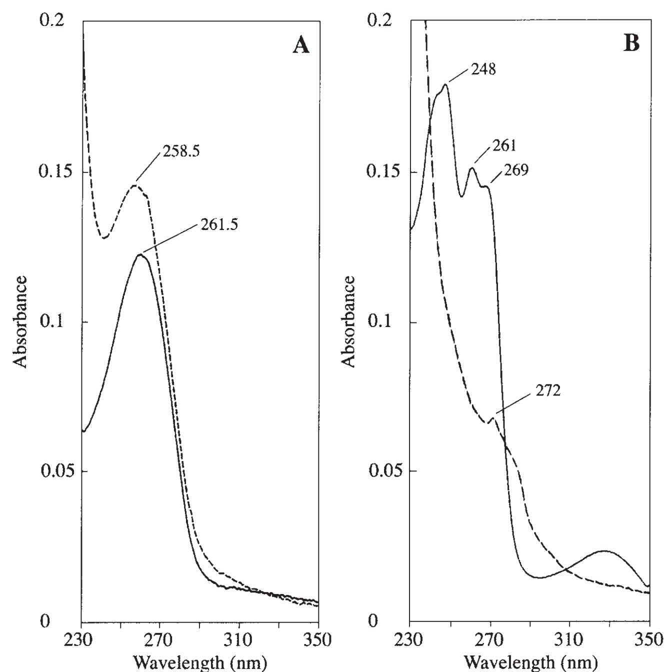


Fig. 7. Absorption spectra of cyanide-insensitive respiratory terminal component **A** and menaquinone **B** purified from *Bacillus* YN-1. **A** Absorption spectra of the purified sample (139 μM) dissolved in water. **B** Menaquinone (8.33 μM) dissolved in ethanol. *Solid lines*, oxidized form; *dashed lines*, reduced by the addition of sodium borohydride

ered the result mainly of the replacement of the heme moiety by another heme species.

In addition to the heme-copper oxidase group, two other classes of respiratory terminal oxidases, cytochrome *bd*-type oxidase and cyanide-insensitive oxidase, are also known. Cytochrome *bd*-type oxidase has been reported from several bacteria, such as *Escherichia coli* (Miller and Gennis 1983), *Azotobacter vinelandii* (Kolonay et al. 1994), *Bacillus stearothermophilus* (Sakamoto et al. 1996), and the alkaliphile *B. firmus* OF4 (Gilmour and Krulwich 1997). Cytochrome *bd* contains only heme *b* and heme *d* as prosthetic cofactors, but no copper atoms in the enzyme molecule. No phylogenetic relationship has been observed between the primary sequences of cytochromes *bd* and heme-copper oxidases despite their similar enzymatic behavior (Green et al. 1988). It has been reported that the activity of cytochrome *bd* is inhibited by antimycin A (Jünemann and Wrigglesworth 1994). In plants and some yeasts, cyanide-insensitive respiration has been reported (Elthon and McIntosh 1987; McIntosh 1994; Moore and Siedow 1991; Moore et al. 1995; Guérin and Camougrand 1986; Minagawa et al. 1990). Also, a novel type of terminal oxidase in cyanide-insensitive respiration has been identified in plant mitochondria and the yeast *Hansenula anomala*, and has been characterized and sequenced (McIntosh 1994; Moore and Siedow 1991; Moore et al. 1995; Sakajo et al. 1991). The enzyme consists of a single 36-kDa protein having nonheme iron as a cofactor, and its activity is specifically inhibited by salicylhydroxamic acid (Yoshimoto et al. 1989). In spite of the structural variety among the three categories of respiratory terminal oxidases, all of these enzymes catalyze 4-electron reduction of oxygen to produce water molecules.

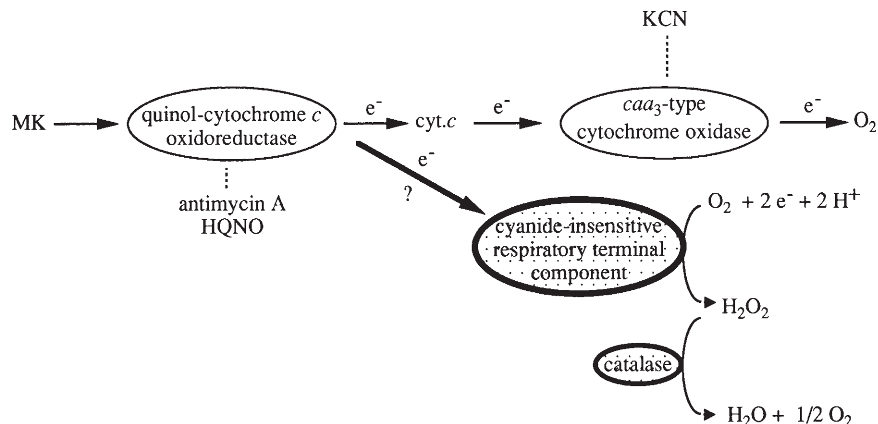
In this study, a branching respiratory system including two terminal components was found in an obligate alkaliphile, *Bacillus* YN-1. One of the two respiratory terminal components was identified as a usual *caa*$_3$-type heme-copper oxidase. The other respiratory terminal component was cyanide-insensitive and was found to be unique because of its nonproteinaceous nature and high oxygen-reducing activity. Cyanide-insensitive respiration accounted for a major part of the activity in *Bacillus* YN-1. This was also consistent with the cyanide-tolerant growth of the bacterium.

The effect of catalase, which catalyzes the decomposition of 2 mol of H_2O_2 to produce 1 mol each of O_2 and H_2O, on the reaction of the nonproteinaceous material clearly showed that the product of the oxygen reduction was not H_2O but H_2O_2. The isolated bacterial membranes also generated H_2O_2 with oxygen consumption coupled with oxidation of physiological respiratory substrates, such as NADH and succinate. The cytotoxicity of active oxygen species such as H_2O_2 has been well established. Most aerobic organisms contain superoxide dismutase and catalase for diminishing the levels of O_2^- and H_2O_2 that are unavoidably generated by various biological processes within the living cells. As reported for the alkaliphilic *Bacillus* YN-2000 (Yumoto et al. 1990), the catalase content in *Bacillus* YN-1 is also very high. Therefore, H_2O_2 generated during the respiratory process would be promptly converted into oxygen by catalase. It should be noted that the H_2O_2 generated by photorespiration in the peroxisomes of plant and cyanobacterial cells is also degraded immediately by catalase (Chollet 1977).

Quinones, due to their self-oxidative character, are known to consume some oxygen in the presence of a proper reductant. However, as summarized in Table 4, the molecular and catalytic properties of menaquinones extracted from *Bacillus* YN-1 were different from those of the nonproteinaceous material. The oxygen-reducing activity of the nonproteinaceous material was about 4-times stronger than that of menaquinone. Furthermore, the two also showed differences in their hydrophobicity, absorption spectrum, and molecular weight. We therefore conclude that the nonproteinaceous material purified in the present study was neither a menaquinone nor a menaquinone derivative.

Based on the present results, we propose the novel respiratory oxygen-reducing mechanism shown in Fig. 8. The cyanide-insensitive pathway diverges at complex III and terminates with two elementary reactions. First, the nonproteinaceous component catalyzes the 2-electron reduction of oxygen to produce H_2O_2. Secondly, the toxic product is diminished by catalase. This combination of two reactions would generate water molecules as a final product of respiration in this system. It is still not clear whether complex III donates electrons to cyanide-insensitive respi-

Fig. 8. Proposed model of the respiratory chain in alkaliphilic *Bacillus* YN-1. *MK*, menaquinone' *HQNO*, 2-heptyl-4-hydroxyquinoline-*N*-oxide



ratory terminal component directly or indirectly. Because cyanide-insensitive respiration is dominant in *Bacillus* YN-1, a high tolerance against cyanide is expected during bacterial growth. Indeed, 100 μM potassium cyanide exerted no influence on the growth of the obligate alkaliphilic *Bacillus* YN-1, whereas it markedly affected growth of the facultative alkaliphilic *Bacillus* YN-2000.

It is very important to elucidate the biological significance of this novel type of respiratory system in terms of the alkaliphilicity of these bacteria. Gilmour and Krulwich have reported that no component oxidizing TMPD exists in the membrane fraction of a facultative alkaliphile, *Bacillus firmus* OF4, with the exception of caa_3-type cytochrome oxidase (Gilmour and Krulwich 1997). In the present study, the new nonproteinaceous material with oxygen-reducing activity was found to exist in alkaline-cultured *Bacillus* YN-2000. The nonproteinaceous material with oxygen-reducing activity has also been found in *Bacillus alcalophilus* (data not shown). These results seem very significant, because they suggest that this nonproteinaceous material is not specific to *Bacillus* YN-1, but widely distributed among the alkaliphilic bacteria. The structure of the nonproteinaceous material and the biological significance of this novel respiratory mechanism in alkaliphilic organisms will require further investigation.

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