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著者(和文)	東端晃
Author(English)	Akira Higashibata
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Studies on the Respiratory Terminals of Alkaliphilic *Bacillus* YN-1

A DISSERTATION

submitted in partial satisfaction of

the requirements of the degree

DOCTOR OF PHILOSOPHY (Ph. D.)

by

Akira Higashibata

DEPARTMENT OF LIFE SCIENCE

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Contents

Abbreviations

DEAE-	diethylaminoethyl-
DMSO	dimethyl sulfoxide
DSS	sodium 4,4-dimethyl-4-silapentanesulfonate
EDTA	ethylenediaminetetraacetate
EI-	electron ionization-
FAB-	fast atomic bombardment-
FCCP	carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone
HPLC	high-performance liquid chromatography
HOQNO	2-heptyl-4-hydroxyquinoline-*N*-oxide
MK	menaquinone
NADH	nicotinamide adenine dinucleotide, reduced form
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
SHAM	salicylhydroxamic acid
SDS	sodium dodecyl sulfate
TLC	thin-layer chromatography
TMPD	*N,N,N',N'*,-tetramethyl-*p*-phenylenediamine
Tris	tris (hydroxymethyl) aminomethane

Section I

General Introduction

Literature Review

It has been reported that several kinds of microorganisms have ability to live under extreme environments such as high temperature, high pressure, high salt concentration and high acidic or alkaline pH conditions. Such organisms are classified as "extremophiles". Microorganisms that can grow in an alkaline pH condition are designated "alkaliphiles". Many researchers have reported that the extremophile has special abilities which are not present in neutralophiles; some of them produce alkaline-tolerant extracellular enzymes, such as cellulases and proteinases [1]. Therefore, it seems likely that alkaliphilic microorganisms have a number of potential applications.

Alkaliphilic bacteria have been isolated from indigo dye balls [2, 3], potato-processing plant-effluents [4], alkaline lakes [5-7], and enrichment culturing soil [8], and mostly belong to *Bacillus* species. Generally, alkaliphiles are classified into two categories: the alkaline-tolerant bacteria which show their optimal growth in the pH region from 7 to 9 but cannot grow at pH above 9.5, and the alkaliphilic bacteria which show their optimal growth in the pH region between 10 and 12. The alkaliphilic organisms can be further divided into facultative alkaliphiles and obligate alkaliphiles. The former bacteria show good growth not only at pH 10 but also in the neutral pH range. On the other hand, the latter bacteria show optimal pH of the growth at above 10 but cannot grow below 8.5-9.0 of pH. *Bacillus* YN-1 used in this study is an obligate alkaliphilic Gram-positive bacteria that can grow optimally at about pH 10.

Despite the bioindustrial advancements of alkaliphiles, the bioenergetic mechanism, *i.e.*, the ATP biosynthesis coupled with the respiration under an alkaline pH has not yet been clarified. Mitchell has presented a chemiosmotic model to explain the bioenergetic work in the living cells [9]. His model is now widely confirmed and almost universally accepted. As applied to oxidative phosphorylation by mitochondrial or bacterial respiratory chain, the chemiosmotic model posits that the extrusion of protons across the coupling membrane with respiration establishes an electrochemical proton gradient that is the obligatory energy intermediate between

respiration and ATP synthesis. The proton motive force ($\Delta\mu_{H^+}$) is composed of transmembrane electric potential ($\Delta\psi$) and pH gradient across the cytoplasmic membrane (ΔpH), and bacteria utilize the proton motive force as the energy source for their cellular functions such as active transport and flagellar motor. On the other hand, the cytoplasmic pH of an obligate alkaliphile is usually about 8.5 which is maintained by the effect of Na^+/H^+ antiporters that catalyze the electrogenic exchange of extracellular H^+ for intracellular Na^+ [10, 11]. Therefore, the cytoplasmic pH is lower than extracellular pH in an obligate alkaliphile. However, ATP that is necessary for life in the alkaliphile is also synthesized by proton motive F_1F_o-ATPase [12]. These evidences indicate that the proton gradient produced by respiratory chain is essentially required for the growth of the alkaliphiles as the same as for neutralophiles (Figure 1). Krulwich *et al.* have suggested that the direct proton transfer for alkaliphilic oxidative phosphorylation might occur between a proton translocating respiratory complex and the ATP synthase in the cytoplasmic membrane of alkaliphile (Figure 2) [13].

Recently, several respiratory components were purified and characterized from the variety of alkaliphilic bacteria [14]. Quirk *et al.* [15] have succeeded in cloning and sequencing of the *cta* gene encoding the caa_3-type cytochrome oxidase from alkaliphilic *Bacillus firms* OF4 and Gilmour *et al.* [16] have purified and characterized cytochrome *bd* from *Bacillus firms* OF4. Xu *et al.* determined the *ndh* gene which encodes the NADH dehydrogenase of alkaliphilic *Bacillus* sp. strain YN-1 [17]. Also, Yumoto *et al.* [18, 19] and Qureshi *et al.* [20] have reported that two kinds of membrane bound cytochrome *c* and a novel *aco*-type cytochrome oxidase are present in a facultative alkaliphilic *Bacillus* YN-2000, respectively.

Despite so much works in this field, it has not been clarified that what respiratory components of alkaliphilic bacteria pump the protons across the cytoplasmic membrane and how the proton gradients established under high alkaline conditions. Therefore, it is important to characterize the respiratory chain of alkaliphilic bacteria.

In the present study, I have investigated the respiratory system of an obligate alkaliphilic

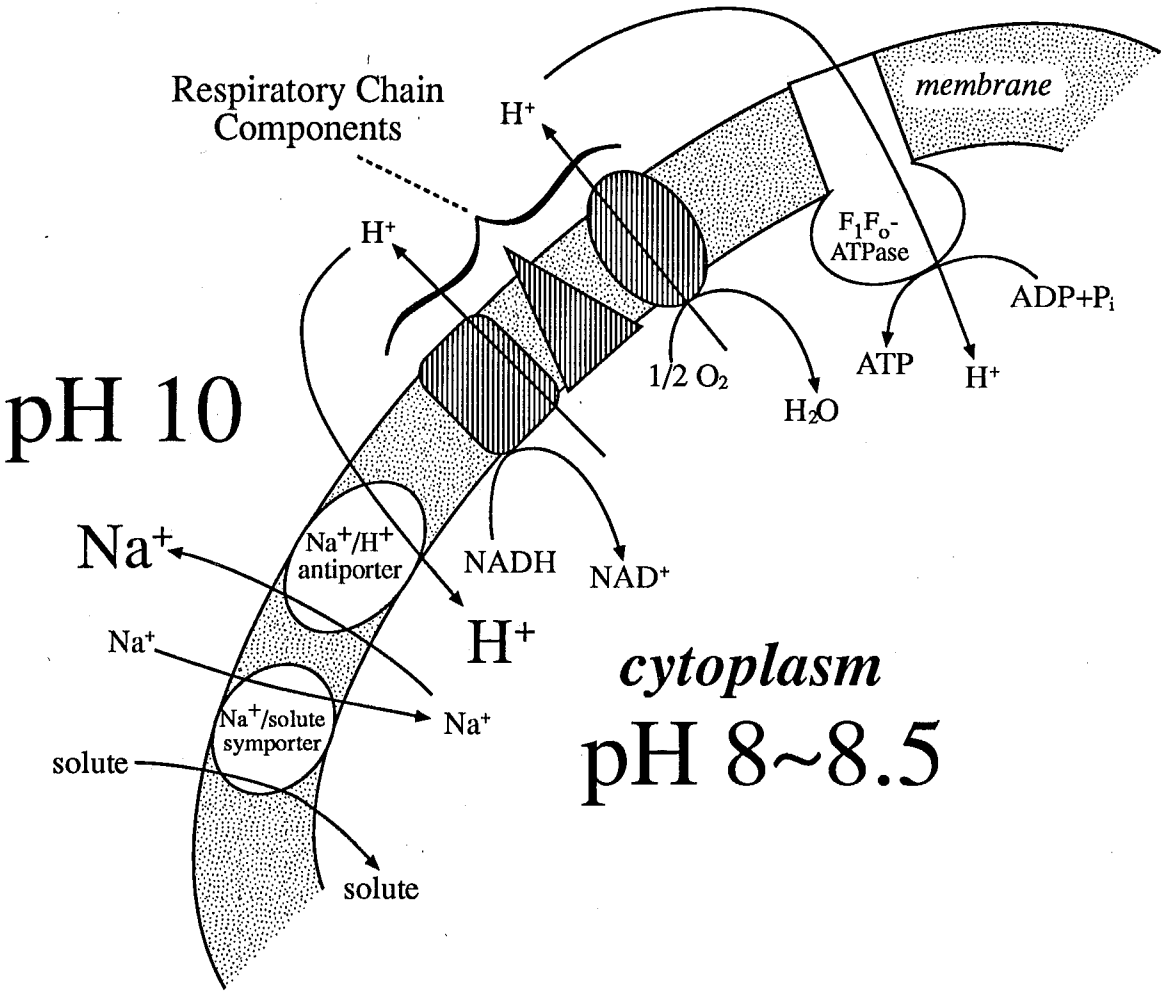

Figure 1 **A schematic representation of Na^+ and H^+ fluxes across the membrane of an extreme alkaliphile.** (Ref. [21])

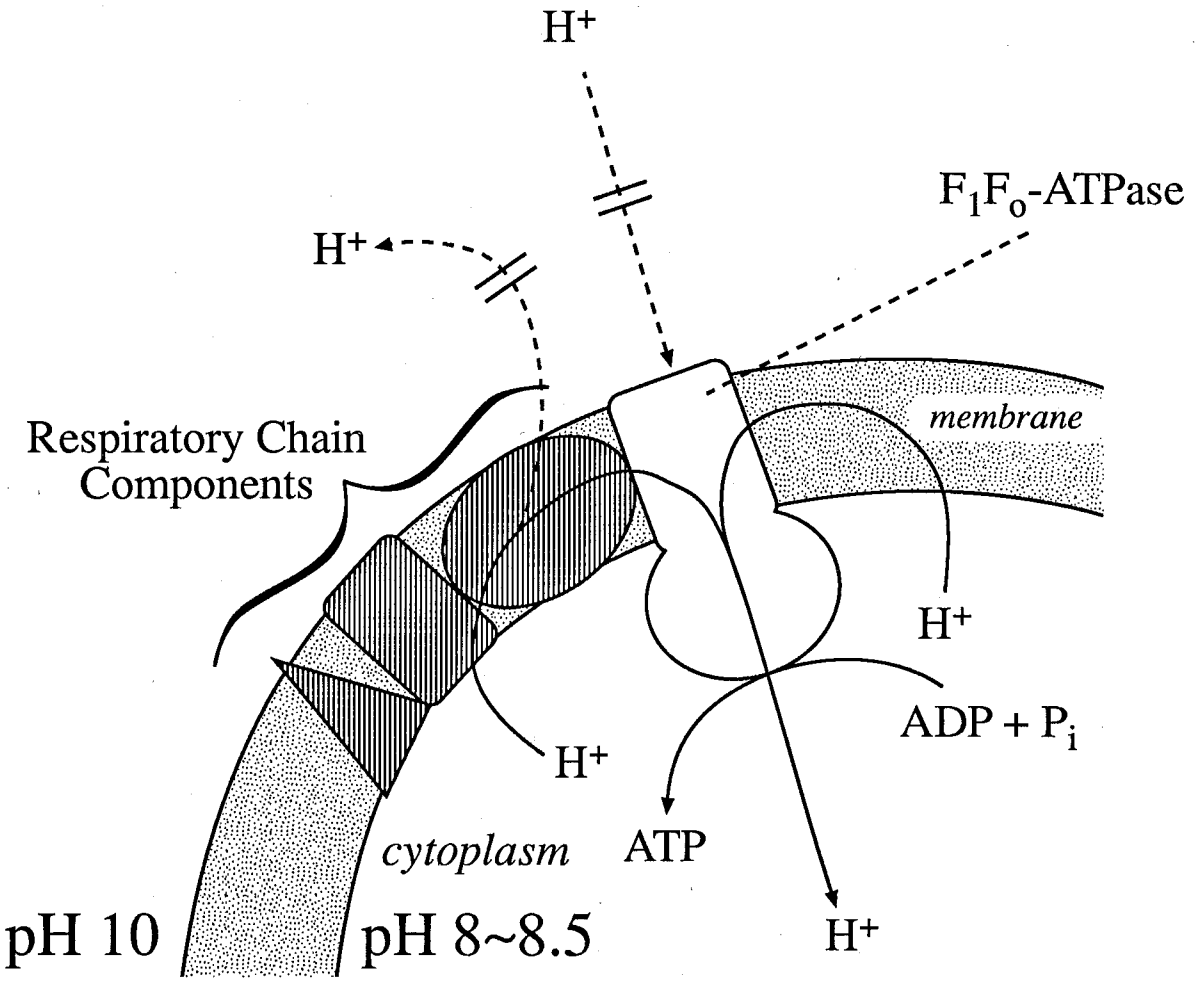

Figure 2 **Schematic representation of a hypothesis that accounts for the bioenergetic properties of extreme alkaliphiles.** (Ref. [12])

Bacillus YN-1 which grows optimally at pH 10 and found two respiratory terminals: a cyanide-sensitive component and a cyanide-insensitive component. Furthermore, I have purified two terminal components of the alkaliphile and studied their molecular features. Finally, I have proposed a new electron transfer system present in an obligate alkaliphilic *Bacillus* YN-1.

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Section II

Identification of the Respiratory Terminals of Alkaliphilic *Bacillus* YN-1

Introduction

An obligate alkaliphilic bacterium, *Bacillus* YN-1, grows optimally under high pH condition as pH 10. In the case of neutralophiles, the cytoplasmic pH of the cell is usually maintained to be higher than the extracellular pH, and then, ATP is synthesized by F_1F_o-ATPase derived by proton motive force. On the other hand, the cytoplasmic pH of alkaliphilic bacteria is lower than the external pH as described in Section I [1, 2]. However, ATP is also synthesized with F_1F_o-ATPase energized by proton motive force in alkaliphilic bacteria [3]. The F_1F_o-ATPase mainly utilizes the proton which is pumped out from cytoplasm by several components of respiratory chain. Almost all of enzymes involved in the respiratory chain contain flavins, hemes, and non-heme irons as cofactors that function as redox reaction center. In this section, I have investigated the effects of the respiratory inhibitors such as antimycin A, HOQNO, and cyanide upon the cytochrome *c* oxidase activity and the oxygen-reducing activity of the membrane prepared from the alkaliphilic *Bacillus* YN-1, and found two kinds of respiratory terminals, a cyanide-sensitive cytochrome *c* oxidase containing heme *a*, and a cyanide-insensitive non-proteinous material oxidizing TMPD. Furthermore, the cyanide-insensitive terminal has been also found in the facultative alkaliphilic *Bacillus* YN-2000 cultivated under an alkaline condition. These results suggest that cyanide-insensitive respiratory terminal component has an important role in the growth of these alkaliphilic bacteria under an alkaline condition.

Materials and Methods

Organism

Obligate alkaliphilic *Bacillus* YN-1 and facultative alkaliphilic *Bacillus* YN-2000 were both kindly provided by Dr. Y. Nosoh (Iwaki-Meisei University, Fukushima, Japan) and Dr. N. Koyama (Chiba University, Chiba, Japan). The organisms were aerobically grown in medium containing 10 g of polypeptone, 4.5 g of yeast extract, 1 g of glucose, 1 g of K_2HPO_4, 0.1 g of $MgCl_2 \cdot 6H_2O$, and 10 g of Na_2CO_3 per liter according to the methods of Qureshi *et al.* [4]. The pH of the medium was adjusted to 10.0 for alkaline cultivation, or to 8.0 for neutral cultivation by adding $NaHCO_3$ or NaH_2PO_4. The bacterial cells were harvested at the early-stationary growth phase and stored at -80°C until use.

Preparation of the membranes from *Bacillus* YN-1

About 100 g (wet weight) of the frozen cells was suspended in 300 ml of 10 mM Tris-HCl buffer, pH 8.0 containing 0.1 mM EDTA and 10 μM PMSF. DNase (1 mg/ml) was added to the suspension, and the suspension was treated with French pressure (1000 kgf/cm^2). Then, the suspension was centrifuged at 6,000 x *g* for 15 min to remove the unbroken cells. The supernatant was centrifuged at 187,000 x *g* for 1 h to remove the soluble proteins. The pellets were washed with 100 mM Tris-HCl buffer, pH 8.0 containing 0.3 M KCl, 1 mM EDTA, and 10 μM PMSF, and then centrifuged at 187,000 x *g* for 1 h. The pellets was suspended in 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA and 10 μM PMSF, and used as the membranes fraction.

Measurement of the cytochrome *c* oxidase activity

The cytochrome *c* oxidase activity was measured spectrophotometrically at room temperature by following the decrease in the absorbance at 550 nm with a spectrophotometer (model MPS-2000, Shimadzu, Kyoto, Japan), using 1 cm light path cuvettes. The reaction

mixture contained 25 mM sodium phosphate buffer, pH 6.5, *Saccharomyces cerevisiae* ferrocytochrome *c* (11.5 μM) and the sample in the total volume of 1 ml.

Measurements of the respiratory activities of the membranes

The oxygen consuming activity of the membranes were measured polarographically with a Clark-type oxygen electrode (model Mp-1000, Iijima Products M.F.G. Co., Ltd., Aichi, Japan) at 25°C. The standard reaction mixture contained the membranes (0.8 mg/ml) suspended in 25 mM Tris-HCl buffer, pH 8.0 in the total volume of 2.0 ml. The reaction was started by the addition of the respiratory substrates such as 10 mM NADH or 5 mM ascorbate *plus* 0.1 mM TMPD. The inhibitors were added to the reaction mixture before the addition of substrate. HOQNO and antimycin A were dissolved in DMSO, because DMSO had no effect on the oxygen consuming activity of the membranes. When ascorbate-TMPD was used as substrate, a control activity in the absence of the membranes was subtracted from the value in the presence of the membranes. The concentration of oxygen in the reaction mixture was determined to be 259 μmol/ml at 25°C according to the method of Winkler [5].

Other determinations

Protein concentration was determined by using BCA Protein Assay Reagents (Pierce, Rockford, Ill., USA).

Reagents

HOQNO, antimycin A, *S. cerevisiae* cytochrome *c* and bovine liver catalase were purchased from Sigma Chemical Company (St. Louis, Mo., USA), NADH, TMPD, sodium ascorbate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Potassium cyanide was purchased from Koso Chemical Co., Ltd. (Tokyo, Japan). All chemicals were of the highest grade commercially available.

Results and Discussion

Effects of the inhibitors on the respiratory activity of the cytoplasmic membranes prepared from *Bacillus* YN-1

Figure 1 shows the effects of cyanide on the cytochrome *c* oxidase activity of the membranes prepared from *Bacillus* YN-1. Ferrocytochrome *c* oxidase activity of the membranes was strongly inhibited by cyanide, and the K_i value was determined to be 2 μM. This result indicates that *Bacillus* YN-1 has the cyanide-sensitive cytochrome *c* oxidase in the cytoplasmic membranes.

The effects of cyanide on the oxygen consuming activities of the membranes prepared from *Bacillus* YN-1 and *Bacillus* YN-2000 were analyzed with the oxygen electrode as described in "Materials and Methods". As shown in Figure 2, the oxygen consuming activity of the membranes prepared from *Bacillus* YN-2000 was effectively inhibited by cyanide because the facultative alkaliphilic bacterium, *Bacillus* YN-2000 has also a cyanide-sensitive terminal oxidase, *aco* -type cytochrome *c* oxidase [4]. These results strongly indicate that cytochrome *aco* functions as a physiological terminal oxidase in the aerobic respiratory chain of *Bacillus* YN-2000. On the other hand, neither of the oxygen consuming activity nor the ascorbate-TMPD oxidizing activity of the membranes prepared from *Bacillus* YN-1 were inhibited by cyanide. Therefore, the cyanide-insensitive component seems to be mainly involved in the respiratory pathway from NADH to O_2 of *Bacillus* YN-1 as terminal component. Thus the respiratory systems of an obligate alkaliphilic *Bacillus* YN-1 contains two components as respiratory terminals, one is a cyanide-sensitive cytochrome *c* oxidase and the other is a cyanide-insensitive component which catalyzes reduction oxygen molecules.

To elucidate the branched point between the cyanide-sensitive and the cyanide-insensitive respiratory chain, the effects of the respiratory inhibitors on the oxygen consuming activities of the membranes prepared from *Bacillus* YN-1 were investigated (Figure 3). Antimycin A and

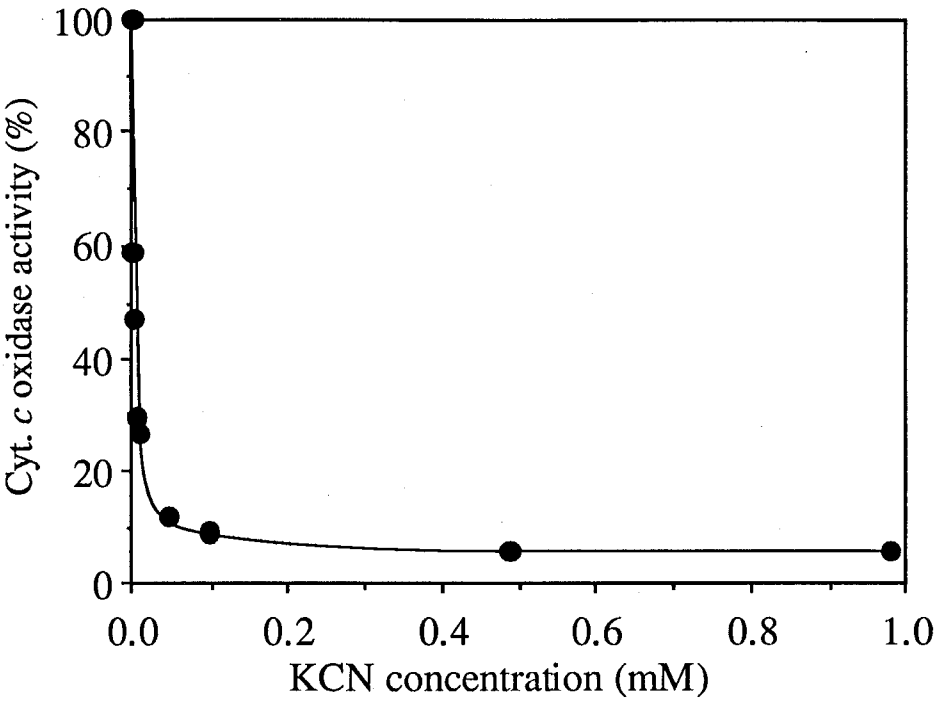

Figure 1 **Effect of cyanide on the cytochrome *c* oxidase activity of the membranes prepared from *Bacillus* YN-1.** The reaction mixture contained 25 mM sodium phosphate buffer, pH 6.5, 11.5 μM *S. cerevisiae* ferrocytochrome *c*, and the membranes containing 50 μg of protein.

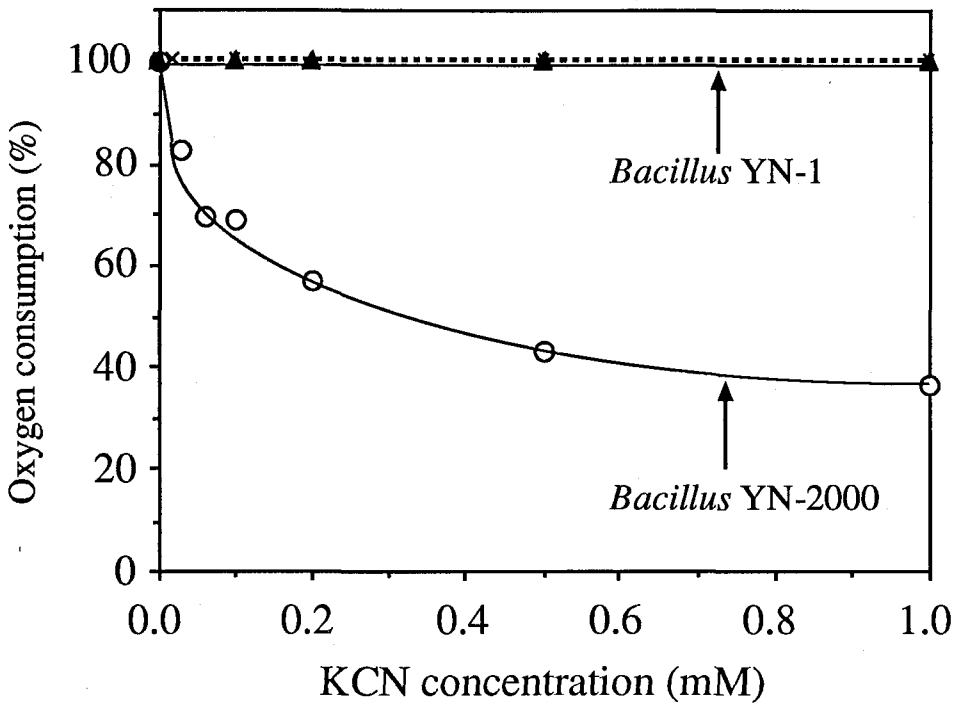

Figure 2 **Effects of cyanide on the oxygen consuming activity of the membrane prepared from *Bacillus* YN-1 (▲) and *Bacillus* YN-2000 (○).** The reaction mixture contained 25 mM Tris-HCl buffer, pH 8.0, 10 mM NADH, and the membranes. NADH was used as substrate. Dashed line indicates the oxygen consuming activity of the membranes prepared from *Bacillus* YN-1 in the presence of 5 mM ascorbate and 0.1 mM TMPD. *Bacillus* YN-2000 was cultivated at pH 10.0 and the membranes were prepared according to the method as described in "Preparation of the membranes from *Bacillus* YN-1".

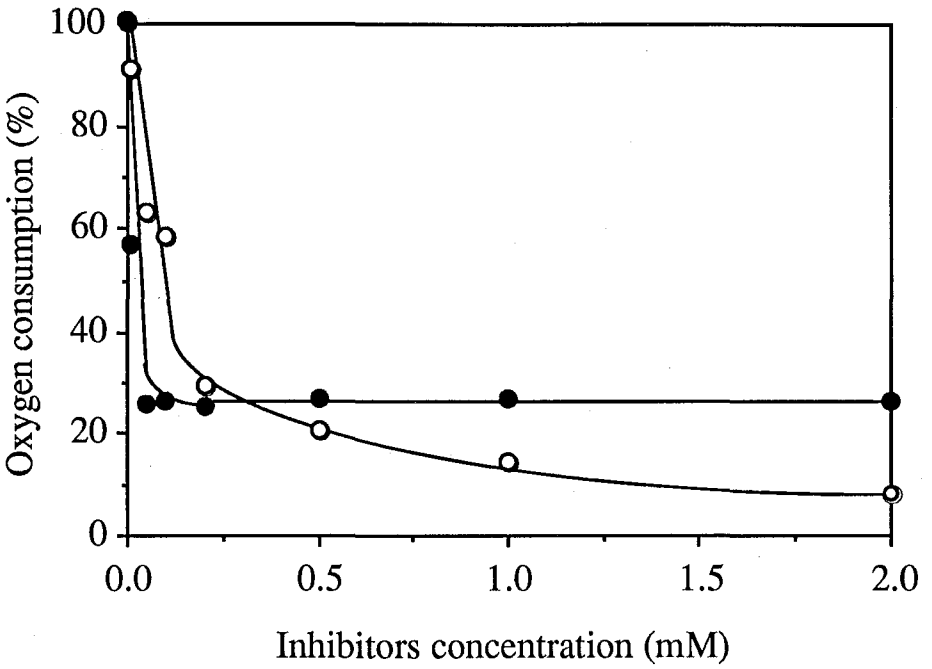

Figure 3 **Effects of antimycin A and HOQNO on the respiratory activity of the membranes prepared from *Bacillus* YN-1.** The reaction mixture contained 25 mM Tris-HCl buffer, pH 8.0, 10 mM NADH, inhibitors and the membranes. NADH was used as substrate. The experimental condition was described in "Materials and Methods". The symbols indicate that: (○) antimycin A, (●) HOQNO.

HOQNO which are specific inhibitors of quinone-cytochrome *c* oxidoreductase (complex III) strongly inhibited the oxygen consuming activities of the membranes. The K_i values of antimycin A and HOQNO were 92 and 78 μM, respectively. These results suggest that the respiratory chain of alkaliphilic *Bacillus* YN-1 is consisted of the cyanide-sensitive respiratory chain and the cyanide-insensitive respiratory chain, and is branched at complex III. The effects of cyanide, antimycin A, and HOQNO on the respiratory activity of *Bacillus* YN-1 are summarized in Table 1.

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

Activities	Electron donor	Concentration of inhibitors	Residual activity		
			inhibitors		
			cyanide	amtimycin A	HOQNO
cytochrome *c* oxidase activity	cyt. *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%

The oxygen consuming activities with ascorbate-TMPD and NADH were measured by the methods as described in "Materials and Methods". 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.
* not determined.

From the inhibition experiments as described above, the growth of the bacterium is expected to be highly cyanide-tolerant. Figure 4 shows the growth curves of *Bacillus* YN-1 in the presence of several concentrations of cyanide. No effect was observed on bacterial growth

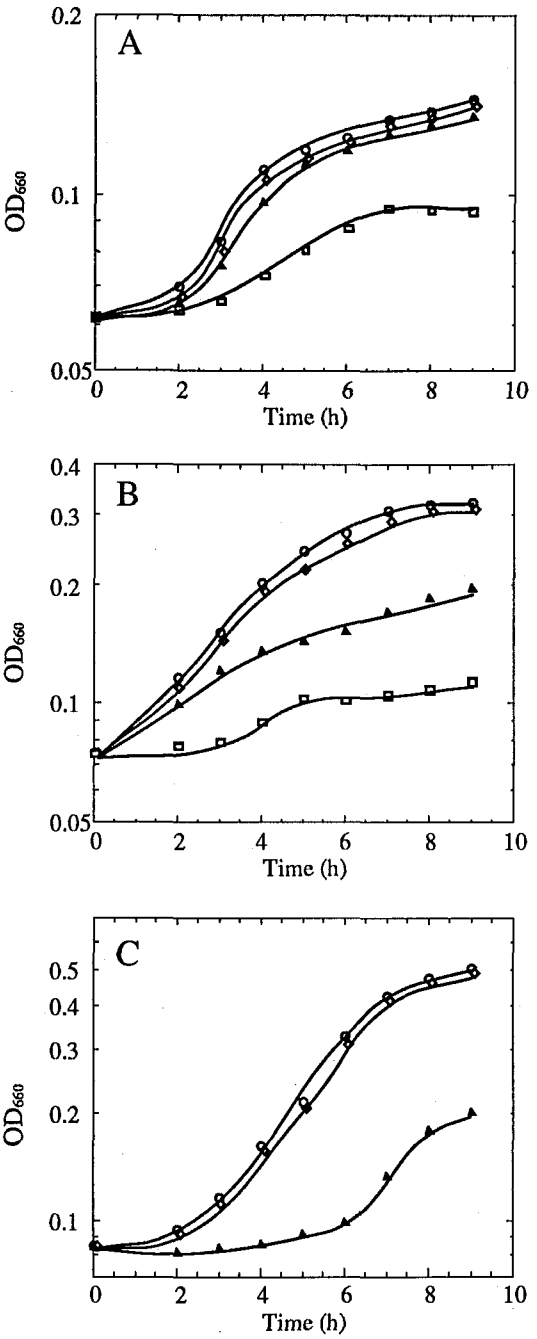

Figure 4 **Effects of cyanide on the growth of *Bacillus* YN-1 and *Bacillus* YN-2000.** (A) *Bacillus* YN-1, (B) *Bacillus* YN-2000 cultivated at pH 10.0, (C) *Bacillus* YN-2000 cultivated at pH 8.0. The symbols indicate that: (○) 0 μM KCN, (◇) in the presence of 10 μM KCN, (△) in the presence of 100 μM KCN, (□) in the presence of 1 mM KCN. Growth was monitored by measuring optical density of the medium at 660 nm.

in the presence of 100 μM cyanide. Even 1 mM of cyanide did not inhibit the growth. On the other hand, cyanide strongly inhibited the growth of facultative alkaliphilic *Bacillus* YN-2000. The growth rate was 63% decreased by an addition of 100 μM cyanide at pH 10, and interestingly, the same concentration of cyanide completely inhibited the growth of the cells cultivated at pH 8.0 as shown in Figure 4B and C. Therefore, it is concluded that the cyanide-insensitive respiration in alkaliphilic *Bacillus* YN-1 and *Bacillus* YN-2000 is physiologically important for growth under an alkaline condition.

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

Respiratory terminal components of *Bacillus* YN-1 were solubilized from the cytoplasmic membranes by the detergent, then fractionated by anion-exchange chromatography. The bacterial membrane fraction prepared from the 100 g (wet weight) cultured cells as described in "Materials and Methods" was suspended in 420 ml of 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM disodium EDTA and 10 μM PMSF (buffer A), and Triton X-100 was added to a concentration of 1.5%. The suspension was stirred gently for 2 h and then centrifuged at 187,000 x *g* for 1 h. In this solubilization step, 96% of total TMPD-dependent oxygen consuming activities were retained in the supernatant. The resulted reddish supernatant was applied to a chromatography on a DEAE-Toyopearl column (4.0 x 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 5A shows the elution profile of the solubilized membranes prepared from *Bacillus* YN-1 on the anion-exchange column chromatography. The terminal respiratory activity was measured by oxidation of TMPD as described in "Materials and Methods". Three fractions showed high TMPD-oxidizing activity. The 14%, 55%, and 21% activities of the total activities originated from the membranes were recovered in the first, the second and the third fractions,

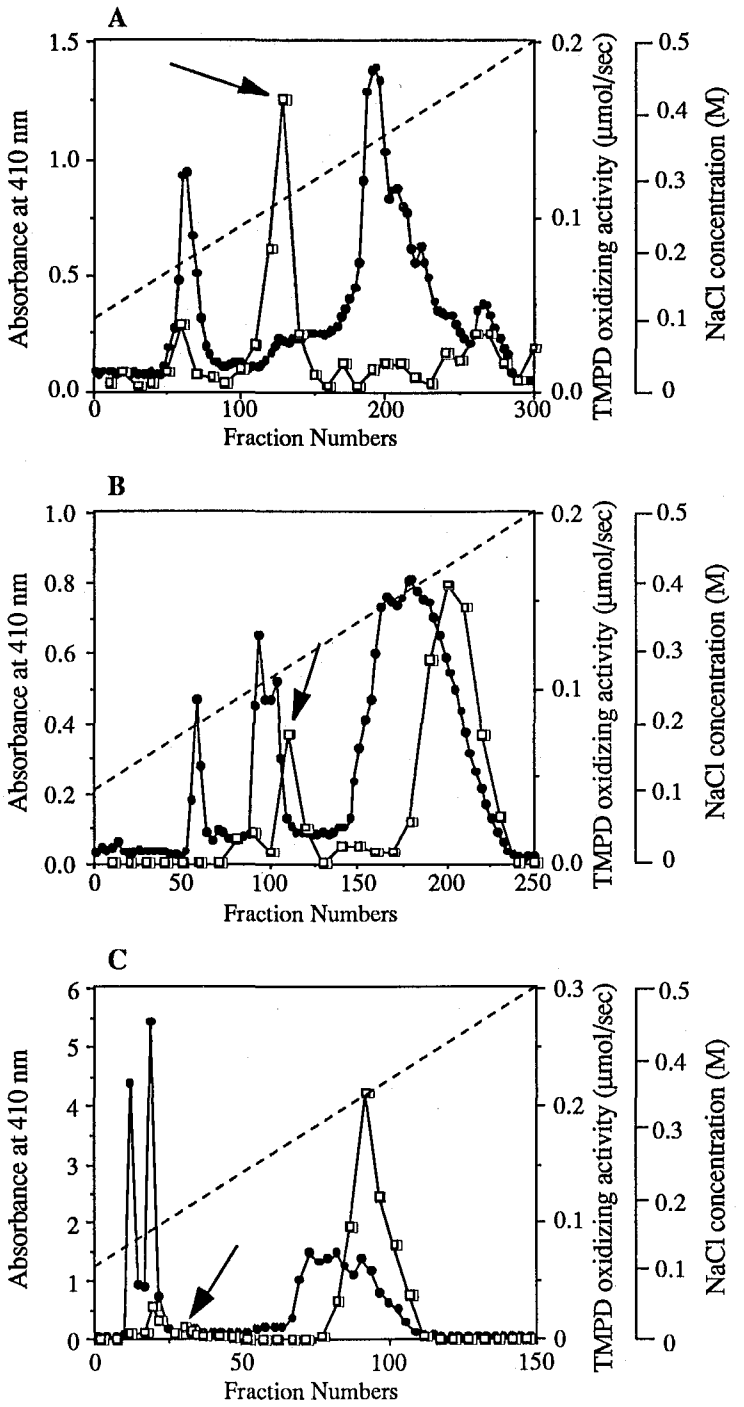

Figure 5 **Fractionation of respiratory terminal components by anion-exchange chromatography.** (A) *Bacillus* YN-1, (B) *Bacillus* YN-2000 grown at pH 10.0, (C) *Bacillus* YN-2000 grown at pH 8.0. (●) absorbance at 410 nm; (□) TMPD oxidizing activity; dashed line, NaCl concentration. The arrows indicate the fraction with the cyanide-insensitive TMPD-oxidizing activity.

respectively. As described above, the membranes prepared from *Bacillus* YN-1 showed cyanide-sensitive and cyanide-insensitive oxygen-consuming activities. Here, I examined the effects of cyanide on the TMPD-oxidizing activities of three fractions. Consequently, I found that only the third fraction showed cyanide-sensitive TMPD-oxidizing activity. Furthermore, the third fraction showed the cytochrome *c* oxidase activity and the spectral properties are very similar with those of caa_3-type cytochrome *c* oxidase, suggesting that the cyanide-sensitive oxygen-consuming activity of the membranes is catalyzed by the heme *a*-containing cytochrome *c* oxidase. On the other hand, the first and second fractions showed the cyanide-insensitive TMPD-oxidizing activities. The absorption spectrum of the first fraction indicated the presence of protoheme. The protohemes showed the TMPD-oxidizing activity at a turnover number 0.94 sec^{-1}. Therefore, it seems likely that the TMPD-oxidizing activity of the first fraction may be caused by the contaminated free protoheme molecules released from the denatured *b*-type cytochromes. The second fraction also showed the cyanide-insensitive TMPD-oxidizing activity. However, no hemoprotein including cytochromes were detected in the fraction. Surprisingly, the activity was not affected even treatment with boiling water (100°C for 5 min) or 1% SDS, suggesting that the TMPD-oxidizing activity found in the second fraction may be catalyzed by a stable non-proteinous material.

The respiratory terminal components of facultative alkaliphilic *Bacillus* YN-2000 cultivated at pH 10.0 and at pH 8.0 were investigated. The cells cultivated at pH 10.0 had two TMPD-oxidizing components as shown in Figure 5B. The fraction eluted at around 0.4 M NaCl was cyanide-sensitive, and identical with that of *aco*-type cytochrome *c* oxidase which had been reported previously [4]. The other TMPD-oxidizing component eluted at about 0.28 M NaCl was heat-stable as that of an obligate alkaliphilic *Bacillus* YN-1. However, it should be noted that the cyanide-insensitive TMPD-oxidizing component occupied only a small part of total activities in *Bacillus* YN-2000. Figure 5C shows the elution profile of the respiratory components prepared from the *Bacillus* YN-2000 cells cultivated at pH 8.0. The cyanide-insensitive TMPD-

oxidizing component was absent in the elution profile. From these studies, the cyanide-insensitive TMPD-oxidizing component is considered to constitute a major respiratory terminal in cyanide-insensitive pathway of the bacterium under high alkaline conditions.

Recently, the alternative respiratory chain has been reported in many higher plants [6-9] and also in some yeasts [10-12]. Figure 6A shows the schematic model of the alternative respiratory chain in higher plants and yeasts. These alternative respiratory pathways showed cyanide-insensitive oxygen reducing activity and usually branched at quinone pool. The enzymes involved in the alternative respiratory chain have been purified and characterized. The molecular mass of the enzyme is 36 kDa, and the oxygen reducing activity is inhibited by specific inhibitor SHAM [13]. However, although the respiratory activity of alkaliphilic *Bacillus* YN-1 was inhibited by antimycin A or HOQNO, that was not inhibited by 100 μM SHAM. Furthermore, the cyanide-insensitive TMPD-oxidizing component is a non-proteinous material, and there is no reports referring to non-proteinous material that function as the respiratory terminal component. Therefore, the alternative respiratory chain of alkaliphilic *Bacillus* seems to be different from those of high plants and yeasts, and to be a new type (Figure 6B). In the following sections, the purifications and characterizations of two respiratory terminals were described.

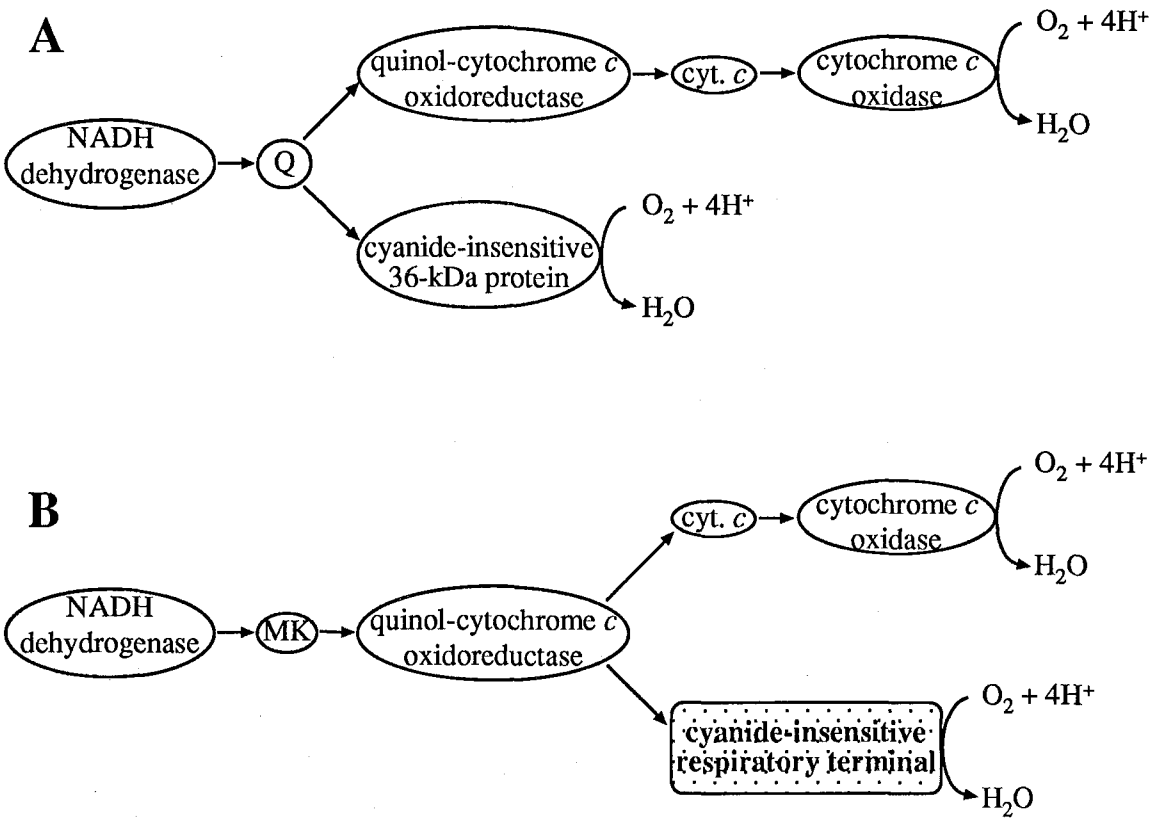

Figure 6 **Alternative electron transport system of higher plants and yeasts (A), and alkaliphilic *Bacillus* YN-1 (B).** Q; ubiquinone, MK; menaquinone, cyt; cytochrome.

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Section III

Purification and Characterization of *caa*$_3$-type Cytochrome Oxidase from Alkaliphilic *Bacillus* YN-1

Introduction

As described in Section I, alkaliphilic bacteria require proton gradient for ATP synthesis. It is generally accepted that proton gradient is produced by respiratory components. In the mitochondrial respiratory chain, complex I, III and IV can translocate protons across the inner membrane [1-3]. It is notable that alkaliphilic bacteria are found to have very high concentrations of membrane-associated respiratory components [4] and that the array of cytochromes, as distinguished by redox potentials, is complex. However, we have not yet known whether these respiratory components can drive protons across the membrane for the synthesis of ATP under extremely alkaline pH conditions.

Cytochrome *c* oxidase (EC 1.9.3.1), the terminal enzyme of the mitochondrial and the bacterial aerobic respiratory chains have been studied extensively during past two decades. The enzyme mediates electron transport between ferrocytochrome *c* and molecular oxygen, pumping protons across the mitochondrial inner membrane or the bacterial cytoplasmic membrane [3].

Recently, Qureshi *et al.* have purified *aco*-type cytochrome *c* oxidase from the facultative alkaliphilic bacteria, *Bacillus* YN-2000 [5] and further cloned and sequenced the gene (Denda *et al.*, unpublished results). The primary structure of *aco*-type cytochrome *c* oxidase from *Bacillus* YN-2000 is highly homologous with those of the terminal oxidases of thermophilic bacteria. On the other hand, Krulwich *et al.* have partially purified caa_3-type cytochrome *c* oxidase [6] and *bd*-type quinol oxidase [7] from the facultative alkaliphilic bacterium, *Bacillus firmus* OF4.

In this study, I have purified caa_3-type cytochrome *c* oxidase to an electrophoretically homogeneous state from an obligate alkaliphilic bacterium, *Bacillus* YN-1, and characterized the molecular and enzymatic properties.

Materials and Methods

Organism

Cultivation was performed as described in "Materials and Methods" of Section II.

Physical and chemical measurements

Absorption spectra were recorded with a spectrophotometer (model MPS-2000, Shimadzu, Kyoto, Japan) using a 1 cm light path cuvette at room temperature. The CO-complex of the oxidase was prepared in a cuvette by incubating the reduced enzyme under a 100% CO atmosphere for 5 min or more. Heme *a* was extracted from the enzyme with acidic acetone (1% HCl) according to the method of Drabkin [8]. The contents of hemes *a* and *c* in the purified enzyme were determined on the basis of the millimolar extinction coefficient at the α-peak of the pyridine ferrohemochromes: 26 $mM^{-1}cm^{-1}$ [9] and 29.1 $mM^{-1}cm^{-1}$ [8] for the hemochromes of heme *a* and heme *c*, respectively. The metal contents of the enzyme were determined by inductively coupled plasma atomic emission spectrometry measurements with a plasma spectrometer (model SPS 1500 VR, Seiko Instrument Inc., Tokyo, Japan).

The purity of the enzyme was confirmed with native polyacrylamide gel electrophoresis in the presence of Triton X-100 according to the method of Davis [10]. The apparent molecular masses of the subunits were estimated by SDS polyacrylamide gel electrophoresis by the method of Kadenbach [11]. Heme *c* in the gel was detected with the heme-staining regents [12] and the cytochrome *c* oxidase activity in the gel was detected with the Nadi-regents [13]. Protein concentration was determined by BCA protein assay protocol (Pierce, Rockford, Ill., USA).

Measurement of the cytochrome *c* oxidase activity

The cytochrome *c* oxidase activity was measured spectrophotometrically at room temperature by following the decrease in the absorbance at 550 nm with a spectrophotometer

(model MPS-2000, Shimadzu, Kyoto, Japan) using a 1 cm light path cuvette. The reaction mixture contained 25 mM sodium phosphate buffer, pH 6.5, 6 μM horse heart or *S. cerevisiae* ferrocytochrome *c* and 4~10 nM heme *a* of the enzyme in a total volume of 1.0 ml.

Measurement of the proton pumping activity of cytochrome *c* oxidase

Bacillus YN-1 cytochrome *c* oxidase vesicles were reconstituted according to the method of Kagawa *et al.* [14] with slight modifications. The proton pumping activity of the vesicles was measured according to the method of Sone *et al.* [15].

Immunological analysis

Antibodies of cytochrome *c* oxidases purified form *Bacillus* YN-1 and *Bacillus* YN-2000 were raised in rabbits, respectively. The enzymes for the antigens were emulsified in 1 ml of Freund incomplete Adjuvant and injected into rabbits subcutaneously several times. The antibodies were purified by ammonium sulfate fractionation (30-50% saturation). Immunodiffusion tests were performed according to the methods of Ouchterlony [16]. The gel was composed by 1% agarose in PBS (8 g of NaCl, 0.2 g of KCl, 2.9 g of $Na_2HPO_4 \cdot 12H_2O$, 0.2 g of KH_2PO_4 per liter, pH 7.4). The gel applied the antigens and antibodies were incubated in 37°C for over night.

Measurement of alkaline and thermal stabilities of cytochrome *c* oxidase

Cytochrome *c* oxidases of *Bacillus* YN-1, *Bacillus* YN-2000, and bovine mitochondria were treated with NaOH solution adjusted at pH 12 (room temperature) or were incubated at several temperatures (pH 6.5) in water bath for time course. After treatment, the cytochrome *c* oxidase activity and the TMPD oxidase activity were measured spectrophotometrically by following the decrease in the absorbance at 550 nm and the increase in the absorbance at 606 nm, respectively. The reaction mixture contained 25 mM sodium phosphate buffer, pH 6.5, with

1% Triton X-100, 6 μM *S. cerevisiae* ferrocytochrome *c* or 1 mM TMPD as substrates, and 4-10 nM heme *a* of the enzyme in a total volume of 1.0 ml.

Reagents

Horse heart cytochrome *c* (Type VI), *S. cerevisiae* cytochrome *c*, and soybean L-α-phosphatidylcholine (Type II-S) were purchased from Sigma Chemical Co. (St. Louis, Mo., USA), and deoxyribonuclease (DNase) from Boehringer-Mannheim (Mannheim, Germany). DEAE-Toyopearl 650 (Fractgel TSK DEAE-650M) was purchased from the Tosoh Corporation (Tokyo, Japan). Sephacryl S-300 was purchased from Pharmacia Fine Chemical (Uppsala, Sweden), and PMSF, $(NH_3)_2SO_4$, and EDTA were purchased from Wako Pure Chemical Industries (Osaka, Japan). Cytochrome *aco* was prepared from facultative alkaliphilic *Bacillus* YN-2000 according to the method of Qureshi *et al.* [5] with slight modifications. Cytochrome aa_3 of bovine heart mitochondria was prepared according to the methods of Yonetani [17] with slight modification.

Results

Purification of cytochrome *c* oxidase from *Bacillus* YN-1

All purification steps were conducted at 4°C. Frozen cells (about 100 g wet weight) were suspended in 300 ml of 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM disodium EDTA and 10 μM PMSF (buffer A). After addition of DNase (1 μg/ml), the suspension was compressed in a French pressure apparatus 1000 kgf/cm^2 to disrupt the cells. The resulting suspension was centrifuged at 6,000 x *g* for 15 min to remove the unbroken cells. The supernatant was centrifuged at 187,000 x *g* for 1 h to remove soluble proteins. The pellets were suspended in 420 ml of buffer A, and Triton X-100 of up to 1.5% in total concentration was added. The suspension was stirred gently for 2 h and then centrifuged at 187,000 x *g* for 1 h. The reddish supernatant obtained was applied to chromatography on a DEAE-Toyopearl column (4.0 x 24.5 cm) which 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 enzymes 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. The fractions with the cytochrome *c* oxidase activity was dialyzed against 2 liters of buffer B and fractionated with $(NH_3)_2SO_4$ 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 x 148 cm) which had been equilibrated with buffer B containing 0.25 M NaCl. The eluted fraction was collected and dialyzed against 2 liters of buffer B. The dialyzed enzyme solution was concentrated by adsorption on and elution from a small DEAE-Toyopearl column (2.2 x 5 cm). The dialyzed sample was used as the purified cytochrome *c* oxidase preparation. The representative purification steps are summarized in Table 1.

When the enzyme was subjected to polyacrylamide electrophoresis in the absence of SDS, only one band was stained with Coomassie Brilliant Blue. The band was also stained with the heme-staining reagents and the Nadi-reagents (Figure 1).

Table 1. Purification of cytochrome *c* 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	8.21	26.6	3.21
$(NH_4)_2SO_4$ fractionation	5.44	17.2	3.16
Sephacryl S-300 gel filtration	0.774	5.46 (16.1%)	7.05

Molecular features

The subunit structure was analyzed by SDS-PAGE (Figure 2). The enzyme was consisted of three kinds of subunits. The molecular mass of each subunit was estimated to be 47.5 kDa, 43 kDa and 21 kDa, respectively. The 43-kDa subunit was stained with heme staining reagents, suggesting that heme *c* may be covalently bound to the 43-kDa subunit.

The metal contents of the purified enzyme was analyzed by the methods as described in "Materials and Methods". The enzyme contained 3 atoms of Fe and 1.3 atoms of Cu per one molecule of the structural unit of the enzyme.

Spectral properties

Figure 3A shows the absorption spectra of the oxidized and reduced forms of *Bacillus* YN-1 cytochrome *c* oxidase. In the oxidized form, the enzyme showed a sharp absorption peak at 410 nm and small peaks at 527 and 595 nm, while in the reduced form prepared by an addition of a small amount dithionite, it showed peaks at 416, 441, 522, 550 and 597 nm. The pyridine ferrohemochrome spectrum of the enzyme showed absorption peaks at 550 nm and 587 nm (Figure 4), suggesting the presence of heme *c* and heme *a* molecules in the enzyme. The ratio of

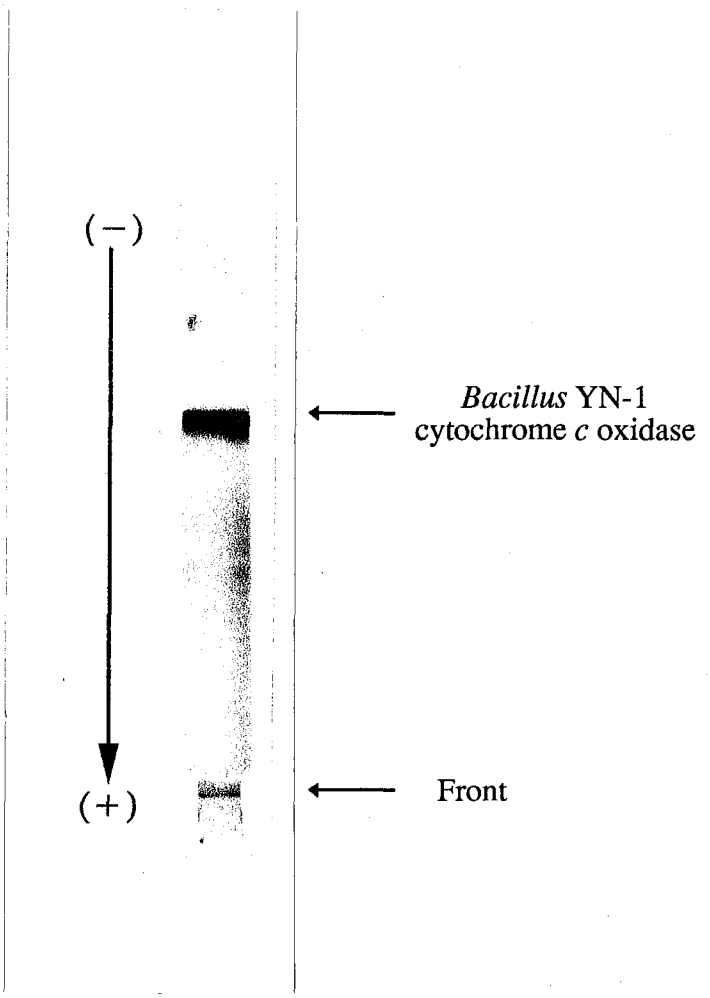

Figure 1 **Polyacrylamide gel electrophoresis of the purified *Bacillus* YN-1 cytochrome *c* oxidase in the absence of SDS.** The purified enzyme (30 μg) was electrophoresed on a 7% polyacrylamide gel in the absence of SDS. The gel was stained with Coomassie Brilliant Blue.

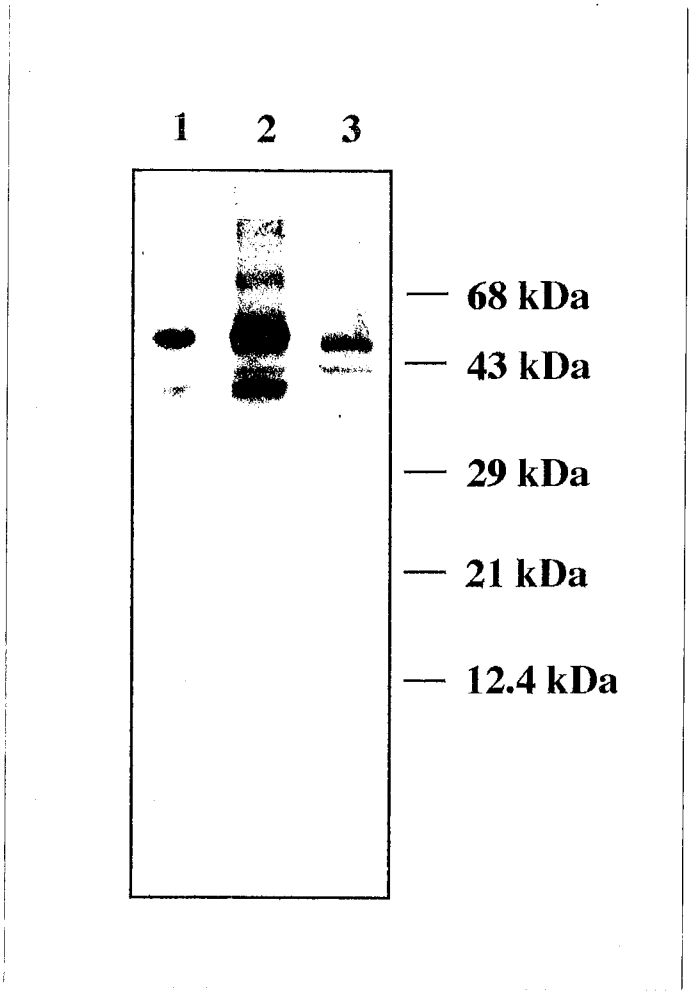

Figure 2 **SDS polyacrylamide electrophoresis of cytochrome *c* oxidases purified from *Bacillus* YN-1 and *Bacillus* YN-2000 (cytochrome *aco*).** The purified enzymes (6 μg *Bacillus* YN-1 cytochrome *c* oxidase and 8 μg *Bacillus* YN-2000 cytochrome *aco*) was electrophoresed on a 14% polyacrylamide gel in the presence of SDS. After the electrophoresis, the gel was stained with Coomassie Brilliant Blue. *Lane 1*, *Bacillus* YN-2000 cytochrome *aco*; *lane 2*, *Bacillus* YN-1 cytochrome *c* oxidase + *Bacillus* YN-2000 cytochrome *aco*; *lane 3*, *Bacillus* YN-1 cytochrome *c* oxidase.

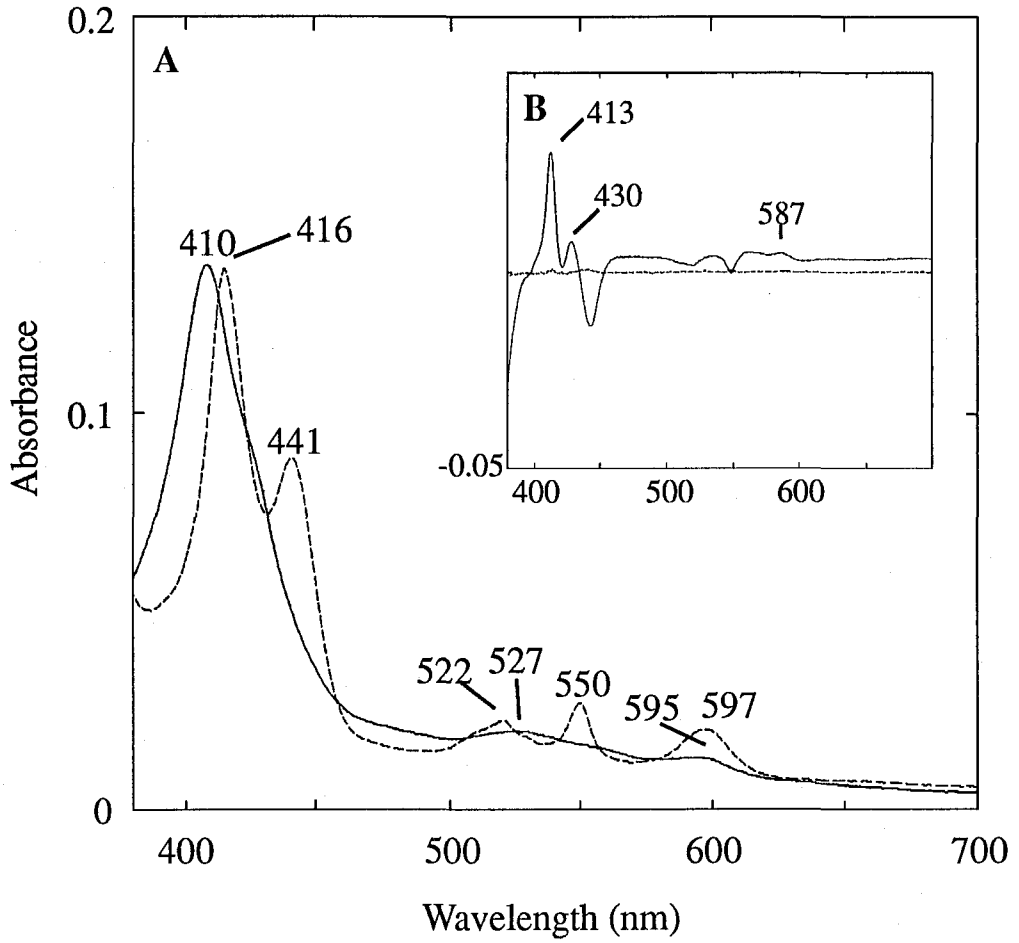

Figure 3 **(A) Absorption spectra of *Bacillus* YN-1 cytochrome *c* oxidase.** The enzyme was dissolved in 10 mM Tris-HCl buffer, pH 8.0, containing 1% Triton X-100. Solid line; oxidized form, dashed line; reduced form. **(B) (Reduced+CO) *minus* (reduced) difference spectrum.**

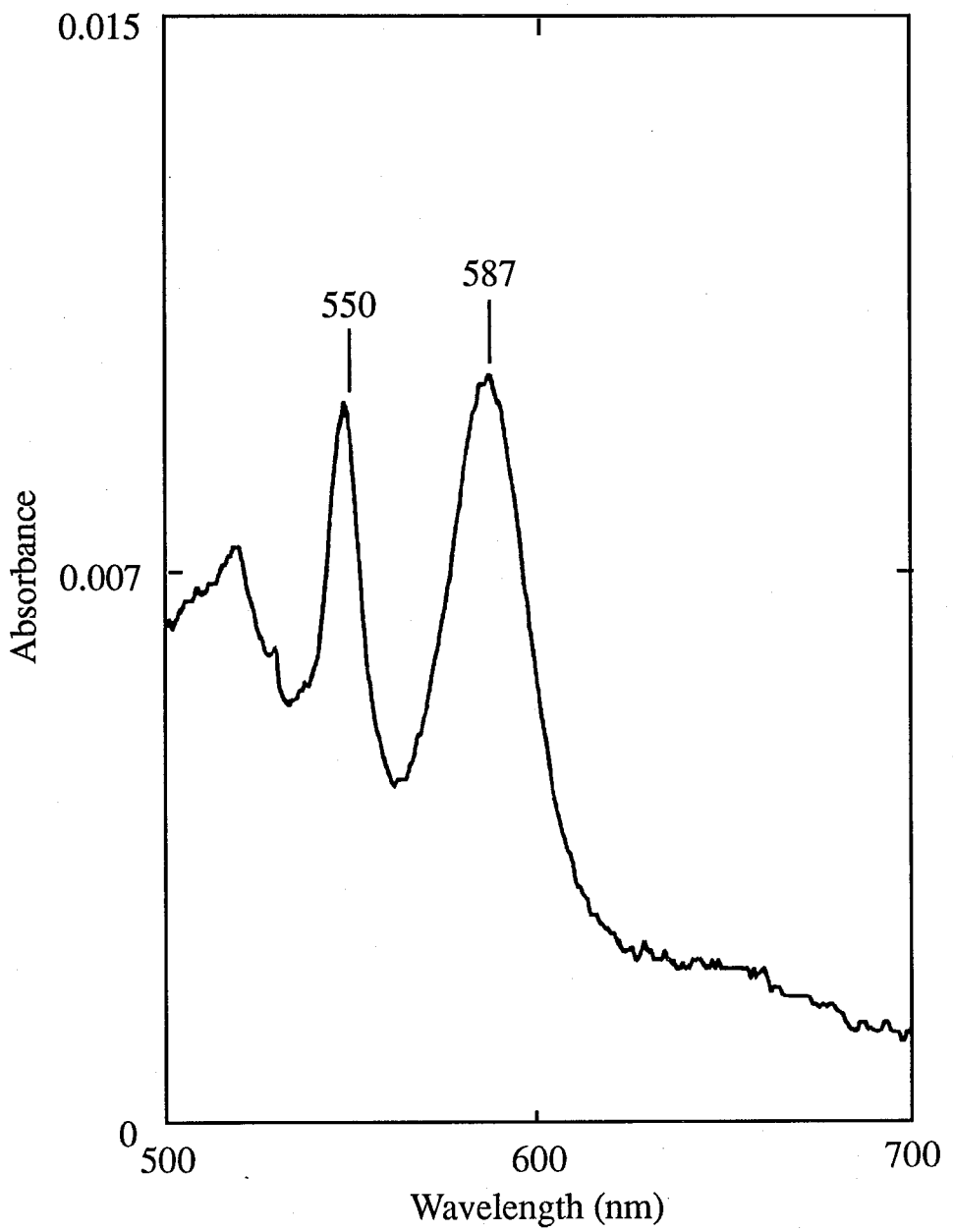

Figure 4 **Absorption spectrum of pyridine ferrohemochrome of *Bacillus* YN-1 cytochrome *c* oxidase.** The enzyme was dissolved in 0.1 N NaOH, 5% pyridine, and a small amount of dithionite at a concentration 3.16 μM heme *a*.

heme *a* to heme *c* was estimated at about 2:1 on the basis of the spectrum. The difference spectrum, (reduced+CO) *minus* (reduced), of the cytochrome *c* oxidase is shown in Figure 3B, suggesting that the enzyme contains CO-binding high spin heme *a* in the molecule.

The absorption spectrum of the oxidized form in the infrared region of the enzyme showed a broad peak at 800 nm, indicating the presence of Cu_A (Figure 5). These results indicate that the purified enzyme was the caa_3-type cytochrome *c* oxidase.

Immunological test

Figure 6 shows the immunodiffusion analysis of cytochrome *c* oxidases purified from *Bacillus* YN-1 and *Bacillus* YN-2000, indicating that cytochrome oxidases from *Bacillus* YN-1 and *Bacillus* YN-2000 have different structural properties although both enzymes have similar subunit structure and prosthetic groups.

Catalytic properties

The cytochrome *c* oxidase activity was measured spectrophotometrically with horse cytochrome *c* and *S. cerevisiae* cytochrome *c* as electron donors. The optimum pH of the cytochrome *c* oxidase activity of the enzyme was found to be 6.5. The K_m values for horse cytochrome *c* of the enzyme was 5.32 μM, while the value for *S. cerevisiae* cytochrome *c* was 8.42 μM. The V_{max} values for horse and *S. cerevisiae* cytochrome *c* were 0.484 and 3.61 sec^{-1}, respectively. The V_{max} for horse cytochrome *c* of the enzyme was fairly lower than that of cytochrome *aco* (the V_{max} and the K_m values of cytochrome *aco* of *Bacillus* YN-2000 for horse cytochrome *c* were 48.8 s^{-1} and 0.9 μM, respectively). The effects of cyanide on the cytochrome *c* oxidase activity and the oxygen consuming activity were examined. As shown in Figure 7, both activities were strongly inhibited by cyanide. The K_i values of cyanide for the cytochrome *c* oxidase activity and the oxygen consuming activity were 3.3 and 4.5 μM, respectively.

The proton pumping activity of the liposomal enzyme was measured by the method

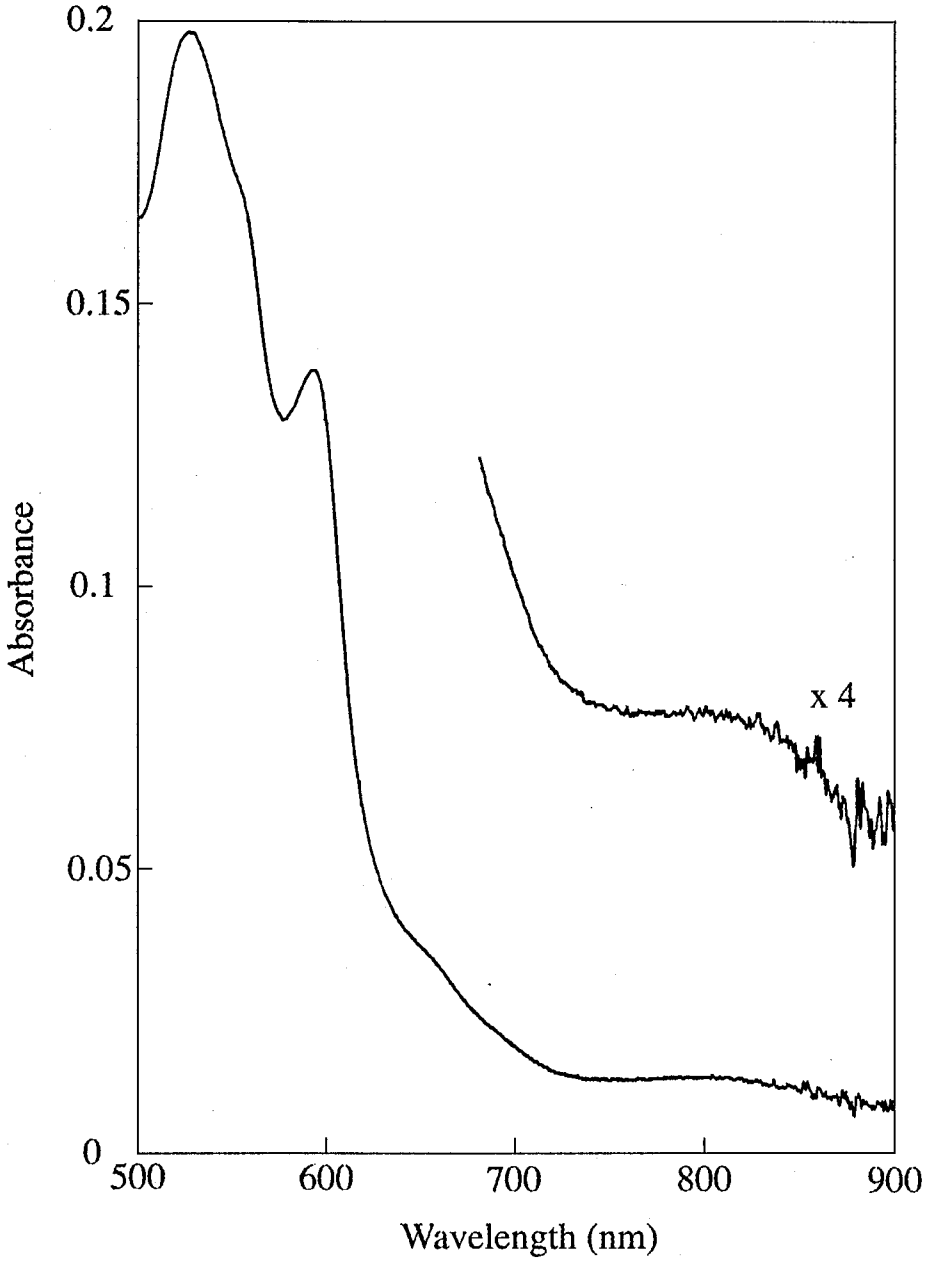

Figure 5 **Absorption spectra in the near infrared region of cytochrome *c* oxidase purified from *Bacillus* YN-1.** The enzyme was dissolved in 10 mM Tris-HCl, pH 8.0, containing 1% Triton X-100 at a concentration 7.96 μM heme *a*.

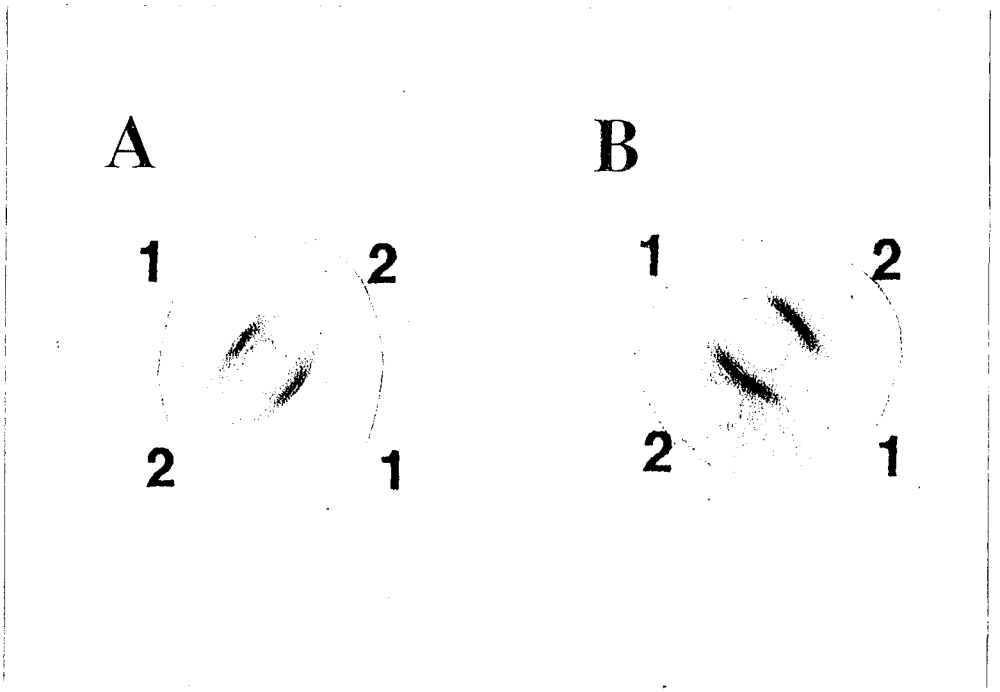

Figure 6 **Immunodiffusion of cytochrome *c* oxidases purified from alkaliphilic *Bacillus* YN-1 and *Bacillus* YN-2000.** (A) Interaction between *Bacillus* YN-1 cytochrome *c* oxidase antiserum and cytochrome *c* oxidases. (B) Interaction between *Bacillus* YN-2000 cytochrome *c* oxidase antiserum and cytochrome *c* oxidases. 1 and 2 indicate *Bacillus* YN-1 and *Bacillus* YN-2000 cytochrome *c* oxidases, respectively. Antiserum was injected in the central holes.

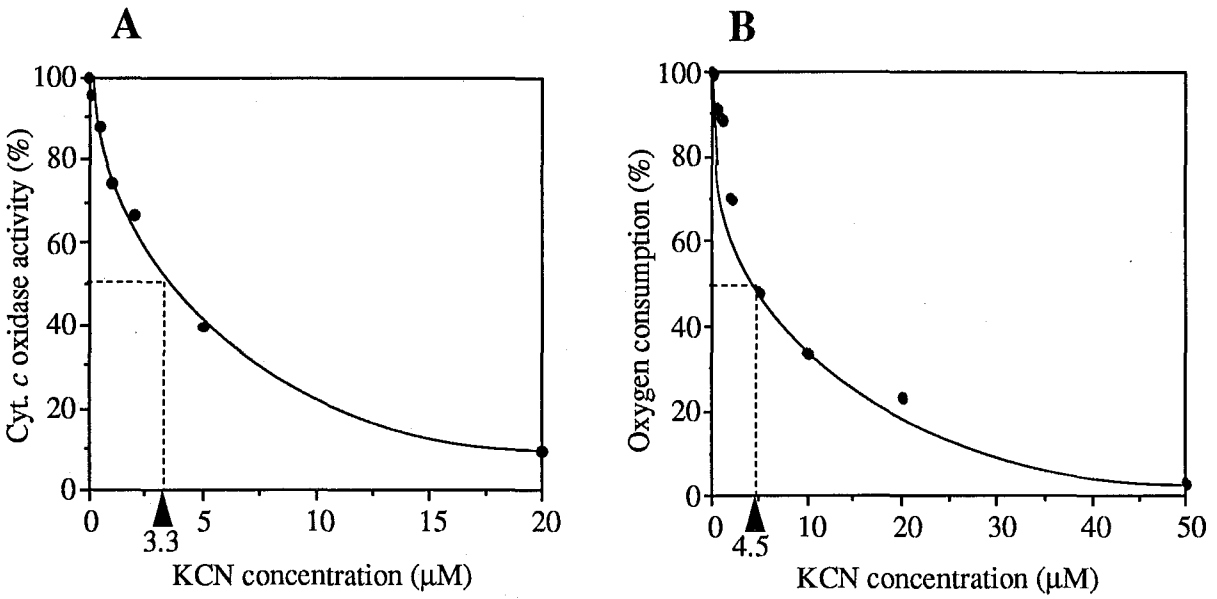

Figure 7 **Effect of cyanide on cytochrome *c* oxidase purified from *Bacillus* YN-1.** (A) the effect of cyanide on cytochrome *c* oxidase activity of the purified enzyme. *S. cerevisiae* cytochrome *c* was used as electron donor. (B) the effect of cyanide on oxygen consumption activity of the purified enzyme. Ascorbate-TMPD were used as electron donating system. (▲); the K_i values of cyanide for each activity.

described in "Materials and Methods". However, the proton pumping activity of the reconstituted liposomes could not be detected at pH 8.0.

Alkaline and thermal stabilities of cytochrome *c* oxidases of alkaliphilic *Bacillus*

Generally, the extracellular enzymes of alkaliphilic organisms show high alkaline-tolerant. Recently, the primary structure of *aco*-type cytochrome oxidase of *Bacillus* YN-2000 was determined by Denda *et al.* (unpublished result). The primary structure is homologous with those of cytochrome *c* oxidase of thermophilic bacteria (Table 2). In the present study, alkaline and thermal stabilities of cytochrome *c* oxidases of alkaliphilic *Bacillus* were examined. Figure 8 shows the time course of the residual cytochrome *c* oxidase activities of the enzymes under alkaline and variable temperature conditions. The cytochrome *c* oxidase of *Bacillus* YN-1 was not denatured at pH 12 (room temperature) or at 50°C for 15 min (pH 6.5), while the mitochondrial cytochrome *c* oxidase lost almost the activity at those conditions (Figure 9). These results indicate that cytochrome *c* oxidases of alkaliphiles are stable under high alkaline and thermal conditions.

Table 2. Comparison of the primary structures of cytochrome oxidases subunit I

	Bacillus YN-2000 cytochrome *aco*	*Bacillus* PS3 cytochrome caa_3	*Bacillus firmus* cytochrome caa_3	*Bacillus subtilis* cytochrome aa_3-600	mitochondrial cytochrome aa_3
B. YN-2000 cyt. *aco*	—	78.0	64.4	47.6	41.6
	B. PS3 cyt. caa_3	—	63.1	47.9	41.8
		B. firmus cyt. caa_3	—	45.9	40.1
			B. subtilis cyt. aa_3-600	—	37.4
				mit. cyt. aa_3	—

a, *Bacillus* YN-2000 and *Bacillus firmus* are alkaliphilic bacteria.
b, *Bacillus* PS3 is a thermophilic bacterium.
c, *Bacillus subtilis* is a neutralophilic bacterium.
d, The enzyme was purified from bovine heart.

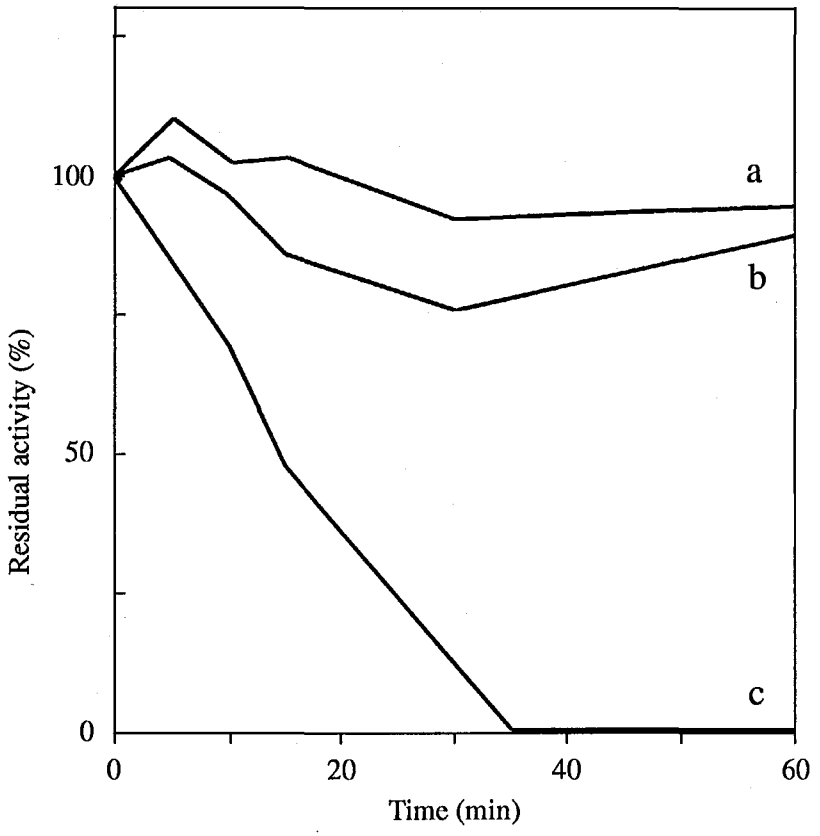

Figure 8 **Alkaline stability of the cytochrome oxidases.** The enzymes were incubated at room temperature and pH 12 for 60 min. a, b, c, indicate *Bacillus* YN-1 (alkaliphile) cytochrome *c* oxidase, *Bacillus* YN-2000 (alkaliphile) cytochrome *aco*, mitochondrial cytochrome aa_3, respectively. *S. cerevisiae* cytochrome *c* was used as substrate.

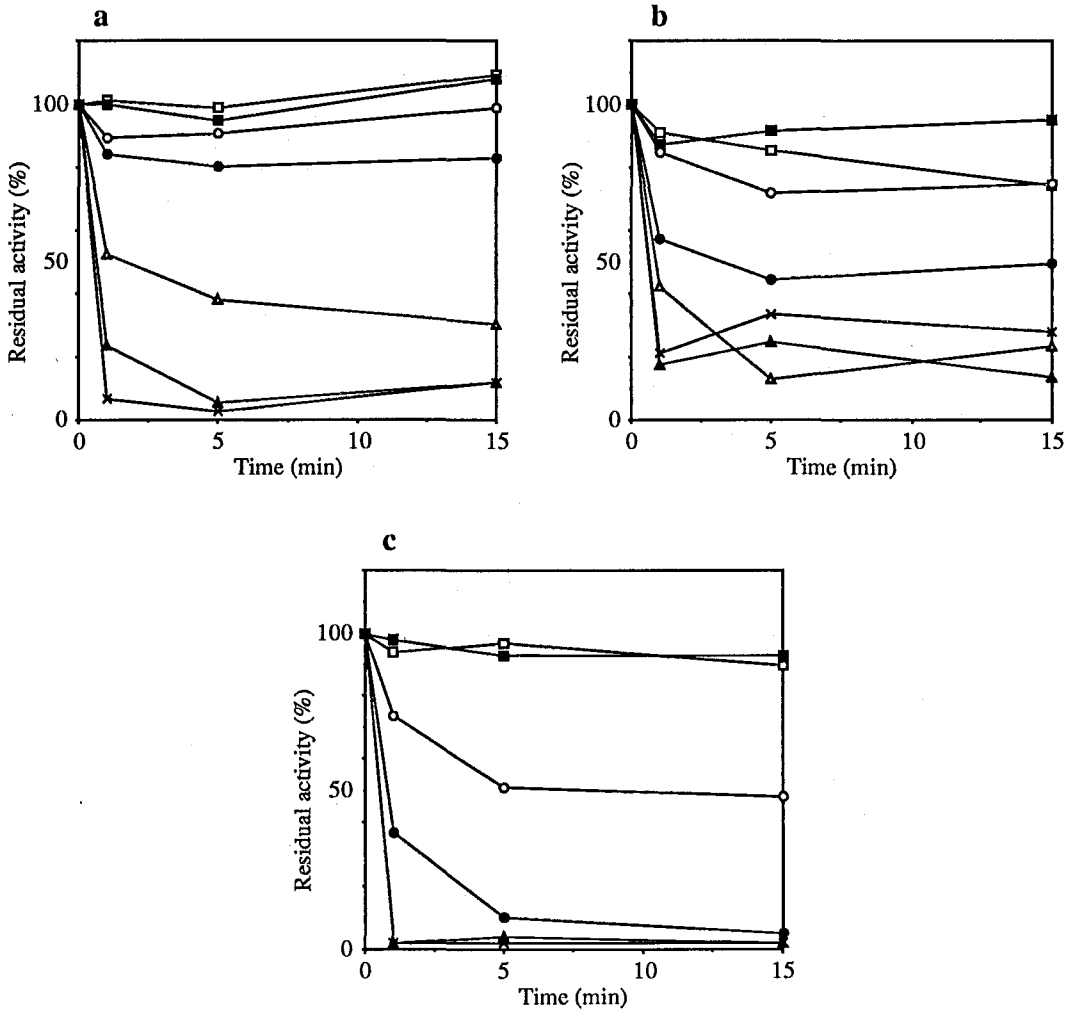

Figure 9 **Thermal stability of the cytochrome oxidases.** a, b, c, indicate *Bacillus* YN-1 (alkaliphile) cytochrome *c* oxidase, *Bacillus* YN-2000 (alkaliphile) cytochrome *aco*, mitochondrial cytochrome aa_3, respectively. *S. cerevisiae* cytochrome *c* was used as substrate. The enzymes were incubated at various temperatures and pH 8.0 for 15 min. The symbols indicate that: (□) 20°C, (■) 30°C, (○) 40°C, (●) 50°C, (△) 60°C, (▲) 70°C, (×) 80°C.

Discussion

Recently, three kinds of cytochrome *c* oxidase containing heme *a*, aa_3-type [18], caa_3-type [19, 20] and ba_3-type [21], have been purified from aerobic bacteria. The caa_3-type [6] cytochrome *c* oxidase has been purified from facultative alkaliphilic *Bacillus firms* OF4, and *bd*-type oxidase from the mutant lacking of *cta* gene encoded of caa_3-type cytochrome *c* oxidase of *Bacillus firms* OF4 [7]. Qureshi *et al.* have purified and characterized *aco*-type cytochrome *c* oxidase from facultative alkaliphilic *Bacillus* YN-2000 [5]. All enzymes have a high spin heme *a* (heme a_3) as oxygen binding site in the molecules [22] except for *aco*-type cytochrome *c* oxidase of *Bacillus* YN-2000 (*aco*-type cytochrome *c* oxidase has a high spin heme *o* as oxygen binding site [5]).

The enzyme purified from *Bacillus* YN-1 contains 2 mol of heme *a* and 1 mol of heme *c* per mol of enzyme. Although the enzyme shows the blue-shifted absorption peak at 597 nm in the reduced form, the spectral properties are quite similar to those of caa_3-type cytochrome *c* oxidases which have been purified from the thermophilic bacteria as *Bacillus* PS3 [19] and *Thermus thermophilus* HB8 [20]. Further, (reduced+CO) *minus* (reduced) difference spectrum is similar to that of *Bacillus* PS3 caa_3-type cytochrome *c* oxidase [19], suggesting that *Bacillus* YN-1 cytochrome *c* oxidase has a CO-binding heme *a* in the molecule. Therefore, it is concluded that cytochrome *c* oxidase purified from alkaliphilic *Bacillus* YN-1 is designated as cytochrome caa_3.

Recently, Krulwich *et al.* have purified caa_3-type cytochrome *c* oxidase from facultative alkaliphilic bacterium, *Bacillus firmus* OF4, and reported that the enzyme molecule contains 2 copper atoms, Cu_A and Cu_B, in the minimal structural unit [6]. Cytochrome caa_3 purified from *Bacillus* YN-1 contained 1.3 atoms of Cu per one molecule of enzyme. The Cu contents of the enzyme are slightly lower than those of *Bacillus firms* OF4. Copper may be lost during the purification of the enzyme.

When *S. cerevisiae* ferrocytochrome *c* is used as electron donor, the V_{max} is calculated to be about 3.61 sec^{-1}. Compared with the cytochrome *c* oxidase activity of *T. thermophilus* HB8 cytochrome caa_3 [20], the V_{max} is quite low. However, it should be noted that the V_{max} of *Bacillus firmus* OF4 cytochrome caa_3 is 5.8 sec^{-1} [6] and the V_{max} of *Bacillus* YN-1 membranes is about 2.9 sec^{-1}. Therefore, the low activity of *Bacillus* YN-1 cytochrome caa_3 may be the original feature.

Cytochrome *c* oxidases purified from *Bacillus* YN-1 and *Bacillus* YN-2000 show not only the alkaline stability but also the thermal stability as shown in Figures 8 and 9. These properties are consistent with the homology of cytochrome *c* oxidase subunit I between alkaliphilic bacteria and thermophilic bacteria as shown in Table 2.

Some bacterial aa_3-type cytochrome *c* oxidases translocate proton across the cytoplasmic membrane at pH 7.0. The transmembrane proton gradient generated by the oxidase is utilized for ATP synthesis and secondary active transport. The enzyme terminal oxidase therefore plays a crucial function in aerobic respiratory chain. In the present study, I have reconstituted the purified *Bacillus* YN-1 cytochrome caa_3 into phospholipid vesicles and examined the respiratory control of the liposomes at pH 8.0. The uncoupler, FCCP, did not increase the oxygen consumption rate when horse cytochrome *c* was used as electron donor. Although it cannot be ruled out the possibility that the reconstituted system is not suitable for measuring the proton-pumping activity, this result suggests that *Bacillus* YN-1 cytochrome caa_3 may have no proton-pumping activity. Recently, Krulwich *et al.* have suggested that alkaliphilic *Bacillus* species might transfer electrons from the respiratory components to the F_o moiety of the F_1F_o-ATP synthase within the cytoplasmic membranes [23] (See Section I, Fig. 2).

In the present study, caa_3-type cytochrome *c* oxidase has been purified and characterized from an alkaliphilic bacterium, *Bacillus* YN-1. The enzyme is stable under alkaline condition. As described above, however, the enzymatic activity is very low and, furthermore, the enzyme has no proton pumping activity. Therefore, the cytochrome caa_3 may have little contributions

for ATP synthesis in alkaliphilic *Bacillus* YN-1.

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Section IV

Purification and Characterization of Cyanide-Insensitive Respiratory Terminal Component from Alkaliphilic *Bacillus* YN-1

Introduction

As discussed in Section III, a caa_3-type cytochrome *c* oxidase from an alkaliphilic bacterium, *Bacillus* YN-1, are successfully purified and characterized in detail. The enzyme also showed the TMPD-oxidizing activity. As described in Section II, I found three peaks with the TMPD-oxidizing activity on DEAE ion-exchange chromatography of the solubilized membranes prepared from alkaliphilic *Bacillus* YN-1. The fractions eluted at 0.45 M NaCl showed the cytochrome *c* oxidase activity and also the TMPD-oxidizing activity. The caa_3-type cytochrome *c* oxidase was purified from the fractions. On the other hand, the second fraction showing TMPD-oxidizing activity was eluted at 0.275 M NaCl. However, this fraction showed no cytochrome *c* oxidase activity and the activity was not changed by heat (100°C for 5 min) and 1% SDS treatments. The TMPD-oxidizing activity of this fraction was not inhibited by an addition of cyanide. The component was more purified with reverse-phase and gel filtration HPLC. Furthermore, the TMPD-oxidizing fraction with no cytochrome *c* oxidase activity was also found during the purification of *aco*-type cytochrome *c* oxidase from a facultative alkaliphilic bacterium, *Bacillus* YN-2000. These findings suggest that an alternative terminal component may participated in the respiratory chain of alkaliphilic bacteria.

In this study, I tried to isolate a cyanide-insensitive respiratory terminal component and to infer the structure of this component. It seems that the component include several peptides as basic structure. And I have investigated the physiological functions in the aerobic respiratory chain of the alkaliphilic bacterium, *Bacillus* YN-1.

Materials and Methods

Organism

Cultivation of alkaliphilic *Bacillus* YN-1 was performed as described in "Materials and Methods" of Section II.

Preparation of membrane from *Bacillus* YN-1

Membrane fraction of alkaliphilic *Bacillus* YN-1 was prepared as described in "Materials and Methods" of Section II.

Preparation of the cell walls from *Bacillus* YN-1

The cell walls was prepared from 10 g of frozen cells according to the method of Aono *et al* [1].

Measurements of the membranes respiratory activities

The oxygen consuming activity of the membrane were measured polarographically with a Clark-type oxygen electrode (model Mp-1000, Iijima Products M.F.G. Co., Ltd., Aichi, Japan) at 25°C. The standard reaction mixture contained the membranes (0.8 mg/ml) suspended in 25 mM Tris-HCl buffer, pH 8.0 in a volume of 2.0 ml. The reaction was started by addition of the respiratory substrates such as 10 mM NADH or 5 mM ascorbate *plus* 0.1 mM TMPD. Potassium cyanide was added to the reaction mixture before the addition of substrate. When ascorbate-TMPD was used as substrate, a control activity in the absence of membranes was subtracted from the value in the presence of membranes. The concentration of oxygen in the reaction mixture was determined to be 259 μmol/ml at 25°C according to the method of Winkler [2].

Measurement of the cytochrome *c* oxidase activity

The cytochrome *c* oxidase activity was measured spectrophotometrically at room temperature by following the decrease in the absorbance at 550 nm with a spectrophotometer (model MPS-2000, Shimadzu, Kyoto, Japan) using 1 cm light path cuvettes. The reaction mixture contained 25 mM sodium phosphate buffer, pH 6.5, 11.5 μM *S. cerevisiae* ferrocytochrome *c* and the sample in a total volume of 1 ml.

Measurement of the TMPD-oxidizing activity

TMPD oxidizing activity was measured spectrophotometrically at room temperature by following the increase in the absorbance at 606 nm. The reaction mixture contained 25 mM Tris-HCl buffer, pH 8.0, 1 mM TMPD and the sample in a final volume of 1 ml. The reaction was started by the addition of sample.

Spectroscopic analysis

Absorption spectrum of the sample was recorded by a spectrophotometer (model MPS-2000, Shimadzu, Kyoto, Japan) using 1 cm light path cuvettes at room temperature. FAB- and EI-mass spectra of the sample were recorded with a mass spectrometer (model JMS-AX505HA, JEOL Ltd., Tokyo). Glycerol was used as matrix. The sample was bombarded by Xe atoms at an acceleration voltage of -10 kV and a fast atom bombardment gun voltage of 3 kV, using the positive ion mode. The scan range was 25~1000 *m/z*. ^{1}H and ^{13}C-NMR spectra of the sample were measured with a spectrometers (model JNM-EX270, JEOL Ltd., Tokyo) at 270 and 67.8 MHz, respectively. DSS and 1,4-dioxane were used as the external references in the case of ^{1}H and ^{13}C, respectively. The lyophilized material was dissolved in D_2O for these NMR analyses.

Other determinations

Protein concentration was determined by using BCA Protein Assay Reagents (Pierce, Rockford, Ill., USA).

Reagents

Menaquinone of *Bacillus* YN-1 were extracted by hexane from the bacterial membranes, and were partially purified by according to the method of Wan *et al.* [3]. DEAE-Toyopearl 650 (Fractgel TSK DEAE-650-M) was purchased from the Tosoh Corporation (Tokyo, Japan). The packed column for HPLC, the Cosmosil $5C_{18}$ (4.6 x 250 mm) and the Polyhtdroxyethyl aspartamide were purchased from Nacalai Tesque (Kyoto, Japan) and Poly LC (Columbia, SC, USA), respectively. TLC pre-coated plates (glass) Cellulose F were purchased from Merck (Darmstadt, Germany). D_2O were purchased from SCETI Co.,Ltd. (Saint Aubin, France). All chemicals were of the highest grade commercially available.

Results and Discussion

Purification and characterization of cyanide-insensitive respiratory terminal component from *Bacillus* YN-1

The cell walls and the cytoplasmic membranes were prepared from the Gram positive bacterium, *Bacillus* YN-1 according to the method of Aono *et al.* [1] and by the methods as described in section of "Materials and Methods", respectively. The TMPD-oxidizing activity was not found in the cell walls fraction and in the soluble fraction. Only the cytoplasmic membranes showed high TMPD-oxidizing activity. After the cytoplasmic membranes were solubilized with 1% Triton X-100, the suspension was applied on a DEAE-Toyopearl column. Figure 1 shows the elution profile from first DEAE ion-exchange chromatography during the purification of caa_3-type cytochrome *c* oxidase from *Bacillus* YN-1. Cytochrome caa_3 was eluted at 0.45 M NaCl, while most of the TMPD-oxidizing activities were retained in the fraction eluted at 0.275 M NaCl. To find out whether the fraction eluted at 0.275 M NaCl is protein in nature or not, the fraction was incubated with 1% SDS at 100°C for 5 min. Surprisingly, the TMPD-oxidizing activity of the fraction was not affected by this treatment. Furthermore, the organic solvent such as pyridine had no effect on the TMPD-oxidizing activity of the fractions. Therefore, it is concluded that cyanide-insensitive respiratory terminal component is a stable non-proteinous material, and located in the cytoplasmic membranes.

After the anion-exchange chromatography, the component was further purified with reverse-phase HPLC column which had been equilibrated with H_2O containing 1% HCl as shown in Figure 2A. The active fraction was not adsorbed on the column, suggesting the hydrophilic property of the material. The active fraction was collected, concentrated by evaporating, then was subjected to the second HPLC equipped with a polyhydroxyethyl aspartamide gel filtration column (Figure 2B). The active fraction thus obtained was lyophilized and used as purified sample. The purity of the sample was confirmed with TLC analysis in BuOH-acetone-H_2O

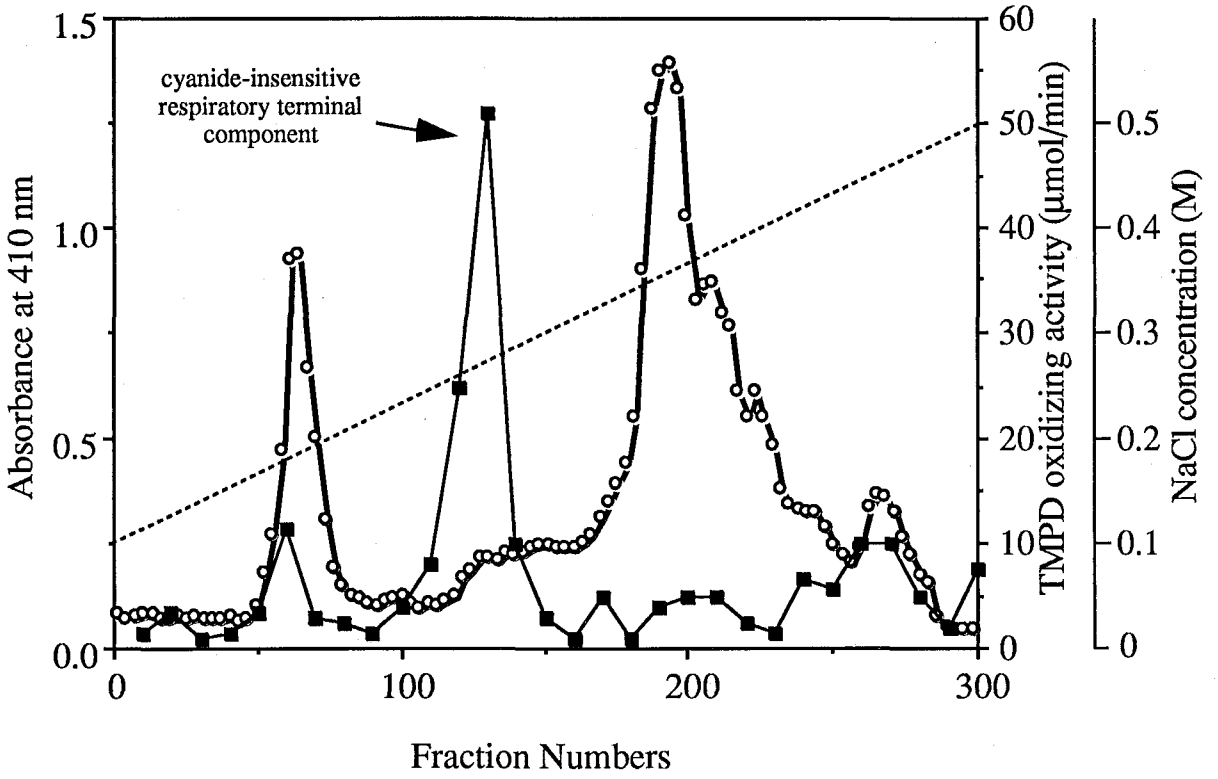

Figure 1 **Elution profile of DEAE ion-exchange chromatography during the purification of cytochrome *caa*$_3$ from *Bacillus* YN-1.** The column was equilibrated with 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM disodium EDTA, 10 μM PMSF and 1% Triton X-100. Cyanide-insensitive respiratory terminal component was eluted with a linear gradient of NaCl (0.1-0.5 M). (○), Absorbance at 410 nm; (■), the TMPD oxidizing activity (μmol/min); dashed line, NaCl concentration (M).

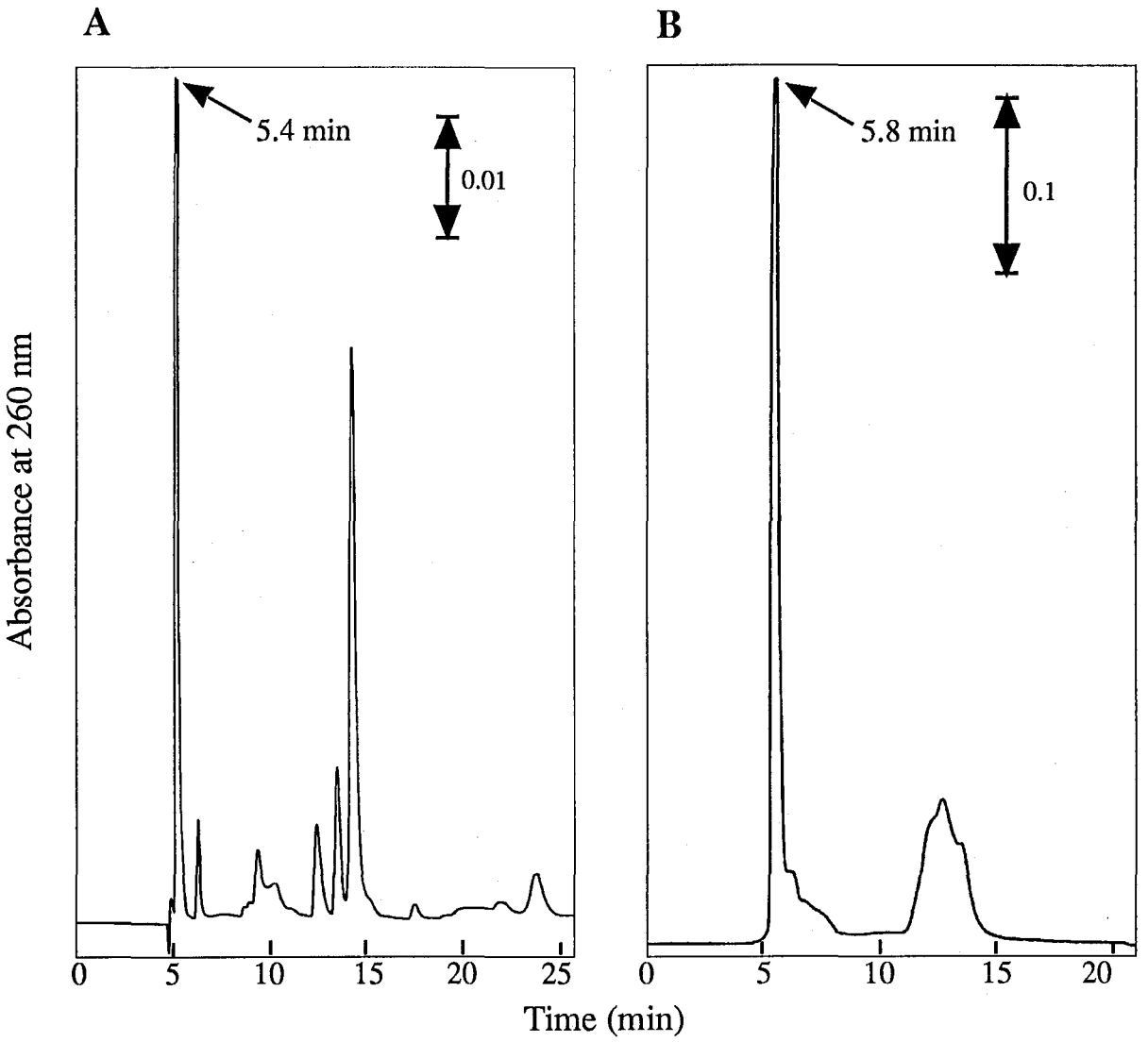

Figure 2 **Purification of cyanide-insensitive respiratory terminal component by HPLC.** (A) The elution profile by Cosmosil $5C_{18}$ reverse-phase HPLC. The elution was followed by the absorbance at 260 nm, the flow rate was 0.5 ml/min. The activity was eluted at 5.4 min. (B) The elution profile by gel filtration HPLC. The activity was eluted at 5.8 min.

(1:1:1, v/v). The average contents of this component in *Bacillus* YN-1 cells was estimated to be 2.0 mg per 1 g of cells (wet weight) in three individual experiments.

Molecular and catalytic properties of cyanide-insensitive respiratory terminal component

Figure 3A shows the effects of cyanide and catalase on the oxygen consuming activity catalyze by the purified cyanide-insensitive respiratory terminal component. When the ascorbate *plus* TMPD was used as electron donating system, the oxygen consuming rate was not affected by 5 mM cyanide. But, by an addition of catalase into the reaction mixture, the oxygen consuming rate was decreased by half as shown in Figure 3A. Figure 3B shows the oxygen-consuming activity of the membranes in the presence of 1 mM NADH and 5 mM cyanide. The activity was also decreased by half with an addition of catalase. These observations revealed that the cyanide-insensitive TMPD-oxidizing activity is not artificial, and hydrogen peroxide is produced during the reaction catalyzed by cyanide-insensitive respiratory terminal component.

Bacillus species have been known to contain menaquinones as respiratory electron carrier which mediate electron transferring reaction from NADH-quinone oxidoreductase (complex I) to quinol-cytochrome *c* oxidoreductase (complex III). Similar to the non-proteinous material in the present study, the quinones have also an oxygen-reducing activity to produce hydrogen peroxide [4]. However, the molecular and catalytic characteristics of cyanide-insensitive respiratory terminal component are very different from those of the menaquinones. First, the TMPD-oxidizing activity of the purified non-proteinous material was 0.182 mol of O_2/mol/min, while that of menaquinone isolated from the same bacterial cells was 0.0435 mol of O_2/mol/min (Table 1). Secondly, although the non-proteinous material was extracted from the bacterial membrane by detergent, the purified sample could not dissolve in any organic solvents such as methanol, ethylacetate and xylene. This hydrophilic character was completely different from the highly hydrophobic properties of menaquinone and its biosynthetic precursors. Thirdly, the absorption spectra in the near-ultraviolet region of the purified cyanide-insensitive respiratory

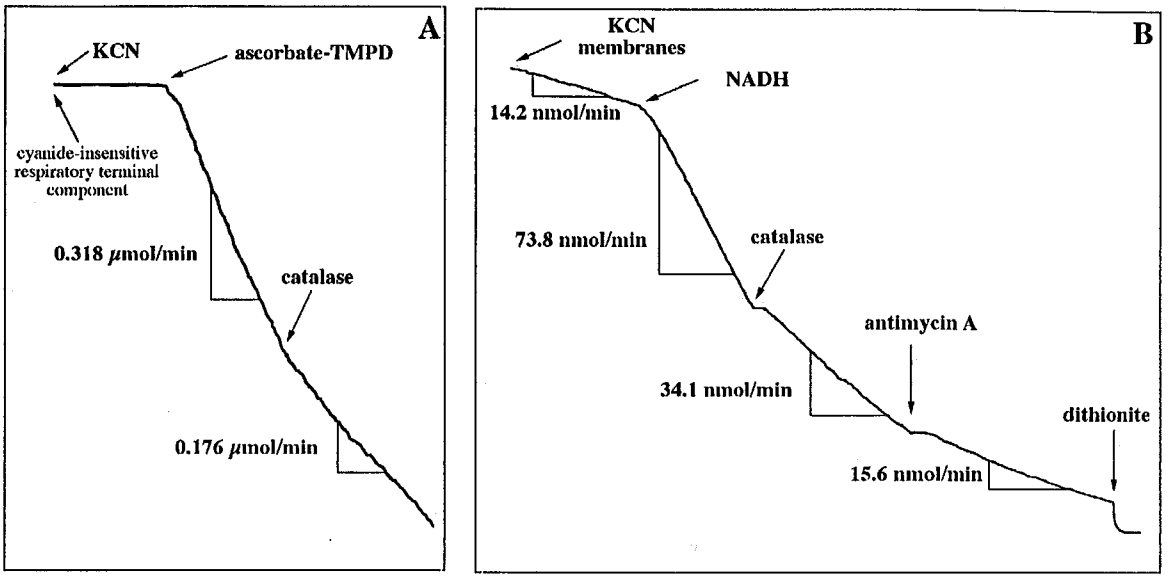

Figure 3 **Effect of catalase on the oxygen consuming activity.** (A) The oxygen consuming activity of the purified cyanide-insensitive respiratory terminal component. Before starting the reaction, 1.1 mg of the purified component was incubated with 5 mM potassium cyanide in the reaction mixture containing 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) The oxygen consuming activity of the membranes prepared from *Bacillus* YN-1. The membranes fraction containing 4.31 mg protein per ml were incubated with 5 mM potassium cyanide for 15 min at 25°C in the reaction mixture contained 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.

terminal component as shown in Figure 4A was considerably different from that of menaquinone which was purified from the same bacterium (Figure 4B). It revealed a symmetrical absorption peak at 261.5 nm in oxidized form, and a peak at 258.5 nm in reduced form which was prepared by the addition of 2.5 mg/ml of sodium borohydride. The millimolar extinction coefficient of cyanide-insensitive respiratory terminal component was estimated to be 0.88.

Table 1. Characteristic comparison between cyanide-insensitive respiratory terminal component and menaquinone purified from *Bacillus* YN-1

	cyanide-insensitive respiratory terminal component	menaquinone
solubility in water	soluble	insoluble
O_2 reduction activity (mol O_2/mol/min)	0.182	0.0453
spectral properties	oxi, 261.5 nm red, 258.5 nm	oxi, 269, 261, 248 nm red, 272 nm
molecular mass	662	MK-6, 580.9 MK-7, 649.0 MK-8, 717.1

Mass spectrometry of cyanide-insensitive respiratory terminal component from *Bacillus* YN-1

The spectrometry yielded a mass of 663.0 to $M+H^+$. Nothing was observed around the large *m/z* region (around 800~1000 *m/z*). Molecular mass of the non-proteinous material was analyzed by FAB- and EI-mass spectrometrical methods as shown in Figure 5A and B, respectively. On FAB-MS, the mass signals were observed at the *m/z* values of 663 and 648

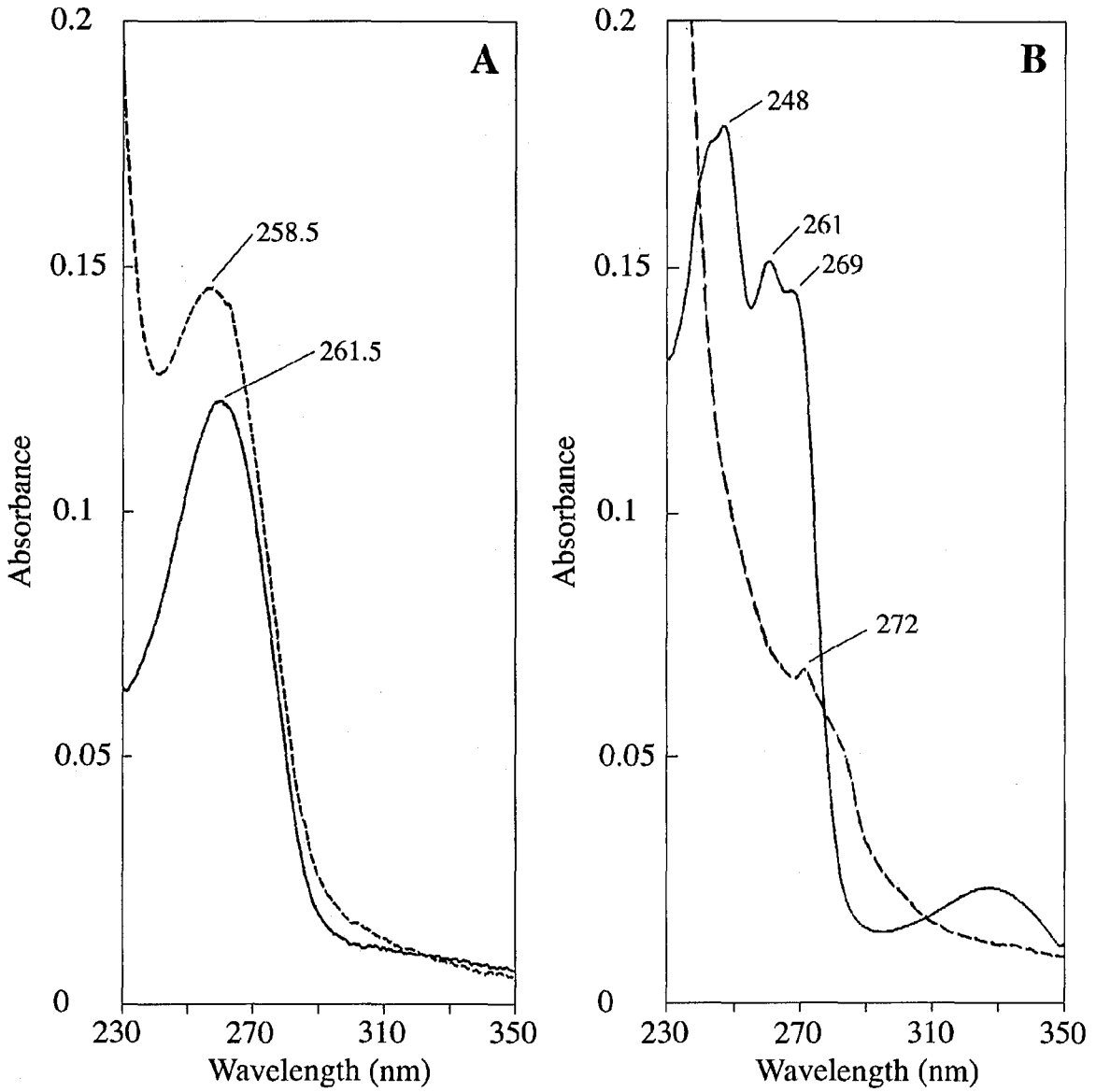

Figure 4 **Absorption spectra of the cyanide-insensitive respiratory terminal component (A) and menaquinone (B) purified from *Bacillus* YN-1.** (A) Purified sample (139 μM) was dissolved in water. (B) Menaquinone (8.33 μM) was dissolved in ethanol. Solid lines; oxidized form, dashed lines; reduced form by the addition of sodium borohydride.

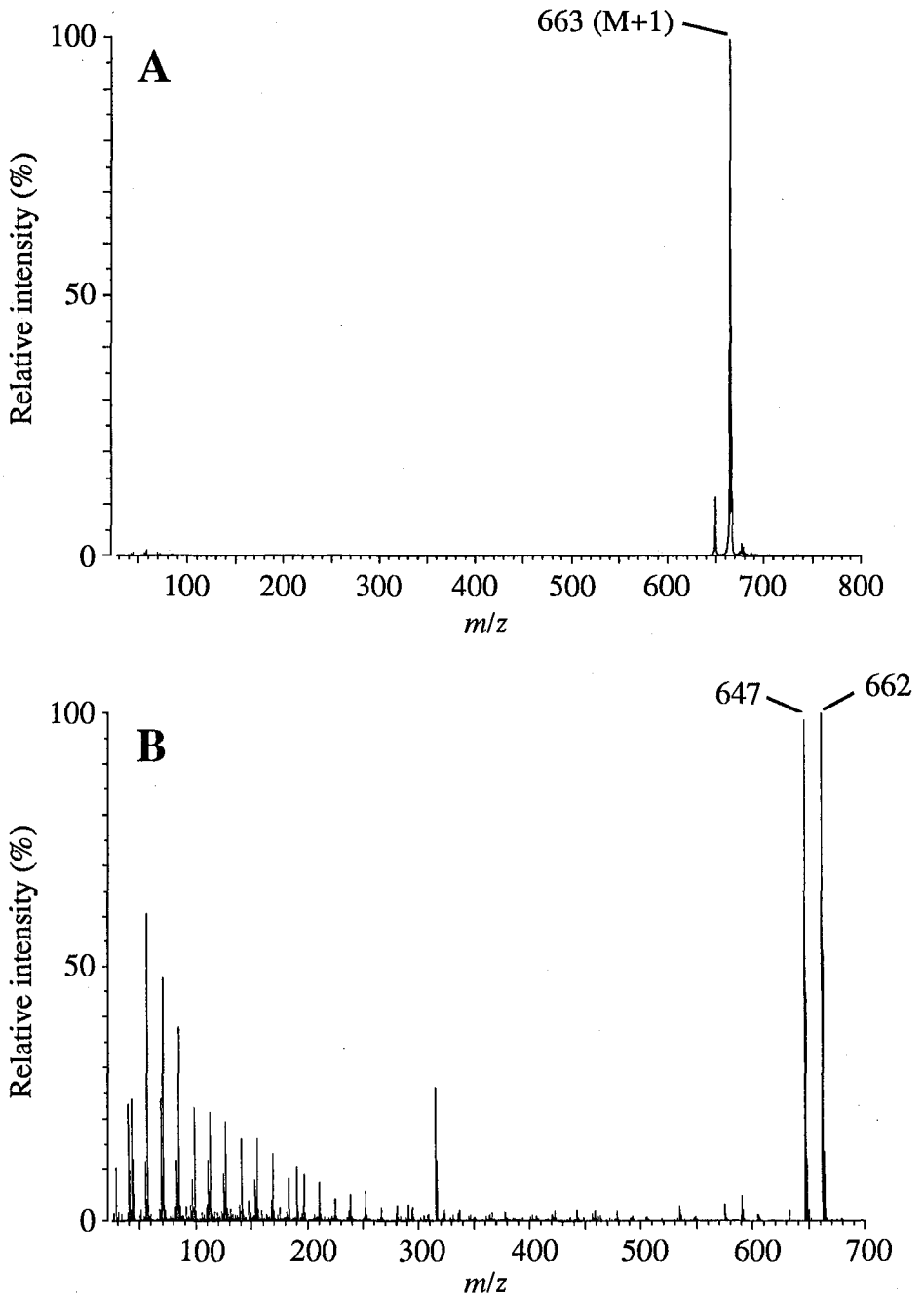

Figure 5 **Mass spectra of cyanide-insensitive respiratory terminal component from *Bacillus* YN-1.** (A) FAB-mass spectrum. Glycerol was used as matrix. The sample was bombarded by Xe atoms at an acceleration voltage of -10 kV and a fast atom bombardment gun voltage of 3 kV, using the positive ion mode. (B) EI-mass spectrum.

(Figure 5A). On EI-MS, mass signals of the fragments were observed mainly at the positions corresponding to the *m/z* values of 662, 647, and 316 (Figure 5B). No signal was observed at the large *m/z* region from 800 to 1000. Therefore, the molecular mass of cyanide-insensitive respiratory terminal component was determined to 662.0. The signal observed at the *m/z* of 647 might be the degraded fragment resulting from the removal of a methyl group from the component. Any menaquinones or its chemical derivatives with the molecular mass of 662 have not yet been reported.

NMR spectrometry of cyanide-insensitive respiratory terminal component from *Bacillus* YN-1

Figure 6A and B shows ^{1}H-NMR and ^{13}C-NMR spectra of cyanide-insensitive respiratory terminal component. On ^{1}H-NMR spectra, two large single peaks were observed at 3.61 and 3.87 ppm. These peaks were ignored for the analysis because these were extremely larger than other signals. The coupled double peaks were clearly observed at 1.38, 2.66, 4.29 ppm regions, respectively. On ^{13}C-NMR, Three large single peaks were observed at 51.4, 57.7, and 171.4 ppm. These peaks were also disregarded because of their excess. At around 180 ppm, noteworthy peaks were observed. These peaks may be originated with the carbonyl carbon of peptide group (-CO-NH-). Therefore, it seems likely that cyanide-insensitive respiratory terminal component contains peptides. The inference of cyanide-insensitive respiratory terminal component was described in following paragraphs.

The structural inference of cyanide-insensitive respiratory terminal component

Figure 7 shows the two dimensional (2D) NMR spectra (^{1}H-^{1}H COSY) in D_2O. Several cross peaks was observed. Notable cross peaks were observed between 1.38 ppm (dd, J=7.2 Hz, J=20.6 Hz) and 4.12 ppm or 4.38 ppm; 2.38 ppm or 2.66 ppm (dd, J=3.42 Hz, J=15.8 Hz) and 4.29 ppm (dd, J=3.05 Hz, J=10.0 Hz), indicated by circles as a and b, respectively. The signal at

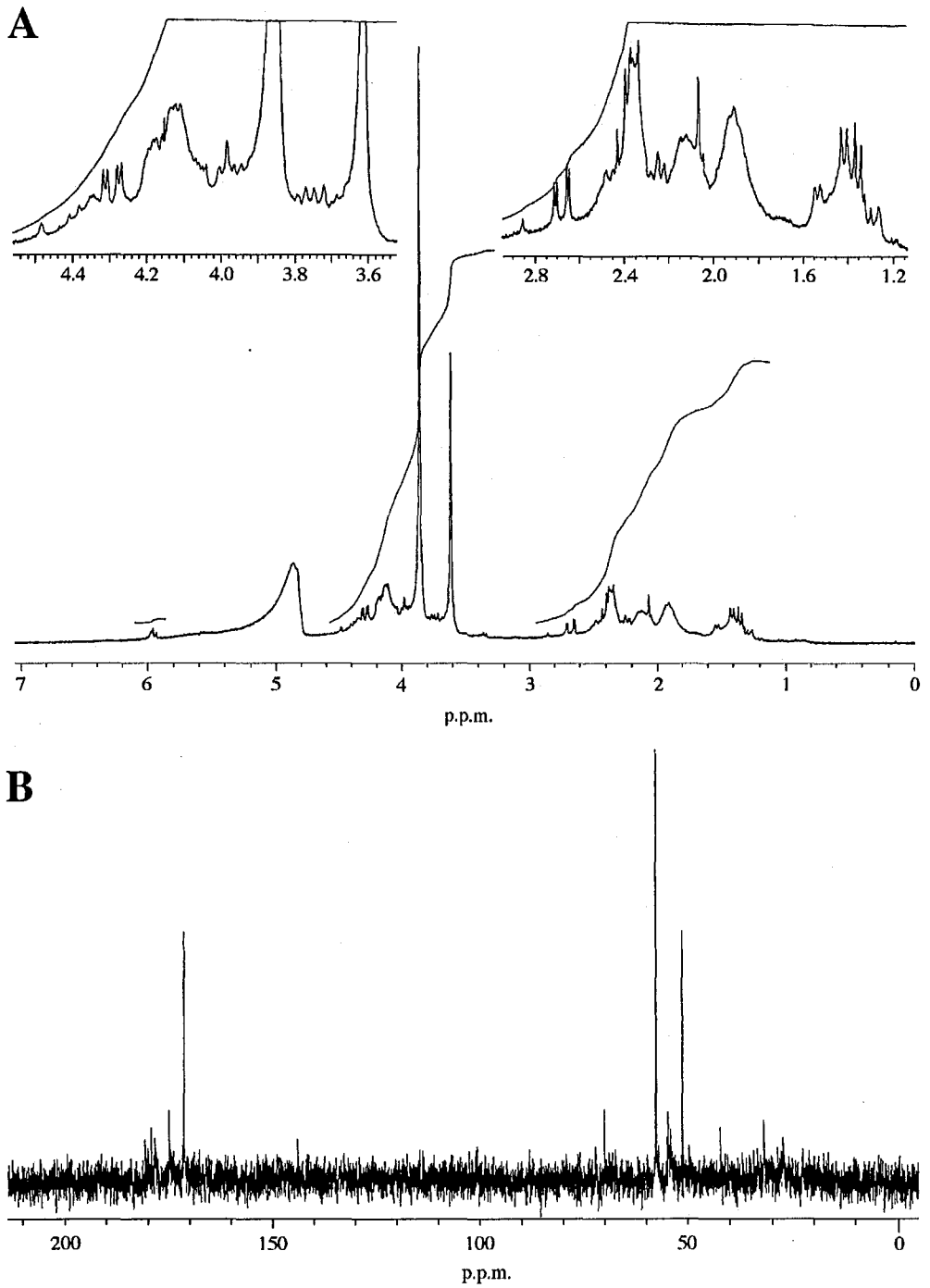

Figure 6 **NMR spectra of cyanide-insensitive respiratory terminal component purified from *Bacillus* YN-1.** (A) ^{1}H-NMR spectrum of cyanide-insensitive respiratory terminal component purified from *Bacillus* YN-1 in D_2O. (B) ^{13}C-NMR spectrum of cyanide-insensitive respiratory terminal component purified from *Bacillus* YN-1 in D_2O. The concentration of the sample was 20.7 mg/ml.

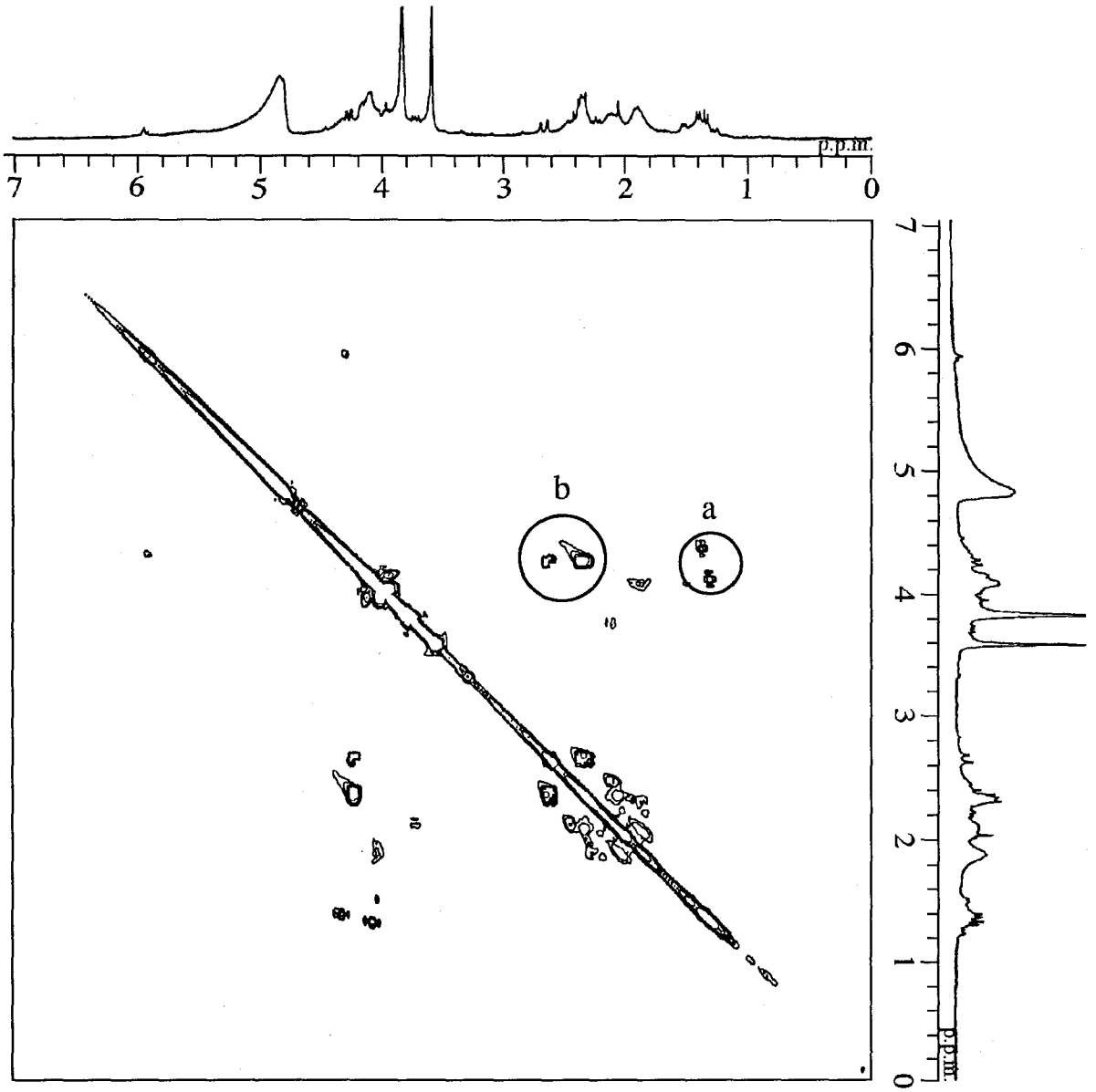

Figure 7 **Two dimensional NMR spectrum (^{1}H-^{1}H COSY) of cyanide-insensitive respiratory terminal component purified from *Bacillus* YN-1.** The sample was dissolved in D_2O. Cross peak assignments: (a) 1.38 ppm (dd, J=7.2 Hz, J=20.6 Hz) and 4.12 ppm or 4.38 ppm, (b) 2.38 ppm or 2.66 ppm (dd, J=3.42 Hz, J=15.8 Hz) and 4.29 ppm (dd, J=3.05 Hz, J=10.0 Hz).

1.38 ppm was only coupled with the protons at 4.12 or 4.38 ppm. These peaks may originate with the methyl group of Ala because there was no cross peak with 2.4~2.7 ppm region which indicate a methylene group protons of amino acids. This results indicates also that cyanide-insensitive respiratory terminal component does not contain the amino acids with methyl group at the end of side chain. The cross peaks of region b indicates the coupling between the protons of α-carbon and methylene groups. Furthermore, there is no signal caused by aromatic rings, imidazole rings and sulfur. Moreover, no specific signal which indicate the existence of sugar ring, Gly, Cys is observed at around 3~3.6 ppm region. From these results, the possible amino acid contained in cyanide-respiratory terminal component are Asx, Glx, Lys, Arg, and Pro. On the ^{13}C-NMR spectrum, the signal at 174.9 ppm which is originated with the carbon of carboxyl group. This result indicate that cyanide-insensitive respiratory terminal component may contain acidic amino acid Asp and/or Glu, corresponding to the properties which is able to adsorb on an anion-exchange column. Inferring from these all results, it seems likely that cyanide-insensitive respiratory terminal component is peptidic material consisted of 5~6 amino acids containing Ala, Asp and/or Glu.

The bacterial cell walls are consisted of peptidoglycan layer which is polymer of *N*-acetylmuramic acid and *N*-acetylglucosamine bridged by peptide.

In the case of Gram-positive bacteria, the peptide chain is consisted of tetrapeptide, L-Ala-D-Glu (D-isoGlu)-diamino acid (L-Lys or m-diaminopimelic acid)-D-Ala. These amino acid is similar to those of cyanide-insensitive respiratory terminal component. As described above, however, the cell walls prepared from *Bacillus* YN-1 did not show the TMPD-oxidizing activity. Therefore, cyanide-insensitive respiratory terminal component is different from tetrapeptide contained the cell walls.

In this Section, I have described about purification, catalytic property, and structural properties of cyanide-insensitive respiratory terminal component of *Bacillus* YN-1. It seems that the component is new type and essentially functions as terminal component in the respiratory

chain of *Bacillus* YN-1. However, the structural detail have not yet been clarified, although the component is considered to be peptidic material containing Ala and acidic amino acid. To elucidate the bioenergetic problem of alkaliphilic bacteria, it is necessary to clarify the complete structure of cyanide-insensitive respiratory terminal component.

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Section V

Concluding Remarks

Conclusion

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 catalytic center, functions as a terminal enzyme of aerobic 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 it can be considered the result mainly of the replacement of 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, have also been reported. Cytochrome *bd*-type oxidase has been reported from several bacteria, such as *Escherichia coli* [1], *Azotobacter vinelandii* [2], *Bacillus stearothermophilus* [3], and alkaliphilic *Bacillus firmus* OF4 [4]. 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 behaviors [5]. It has been reported that the activity of cytochrome *bd* was inhibited by antimycin A [6]. On the other hand, cyanide-insensitive respirations in plants and some yeasts have been reported [7-12], and a novel type of terminal oxidase in cyanide-insensitive respiration has been identified in plant mitochondria and the yeast *Hansenula anomala* [8-10, 13]. The enzyme consists of a single 36-kDa protein having non-heme iron as a cofactor, and its activity is specifically inhibited by salicylhydroxamic acid [14]. In spite of the structural variety among the three categories of respiratory terminal oxidases (heme-copper cytochrome oxidase, cytochrome *bd*, and cyanide-insensitive terminal oxidase), all of these enzymes catalyze 4-electron reduction of oxygen to produce water molecules.

In this study, a branched respiratory chain including two respiratory terminal components has been found in an obligate alkalophilic *Bacillus* YN-1. One of the two respiratory terminals is a typical caa_3-type heme-copper oxidase. The other respiratory terminal component is a cyanide-insensitive and non-proteinous material with high oxygen-reducing activity. As described in Section II, the oxygen-reducing activity of the membranes is not inhibited with cyanide. It is therefore probable that the cyanide-insensitive respiration occupies major part in the respiratory chain of *Bacillus* YN-1. This is also confirmed by the experimental evidence of low enzymatic activity and no proton-pumping activity of caa_3-type heme-copper oxidase of *Bacillus* YN-1 and rapid growth in the presence of cyanide.

Catalase catalyzes the decomposition of 2 mol of H_2O_2 to produce 1 mol each of O_2 and H_2O. Therefore, we have usually examined the effects of catalase on the reaction to investigate whether the terminal enzyme catalyzes 4-electron reduction of oxygen or not. As described in Section II, an addition of catalase to the reaction mixture catalyzed with the cyanide-insensitive respiratory terminal led to reduction of the on the reaction of the enzymatic activity. These results clearly show that the product of the oxygen reduction by the cyanide-insensitive respiratory terminal catalyze 2-electron reduction of oxygen to produce H_2O but H_2O_2. The isolated bacterial membranes also shows the H_2O_2-generating system with the oxygen consumption that coupled with oxidation of physiological respiratory substrates, such as NADH and succinate. Therefore, it seems likely that the cyanide-insensitive respiratory terminal physiologically functions as respiratory terminal in the aerobic respiratory chain of *Bacillus* YN-1.

How the bacteria protect itself from the cytotoxicity of active oxygen species such as H_2O_2. Most aerobic organisms contain superoxide dismutase and catalase for diminishing the O_2^- and H_2O_2 molecules that are unavoidably generated by various biological processes within the living cells. Similarly as reported in case of alkalophilic *Bacillus* YN-2000 [15], the content of catalase in *Bacillus* YN-1 is also very high (data not shown). Therefore, H_2O_2 generated during the respiratory process may be converted into oxygen by catalase *in vivo*.

Quinones, due to their self-oxidative character, have been known to show oxygen consuming activity under the presence of proper reductant. However, the oxygen reducing activity of the cyanide-insensitive respiratory terminal is about 4-times higher than that of menaquinone. Furthermore, it also shows differences in their hydrophobicities, absorption spectra, and molecular masses. These results strongly indicate that the cyanide- insensitive respiratory terminal purified in this study is neither a menaquinone nor a menaquinone derivative.

It is very important to know whether the cyanide-insensitive respiratory terminals widely distributed among the alkaliphilic bacteria. The similar cyanide-insensitive material with oxygen-reducing activity can be found in alkaline-cultured *Bacillus* YN-2000 and *Bacillus alcalophilus*. On the other hand, this component has not been found in *Bacillus* YN-2000 cultivated at pH 8.0 and in a neutralophilic *Bacillus subtilis*. These results suggests that this non-proteinous material is widely distributed among the alkaliphilic bacteria.

Finally, I would like to propose the novel respiratory oxygen-reducing mechanism of an obligate alkaliphilic *Bacillus* YN-1. As shown in Figure 1, the cyanide-insensitive pathway diverges at complex III and terminates with two elementary reactions. First, the non-proteinous component catalyzes the 2-electron reduction of oxygen to produce H_2O_2. Secondly, the toxic product is degraded by the effect of catalase. This combination of two reactions appears to generate water molecules as a final product of respiration in this system and accelerate the electron transfer between respiratory substrate and oxygen. The ultimate goal of research in this field is probably to reconstitute the respiratory chain with the purified components and produce ATP *in vitro*. The present study will give insights into the bioenergetic problems of alkaliphilic bacteria.

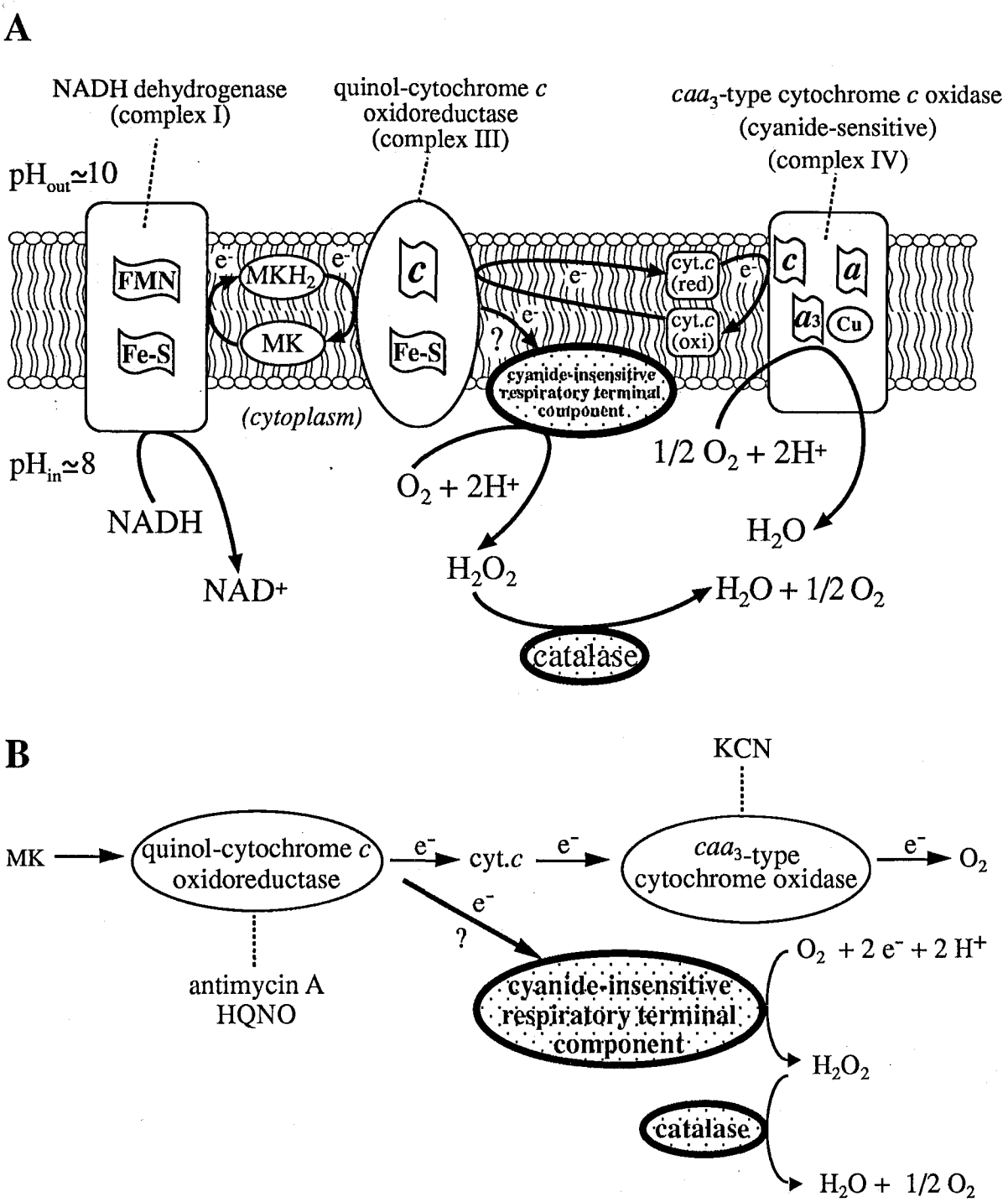

Figure 1 **Proposed model of electron transport system for alkaliphilic *Bacillus* YN-1.** Panel B is the simplified model of the respiratory terminals of *Bacillus* YN-1. Abbreviations used are; MK, menaquinone; MKH_2, menaquinol; cyt., cytochrome.

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A. Higashibata

Akira Higashibata

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