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メタデータ	言語: eng 出版者: 公開日: 2017-10-03 キーワード (Ja): キーワード (En): 作成者: メールアドレス: 所属:
URL	<a href="https://doi.org/10.24517/00010121">https://doi.org/10.24517/00010121</a>

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# Structural analyses of the deduced amino acid sequences of a novel type heme-copper terminal oxidase, cytochrome $aco_3$ , from alkalophilic *Bacillus* YN-2000

Kimitoshi Denda, Akira Oshima, and Yoshihiro Fukumori

**Abstract:** Cytochrome  $aco_3$  from a facultatively alkalophilic bacterium, *Bacillus* YN-2000, was found to be alkaline- and heat-tolerant. To better understand the structural features of *Bacillus* YN-2000 cytochrome  $aco_3$ , the gene encoding this enzyme was cloned and sequenced. Nucleotide sequence analyses of the region neighboring the  $acoI$  (subunit I) gene revealed that the  $acoII$  (subunit II) and  $acoIII$  (subunit III) genes were concomitantly clustered upstream and downstream of the  $acoI$  gene, respectively, forming an operon with transcriptional polarity. The deduced amino acid sequence of subunit I was highly similar to that of cytochrome  $caa_3$  from thermophilic bacterium *Bacillus* PS3 in which the heme  $a_3$  could be replaced with heme  $o$ . Furthermore, a marked paucity of basic amino acid residues was found in the cytochrome  $c$ -binding subunit II, which might be a result of the adaptation to a highly alkaline external milieu.

**Key words:** cytochrome  $c$  oxidase, alkalophile, thermostability, heme  $o$ , *Bacilli*.

**Résumé :** Le cytochrome  $aco_3$  du *Bacillus* YN-2000, une bactérie alcalophile facultative, s'est révélé tolérant à la température et aux conditions alcalines. Pour mieux connaître la structure du cytochrome  $aco_3$  du *Bacillus* YN-2000, le gène qui code cette enzyme a été cloné et séquencé. Les analyses de la séquence des nucléotides de la région avoisinante du gène  $acoI$  (sous-unité I) ont révélé que les gènes  $acoII$  (sous-unité II) et  $acoIII$  (sous-unité III) étaient regroupés de façon concomitante respectivement en amont et en aval du gène  $acoI$ , formant un opéron ayant une polarité de transcription. La séquence des acides aminés obtenue pour la sous-unité I était très semblable à celle du cytochrome  $caa_3$  de la bactérie thermophile de *Bacillus* PS3, dans laquelle l'hème  $a_3$  peut être remplacé par l'hème  $o$ . De plus des résidus d'acides aminés basiques ont rarement été retrouvés dans la sous-unité II de liaison au cytochrome  $c$ , ce qui pourrait être le résultat d'une adaptation à un milieu externe fortement alcalin.

**Mots clés :** cytochrome  $c$  oxydase, alcalophile, thermostabilité, hème  $o$ , *Bacilli*.

[Traduit par la Rédaction]

## Introduction

The membrane-associated oxidase involved in the terminal process of an aerobic respiratory chain catalyzes the reduction of molecular oxygen to water using either ferrocyclochrome  $c$  or quinol as an electron donor (Iwata 1998). In a previous report, we described the functional elements of the redox metal center in subunit I of the heme-copper terminal oxidase superfamily (Denda et al. 1995).

Recently, Yoshikawa (Tsukihara et al. 1996) and Iwata (Iwata et al. 1995) have determined the crystal structures of bovine heart mitochondrial cytochrome  $aa_3$  and *Paracoccus denitrificans* cytochrome  $aa_3$ , respectively, and they have provided a common structural model of the heme-copper respiratory oxidases. On the other hand, a variety of heme coordinations has been found in the various bacterial terminal oxidases (Brown et al. 1993). *Bradyrhizobium japonicum* cytochrome  $cbb_3$  binds two  $b$  hemes (Preisig et al. 1995), *Escherichia coli* cytochrome  $bo$  binds heme  $b$  and heme  $o$  (Chepuri et al. 1990), and *Sulfolobus acidocaldarius* SoxM complex binds heme  $a$  and heme  $b$  in subunit I (Lübben et al. 1994).

A novel cytochrome  $c$  oxidase was purified and characterized from facultatively alkalophilic *Bacillus* YN-2000 (Qureshi et al. 1990). Based on pyridine hemochrome spectra, the enzyme has one molecule each of heme  $c$ , heme  $a$ , and heme  $o$  in the minimal structural unit and, interestingly, functions as the only terminal oxidase at pH 7–10. Stopped-flow and rapid-scan measurements of the turnover of the enzyme demonstrated that heme  $o$  reacts with  $O_2$ , suggesting that electron flow is from heme  $c$  to heme  $o$  to oxygen (Oriei et al. 1991). Electron paramagnetic resonance and resonance

Received July 18, 2001. Revision received September 28, 2001. Accepted October 3, 2001. Published on the NRC Research Press Web site at <http://cjm.nrc.ca> on November 28, 2001.

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**Fig. 1.** Enzymatic stabilities of *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub> and mitochondrial cytochrome *aa*<sub>3</sub> after exposure to alkaline pH and high temperature. (A) Alkaline tolerance of *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub> (□) and mitochondrial cytochrome *aa*<sub>3</sub> (■) at pH 12. (B) Heat tolerance of *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub>. (C) Heat stability of mitochondrial cytochrome *aa*<sub>3</sub>. The incubation temperature is indicated in the figure.

Raman spectra indicated that the complex contained Cu<sub>A</sub>, and that the environment around heme *o* is similar to that observed with heme *a* in mitochondrial cytochrome *aa*<sub>3</sub> (Yumoto et al. 1993). These results suggest that the *Bacillus* YN-2000 complex, provisionally designed as cytochrome *aco*<sub>3</sub>, is closely related to mitochondrial cytochrome *aa*<sub>3</sub>. However, structural analyses of the enzyme complex remain to be achieved.

We report herein the characterization of the alkaline tolerance of *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub>. To investigate the structural and functional features of cytochrome *aco*<sub>3</sub>, we also report cloning of the genes for this cytochrome *aco*<sub>3</sub> and characterization of the deduced amino acid sequences of the enzyme complex.

## Materials and methods

### Bacterial growth

*Bacillus* YN-2000 was cultivated at pH 10.5 by the method of Qureshi et al. (1990). *Escherichia coli* XL1-Blue MRF' was used for PCR analysis, plasmid construction, and DNA sequencing (Sambrook et al. 1989).

### Purification of enzymes

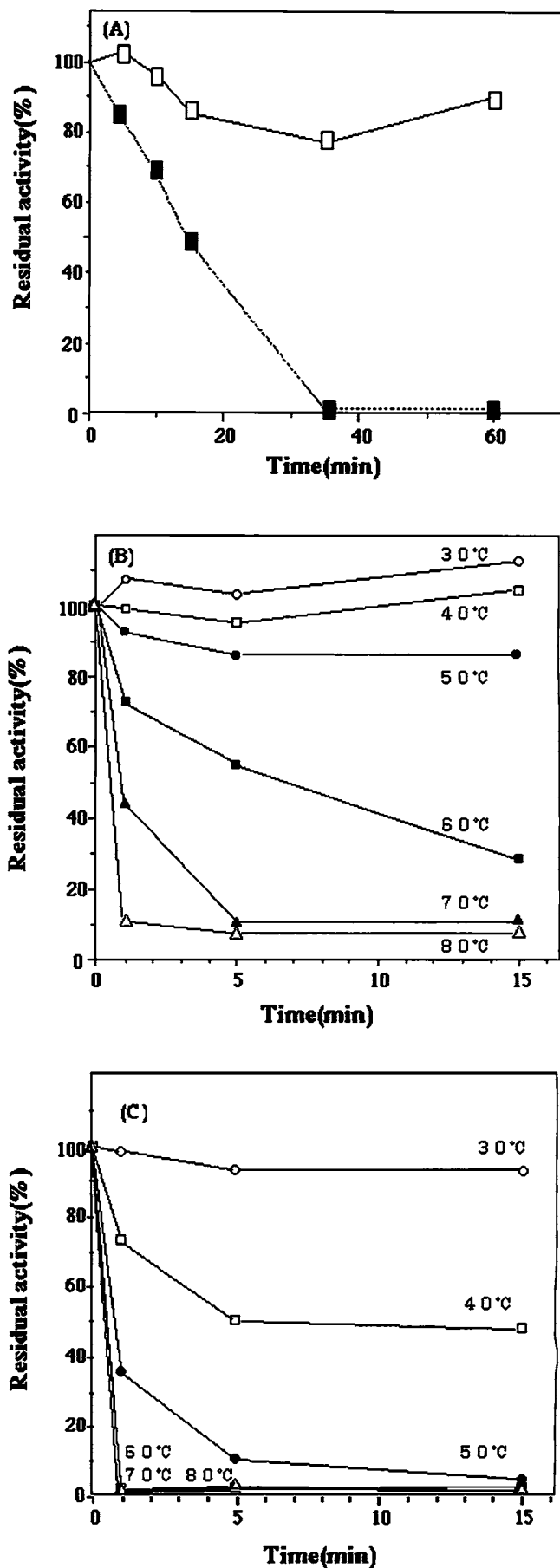
Cytochrome *aco*<sub>3</sub> was purified from *Bacillus* YN-2000, as described previously (Yumoto et al. 1993). Mitochondrial cytochrome *c* oxidase was purified from bovine heart, as described by Yonetani (1960).

### Stability measurements for alkaline and heat tolerance

After the enzymes were incubated at 30–80°C for 0–15 min, the cytochrome *c* oxidase activity of the enzyme was spectrophotometrically measured as follows. The standard reaction mixture contained 4.5 μM *Saccharomyces cerevisiae* ferrocyclochrome *c* and 25 mM sodium phosphate buffer, pH 6.5, with 1% Triton X-100 in a total volume of 1.0 mL. To the reaction mixture, we added 39 nM cytochrome *aco*<sub>3</sub> or 66 nM mitochondrial cytochrome *aa*<sub>3</sub>, and the oxidation of ferrocyclochrome *c* was followed by measuring the decrease of the absorbance at 550 nm over time. Alkaline tolerance was investigated as follows. After the enzyme preparation was incubated with 0.1 M glycine-NaOH buffer, pH 12, for 0–60 min, the cytochrome *c* oxidase activity was measured by the same method described above.

### Design of amplimers

Degenerate primers were designed and synthesized based on the published sequences of *E. coli* cytochrome *bo*<sub>3</sub>, *Paracoccus denitrificans* cytochrome *aa*<sub>3</sub>, *Bacillus subtilis* cytochrome *aa*<sub>3</sub>, and mitochondrial cytochrome *aa*<sub>3</sub> (Saraste 1990). The sequences of the degenerate primers were as follows: 5'ACOI(6F); 5'-TCTGGTTC(TA)TCG(GC)GCACCC-3' and 3'ACOIII(5R); 5'-(TG)CA(AGT)A(AGT)(AGT)AA(TG)GCCA-3'. Primer 5'ACOI(6F) was deduced from the helix VI located sequence FWWFGHP in subunit I. On the other hand, the primer 3'ACOIII(5R) corresponded to the reverse complement from the C-terminal sequence WHFVDV in subunit III.



## DNA manipulations

Standard recombinant techniques were carried out by the method of Sambrook (1989). Genomic DNA from *Bacillus* YN-2000 was prepared by the method of Marmur (1961). PCR was carried out in a reaction mixture containing 200  $\mu$ M deoxy-nucleoside triphosphate (Amersham-Pharmacia, U.K.), 67 mM Tris-HCl buffer (pH 8.8), 6.7 mM MgCl<sub>2</sub>, 0.25  $\mu$ M mixed oligonucleotide primers, and 2 U of DNA polymerase (TOYOBO, Japan) in a total volume of 100  $\mu$ L. The thermal cycling parameters were 30 s denaturation at 92°C, 90 s annealing at 50°C, and 120 s extension at 72°C; 30 cycles were performed.

## Cloning of the cytochrome oxidase locus

The PCR products were used to probe a library of *Bacillus* YN-2000 DNA. Southern blot analysis was carried out according to Sambrook et al. (1989). Consequently, the PCR-generated DNA probe hybridized to a 4.5-kb fragment, which encompassed the *acoII-acoI-acoIII* region of the *Bacillus* genomic DNA completely digested with *Pst*I. The bulk DNA fragments around this length were fractionated, using the Prep-A-Gene DNA purification systems (Bio-Rad, U.S.A.), after agarose gel electrophoresis and were ligated into pUC119. The ligation mixture was used to transform *E. coli* XL1-Blue MRF', and the transformants carrying an insert, which hybridized to the PCR probe, were investigated. All DNA sequences were confirmed by sequencing on both strands. Sequence data obtained were compiled and analyzed, using the BLAST program (Altschul et al. 1990).

## Results and discussion

### Alkaline and heat tolerance of *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub>

*Bacillus* YN-2000 is a gram-positive and facultative alkalophilic bacterium, which can grow at pH 7–11. The taxonomic position of the strain was recently identified (Yumoto et al. 2000). Morphological, biochemical, and chemotaxonomic properties indicated that *Bacillus* YN-2000 was closely related to the mesophilic alkalophile *Bacillus cohnii* (Spanka and Fritze 1993). In the present study, we first investigated the alkaline tolerance of *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub>. Figure 1A shows the effects of alkaline treatment on the cytochrome *c* oxidase activities of *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub> and bovine heart mitochondrial cytochrome *aa*<sub>3</sub>. The mitochondrial cytochrome *aa*<sub>3</sub> was completely denatured after 40 min of incubation at pH 12, while *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub> retained about 90% of the original activity even after 1 h of incubation at pH 12. Figures 1B and 1C show heat stability of *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub> and mitochondrial cytochrome *aa*<sub>3</sub>, respectively. Although the mitochondrial cytochrome *aa*<sub>3</sub> was stable at 30°C for 15 min in the presence of Triton X-100, the enzyme completely lost cytochrome *c* oxidase activity at 50°C. On the other hand, *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub> retained about 85% of the original activity under the same experimental conditions. *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub> was more stable at a higher temperature than was mitochondrial cytochrome *aa*<sub>3</sub>. Therefore, *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub> has unique structural features that are significantly different from mitochondrial cytochrome *aa*<sub>3</sub>. These findings raised a question about the consensus of structural elements that conferred thermal stability and prompted us to clone the gene of the cytochrome *aco*<sub>3</sub> to

**Table 1.** Amino acid composition of *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub>.

Amino acids	Moles percent deduced from DNA		
	YN-2000 <i>aco</i> <sub>3</sub> <sup>†</sup>	PS3 <i>caa</i> <sub>3</sub>	<i>B. subtilis</i> <i>caa</i> <sub>3</sub>
Asx	6.7 (8.9)	7.1	6.8
Thr	6.3 (6.4)	5.7	6.7
Ser	5.3 (5.7)	4.0	4.9
Glx	6.3 (8.8)	6.9	5.9
Pro	3.9 (6.0)	5.0	4.4
Gly	8.9 (9.9)	9.2	8.4
Ala	8.0 (9.4)	8.3	8.0
Cys	0.5 (–)	0.2	0.4
Val	7.6 (5.7)	6.6	7.7
Met	4.1 (2.3)	4.1	3.8
Ile	6.9 (5.9)	6.3	6.5
Leu	11.1 (10.2)	10.9	11.7
Tyr	3.3 (2.8)	3.8	3.2
Phe	9.1 (8.5)	8.7	8.5
Lys	4.1 (4.4)	5.2	5.4
His	2.9 (2.8)	2.7	2.7
Arg	2.3 (2.3)	2.9	2.5
Trp	2.7 (–)	2.4	2.5
Total	100 (100)	100	100

Note: Moles percent of amino acids deduced from protein\* are given in parentheses. YN-2000 *aco*<sub>3</sub>, PS3 *caa*<sub>3</sub>, and *B. subtilis* *caa*<sub>3</sub> denote *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub>, *Bacillus* PS3 cytochrome *caa*<sub>3</sub>, and *B. subtilis* cytochrome *caa*<sub>3</sub>, respectively.

<sup>†</sup>Yumoto et al. 1993.

\*Amount of cysteine and tryptophan was not determined.

directly compare its amino acid sequence with that of other terminal oxidases.

### Cloning and sequencing analysis of the *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub> genes

The cloning method is based on the PCR with the mixed oligonucleotide primers targeted to the highly conserved regions within subunit I and subunit III of the terminal oxidases. A 1.7-kb fragment of *Bacillus* YN-2000 genomic DNA was amplified with two degenerate primers, 5'ACO1(6F) and 3'ACOIII(5R). Subsequently, a 4.5 kb pair fragment, which hybridized to the 1.7 kb PCR amplified DNA fragment, was cloned from *Bacillus* YN-2000 DNA by colony hybridization and the nucleotide sequence determined. Table 1 shows the amino acid compositions of the predicted sequences deduced from *aco* genes and the purified cytochrome *aco* determined in our previous paper (Yumoto et al. 1993). Despite some discrepancies, overall data (see below) suggest that the *aco* gene products and cytochrome *aco*<sub>3</sub> oxidase are identical.

It is possible that the structural genes may be within a single operon and in the order *acoII-acoI-acoIII*, with very short intergenic sequences separating them. Based on the organization of genes coding for cytochrome *aco*<sub>3</sub> from *Bacillus* YN-2000, cytochrome *aco*<sub>3</sub> oxidase resembled that of the *caa*<sub>3</sub>-type oxidase from *B. subtilis*, thermophilic *Bacillus* PS3, and alkalophilic *Bacillus firmus*.

Figure 2 shows the alignments of the deduced amino acid sequences of the *aco* gene products. The *acoI* gene encoding subunit I was predicted to encode a polypeptide with 619

**Fig. 2.** Multiple alignments of amino acid sequences of subunit I (A), subunit II (B), and subunit III (C) of *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub> (B.YN) (accession No. D85547), *Bacillus* PS3 cytochrome *caa*<sub>3</sub> (B.PS3) (accession No. D13955), *Bacillus subtilis* cytochrome *caa*<sub>3</sub> (B.sub) (accession No. X54140), and *Bacillus firmus* cytochrome *caa*<sub>3</sub> (B.firmus) (accession No. M94110). The histidine residues ligated for heme a (o) and Cu<sub>B</sub> in subunit I are emphasized by white letters on the black background. The residues that are conserved in all four oxidases are indicated with asterisks (\*). The heme c binding site in subunit II was also indicated by white letters on the black background.

(A) B.YN-S1  
B.PS3-S1  
B.sub-S1  
B.firmus-S1

```

----MAQKKGFGATVWDYLTYYDHKKIAIMYLIAGGFFFLVGGLEAMFIRIQLKPFESGF
--STIARKKGVGATVWDYLTYYDHKKIAHLYLISGGFFFLGGLEALFIRIQLAKPWNDF
MLNALTEKTRGSMVWDYLTYYDHKKIAILYLVAGGFFFLVGGLEAMFIRIQLAKPENAF
----ATQKQ-EKSVIWDYLTYYDHKKIAIMYLIAGTLFFVKAGVMALFMRIQLMYPENMF
      *      ** ***** ** * ** * * * * * * *

```

B.YN-S1  
B.PS3-S1  
B.sub-S1  
B.firmus-S1

```

LEAGLYNEILTMIGTTMIFLAAMPLIFAFMNAVYPLQIGARDYAFPPFLNSLGFWIFFGGG
LVGGLYNEVLTMIIGTTMIFLAAMPLVFAFMNAVYPLQIGARDYAFPPFLNALGFWIFFGGG
LSAQAYNEVMTMIIGTTMIFLAAMPLIFALMNAVYPLQIGARDYSFPFLNALGFWIFFGGG
LSGQTFNEFITMIIGTTMIFLAATPLLFAMMNAVYPLQIGARDYAFPPVNALGFWIFFGGG
      *      ** ***** ** * ** * * * * * * *

```

B.YN-S1  
B.PS3-S1  
B.sub-S1  
B.firmus-S1

```

VFLNLSWFMGGAPDAGWTSYASLSLASEG-HGVDFYVVLGLQISGIGTLIAGINFLVTTIIN
LFLNCSWFLGGAPDAGWTSYASLSLDSKARHGIDFYTLGLQISGFGTIMGAINFLVTTIIN
IFLNLWFLGGAPDAGWTSYASLSLHSGK-HGIDFSILGLQISGLGTLIAGINFLVTTIIN
LLLNLWFLGGAPDAGWTSYASLSLSSRDYVGLGIDFYVVLGLQVSGIGTLISAINFLVTTIIN
      *      ** ***** ** * ** * * * * * * *

```

B.YN-S1  
B.PS3-S1  
B.sub-S1  
B.firmus-S1

```

MRAPGMTYMRMLPLFTWTFVASALILFAFPALTYGLLLMFDRMFGSAFFDPALGGNTII
MRAPGMTYMRMLPLFTWTFVASALILFAFPPLTYGLIFMMDRLFGGFFNPAAGGNTII
MRAPGMTYMRMLPLFTWTFVASALILFAFPPLTYGLALMLDRLFGTFFNFPALGGNTVI
MRAPGMTYMRMLPLFTWTFVASALILFAFPPLTYGLALMLDRLFEAQYFIPSMGGNTVL
***** ** * * * * * ***** * ** * * * * * * *

```

B.YN-S1  
B.PS3-S1  
B.sub-S1  
B.firmus-S1

```

WEHLFWIFGPEVYIILVLPAGGIFSEIFATFSKKRIFGYSSMVFATYVLIAGLGMVWAWHH
WEHLFWIFGPEVYIILVLPAGGIFSEIFATFSKKRIFGYSSMVFATYVLIAGLGMVWAWHH
WEHLFWIFGPEVYIILVLPAGGIFSEIVPVFARKRIFGYSSMVFATYVLIAGLGMVWAWHH
WQHIFWIFGPEVYIILVLPAGGIISEIVPAFARKRIFGYTAMVFATMIIAGLGMVWAWHH
* * * * ***** * * * * * * * * * * * * *

```

B.YN-S1  
B.PS3-S1  
B.sub-S1  
B.firmus-S1

```

MFTTGLGPIANSIFAVATMAIAPVPTGKIFNWLFTWGGQIKYNTAMLWAIAPISFVVG
MFTTGLGPIANAIFAVATMTIAPVPTGKIFNWLFTWGGQIKFTTPMNYAVAFIPSFVVG
MFTTGLGPIANAIFAVATMAIAPVPTGKIFNWLFTWGGQIKYNTAMLYAFSIFPVVGLG
MFTTGLGPIANSIFAVATMTIAPVPTGKIFNWLFTWGGQIKYNTAMLFASSFVPTFVGLG
*** * * * * ***** * * * * * * * * * * * * *

```

B.YN-S1  
B.PS3-S1  
B.sub-S1  
B.firmus-S1

```

GVTGIMLSAAAADYQVLDYFYVAHFIYVIVGGVVFALFAATHYVWPKMFGKVLDETLGK
GVTGIMLASAAAADYQVLDYFYVAHFIYVIVGGVVFALLAGTHYVWPKMFGKVLDETLGK
GVTGIMLAAAADYQVLDYFYVAHFIYVIVGGVVFGLLAGVHFWPKMFGKILNETMKG
GVTGIMLAMAPYDYLVDYFYVAHFIYVIVGGVILSLFAGLFYVWPKMFGKVLDETLGK
**** * * * * ***** * * * * * * * * * * * * *

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B.YN-S1  
B.PS3-S1  
B.sub-S1  
B.firmus-S1

```

ITFWLFFIGFHLTFPIQHFLGLMGMRRRYTFLPGQGFELGNFISTVGAFFMAAGTIVLL
ITFWLFFIGFHLTFPIQHFLGLTGMRRRYTFLPHQGWETGNLISITGAFFIAAATVILL
ISFVLFVIGFHLTFPIQHFLGLMGMRRRYTFLPGQGLETNLISITGAFFMAARVILL
LFFWVYVIGFHLTFVQHLGLMGMRRRYTFLGQQLDAFNFI STIGTFMAGSILLV
      * * ***** ** * * * * * * * * * * * * *

```

B.YN-S1  
B.PS3-S1  
B.sub-S1  
B.firmus-S1

```

VNIYKTSFSKQ-SVSGDVWGDGRTLEWATASPPLEYNFKQTPVLRGLDPLVWEKMGKKE
INIYVTTAKGE-KVPGDAWGDGRTLEWATASPPVYVNFQTPVLRGLDAFWLEKMGKKE
VNIYVTSYKGE-YVGDVWGDGRTLEWATASPPPEYNFKQTPVLRGLDPLVWEKMGKKS
INIYSAFKGERVTVADPW-DARTLEWATPTVPEYNFQTPVLRGLDPLVWEKMGKGDG
      *      * * * * * * * * * * * * * * * *

```

B.YN-S1  
B.PS3-S1  
B.sub-S1  
B.firmus-S1

```

MKPAEPYGDIMKMPNGSILPFIISLGLFIAAFG-AMYNQERTWGIP--VLIIGLVITFGSM
LTPAEPVGDIMKMPNSFLPFIIFGLFVAAFG-FTYKNDAGWGLP--VAILGLLITLGSIM
MTPAEPVDDIMKMPNGSILPLIISFGLFVAAFG-LLYRSDYAWGLP--VIFIGLVITFITH
MKPAEPVTDIMKMPNGSILPFIISLGLFIAAFG-LMNDKPIINPVIWVIGGLALFPGCM
**** ***** * * * * * * * * * * * * * * * *

```

B.YN-S1  
B.PS3-S1  
B.sub-S1  
B.firmus-S1

```

FFRSVIEDNGYHIHKEDIINDDDKGVGA--
FLRSVIEDNGYHIHKKEVLEL-----
LLRSVIEDNGYHIHKKEELPND-KGVKA--
FVRSIKEDNGYHIFAEQVKADLAELKKG
**** * *

```

Fig. 2 (concluded).

(B)

B. YN-S2 MKRWLPHLRVFLSFLVLAFLAGCG-KPYLSALQPVGEVADMMQMSLILLSTAIMTIVVVY  
 B. PS3-S2 MNKGLCNWRLFSLFGHMALLLAGCG-KPFLSTLQPAGEVADMMQYSLMLLSTSIMVLVIVY  
 B. sub-S2 ---MVKHWRLILLALLVPLLLSGCG-KPFLSTLQPAGEVADKQYDLTVLSTLIMVVVYAV  
 B. firmus-S2 -MKLWKTASRFLPLSFLTLFLTGCLGEEENLTALDPKGFQAQWYIDNMILSIIVMALYSIV  
 \* \* \* \* \*

B. YN-S2 VSIIFVYVVVKFRKRKGDDEFIPKQVEGSHKLEVIWTVPIPIILLLILAVPTVSYTFKLD  
 B. PS3-S2 YALIFVYVIRFRFRKGEENKIPKQVEGSHKLEIIVTVPIPIILLLILAVPTVSYTFKLD  
 B. sub-S2 VSVIFVYVIRFRFRSRVGEENTIPKQVEGSHKLEIIVTVPIPIILLLILAVPTVSYTFKLD  
 B. firmus-S2 VFAIFFIILAKYRRKPGDDE-IPKQVHGNTALEITWTVPIPIILLVILAVPTITGTFMFAD  
 \* \* \* \* \*

B. YN-S2 VEAMHNE-EED--VLVVDYTAELYWFEFYPGYGVVTSQDLVYPTDERIYFNLIASDVYK  
 B. PS3-S2 VKAMNDK-KRDKNVYVYVNRANQYWFEFYPDYGIITSDQLVYPTNEKYVFNLIASDVYK  
 B. sub-S2 TSPMDKGRKAEDALVYVNRANLYWFEFYPDYGIITSQELIYPTDQRYVFNKASDVYK  
 B. firmus-S2 KDPD-P--EVGDNTVYIKVTGHRQFWQFDYENEGFTAGQDVYIYVGEKVIYFELHAQDVLK  
 \* \* \* \* \*

B. YN-S2 SFVWP SAGGKLDTNTDYNVNSFWLSFDSKRAEEAGNLFYKCAELCGPSHALMDFK7QVYS  
 B. PS3-S2 SFWIPAVGGKMDTNTDNKNQFWLFDQKATDKAGGVFYKCAELCGPSHALMDFK7RPLP  
 B. sub-S2 SFWIPSYGGKLDTNTDNENKFFLTFDSKRSKEAGDMFFGKCAELCGPSHALMDFK7KMS  
 B. firmus-S2 SFVWPALGGKIDTVPGITNHRWLEAD-----EPG-VFKGCAELCGPSHALMDFK7LIALE  
 \*\*\* \* \*\*\* \* \* \* \* \*

B. YN-S2 RAEFDQWIEKMQNVEHPTTD-LAQAGEEIFA-QS CIGCN AISG-DAS----SIAPNLAN  
 B. PS3-S2 RDQFDAAWYKMTQNAKPKVYVTDYAKEGEAIFK-KS CIGCNAYTPLDKRPAQRRTAPNLAN  
 B. sub-S2 AKEFQGWTKEMKHYKSTAESK-LAKQGEELFKKMCIGCNAYEPNDKRAEAARTAPNLAN  
 B. firmus-S2 RDEYDAWYEGMS-AEVEEPTETLANQRQVFEENS CIGCNAYGGTGTATA-----AGPAPFN  
 \* \* \* \* \*

B. YN-S2 FGDRSRIAGILEHTPEKRLRLEDPDSVYKPAHEMTGTGTYGELNPDELDAVEYLMDLKFR  
 B. PS3-S2 FGDRSRIAGILEHNEENLKKWLRDPNSVYKPGHEMAGTYGHLTEEQIDALTKYLMKLVY-  
 B. sub-S2 FGERTKYGVEANKENYKAWLKDPSIKPGHEMTGTYPKLSDSETNALYEYKGLKAE  
 B. firmus-S2 FGEREVIAGYLENNDENLEAWIRDPQSLKQGNVQ-PAYPDMSEEDMEALIAVLRSLKVM  
 \*\*\* \* \* \* \* \*

B. YN-S2 KYFEREVTR  
 B. PS3-S2 -----  
 B. sub-S2 K-----  
 B. firmus-S2 -----

(C)

B. YN-S3 MHTEEKLTAETFPDSPEK-ATLEGKKNKFLGFWFLGGETVLFASLFATYALALKDSTAGTK  
 B. sub-S3 MCVQEKFTAETFPASPEK-VTEGKKNKFLGFWFLGGETVLFASLFATYALAAASNSNAGDP  
 B. PS3-S3 MHAEKLTAEFFPAAPERNTALEGKKNKFLGFWFLGGETVLFASLFATYALALKDSTAGTK  
 B. firmus-S3 -MAGAVDTSKGLPSKPER-ATLEGKKNKFLGFWFLGGETVLFATFFGTFLGRGOTADGP  
 \* \* \* \* \*

B. YN-S3 PGAEIFDLGLVFAATMILLTSSLT SVYAMYHMKNFNFRKMLWFMFTWLLGLSFLILEIY  
 B. sub-S3 PTFEMFDVTLVFIATMILLTSSLT SVYAMYHMKNFSFGKMLWLGITILLGAGFLGLEIY  
 B. PS3-S3 SAEEFLQMPVVFHATMILLTSSLT SVYAIYHMKNFDFKMLWFGITVLLGAGFLGLEIY  
 B. firmus-S3 TSADLVADLVFINTMILLTSSLT SVLAMFAHKKNFKAMTITWITVYVLLGLAFLGLEIY  
 \*\* \* \* \* \* \*

B. YN-S3 EFMHYH-LGHTFTSSAFSAFYTLVGTGAGVCFGLLWIGTLLIRYKGGNLYNAPKF  
 B. sub-S3 EFKHYTHFGFTITSSALGSAFYTLVGTGAGVAFGLMWTSTLMIRNAKRGNLYNAPKF  
 B. PS3-S3 EFNHYH-EGHKFTTSAFSAFYTLVGTGAGVSHVAFGLLWILTLMIRNAKRGNLYNAPKF  
 B. firmus-S3 EFMHYVVDYQGFSTSAFSAFYSLVGLHGAHVAFGLSWIIVLLIRYKSGITLTNAPKF  
 \*\* \* \* \* \* \*

B. YN-S3 YVASLYWHFLD????????????????  
 B. sub-S3 YVASLYWHFIDVYVVFIFTVVYLMGVG  
 B. PS3-S3 YVASLYWHFIDVYVVFIFTVVYLMGVG  
 B. firmus-S3 YVASLYWHFIDVYVVFIFTVVYLMGVG  
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residues whose molecular mass is 68 580 Da. As reported previously, the apparent molecular mass of subunit I was approximately 50 000 (Yumoto et al. 1993). As observed for other extremely hydrophobic proteins, including subunit I of *B. firmus* cytochrome *caa*<sub>3</sub>, this polypeptide is thought to migrate anomalously (Quirk et al. 1993). Subunit I is known to be the most structurally conserved subunit in the heme-copper respiratory oxidases. The deduced amino acid sequence was identical to subunit I of *B. firmus* cytochrome *caa*<sub>3</sub>, 64%; *B. subtilis* cytochrome *caa*<sub>3</sub>, 76%; *Bacillus* PS3 cytochrome *caa*<sub>3</sub>, 77%; and *E. coli* cytochrome *bo*, 56%. The hydropathy profile showed that it had 14 transmembrane helices, just like other *caa*<sub>3</sub>-type oxidases. It should be noted that there is little significant structural difference between cytochrome *aa*<sub>3</sub> and cytochrome *aco*<sub>3</sub>, in spite of their different types of heme specificity.

Subunit II was composed of three distinct domains as follows: the N-terminal integral membrane domain anchored to the membrane, the membrane peripheral copper domain, and the C-terminal cytochrome *c* domain. The *acoII* gene was predicted to encode 358 residues with a pI of 7.2 and with a molecular mass of 40 344 Da, which was well consistent with that (41 kDa) of the purified subunit II, as determined by SDS-polyacrylamide gel electrophoresis (Yumoto et al. 1993). Subunit II of *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub> was identical to that of *B. firmus* cytochrome *caa*<sub>3</sub>, 41%; *B. subtilis* cytochrome *caa*<sub>3</sub>, 56%; and *Bacillus* PS3 cytochrome *caa*<sub>3</sub>, 65%. Four invariant carboxylic residues (Glu-145, Asp-173, Asp-188, and Glu-219) involved in cytochrome *c* binding in subunit II were also conserved. The four residues (Cys-270, Cys-273, His-274, and Met-323), experimentally confirmed as the heme *c* binding site in other bacterial cytochrome *c* oxidases, were all conserved in the C-terminal domain of this subunit. This membrane peripheral cytochrome *c* domain contains only a few basic residues because of the replacements of conserved amino acid basic residues in the corresponding region of subunit II of cytochrome *caa*<sub>3</sub>. Consistent with the previous report by Quirk et al. (1993), the region faces the highly alkaline external environment. In general, adaptation to growth at an extremely alkaline pH features the avoidance of basic residues; therefore, substitutions in the peripheral region might assist in maintaining protein function in the highly alkaline exterior.

The hydropathy profile indicates that subunit III of *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub> has five putative transmembrane helices just like those of *caa*<sub>3</sub>-type oxidases. Although we obtained a DNA fragment including the *aco* operon, a few amino acid residues corresponding to the C-terminus in subunit III still remain to be determined completely. However, the amino acid stretch is highly homologous; the C-terminus sequence VVWVFIFTVVYLMGMVG is identical between subunit III of *B. subtilis* cytochrome *caa*<sub>3</sub> and that of *Bacillus* PS3 cytochrome *caa*<sub>3</sub>. Therefore, we tentatively assume that the C-terminal sequence of subunit III is identical. In this case, subunit III of cytochrome *aco*<sub>3</sub> comprised 206 residues with a molecular mass of 23 382 Da, which was consistent with the result from SDS-polyacrylamide gel electrophoresis (22 kDa) (Yumoto et al. 1993). The predicted amino acid sequence was identical to that of *B. firmus* cytochrome *caa*<sub>3</sub>, 64%; *B. subtilis* cytochrome *caa*<sub>3</sub>, 77%; *Bacillus* PS3 cytochrome *caa*<sub>3</sub>, 79%; and *E. coli* cytochrome *bo*, 42%.

Based on the sequence comparisons, subunit III, rather than subunit II, was remarkably identical among *B. subtilis*, *Bacillus* PS3, and *Bacillus* YN-2000, although the third subunit is not thought to be essential for the catalytic activity of the enzyme complex.

The data presented here suggest that the general structural features of the three minimal subunits of the *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub> are fundamentally common to the counterparts of several subclasses in the terminal oxidases (Table 1). However, this cytochrome *c* oxidase is both considerably alkaline- and heat-tolerant. Furthermore, the amino acid sequences of the three subunits are similar to those of the thermophilic bacterium *Bacillus* PS3 cytochrome *caa*<sub>3</sub>. It should be noted that under conditions of slight oxygen limitation, *Bacillus* PS3 synthesizes an alternative isozyme of cytochrome *caa*<sub>3</sub> in which heme *a* is substituted by heme *o* at the high-spin site to produce cytochrome *aco* (Sone and Fujiwara 1991). Therefore, it seems likely that although the gene organization, alignments of amino acid sequences, subunit composition, and hydropathy profile clearly identify this enzyme as a member of the superfamily of heme-copper respiratory oxidases, *Bacillus* YN-2000 cytochrome *aco*<sub>3</sub> may have the structural features widely conserved in the thermophilic enzymes. Recently, Niggemann and Steipe (2000) have suggested that local and nonlocal interactions may contribute to the thermostability of proteins. Therefore, further structural studies on respiratory terminal oxidases from *Bacilli* will provide insight into how the thermostability is correlated with tolerance of the enzyme to high pH.

## Acknowledgements

The authors wish to acknowledge the financial support of a Grant-in-Aid for Scientific Research (C) (09660076) and a Grant-in-Aid for Scientific Research on Priority Areas (10129208) from the Ministry of Education, Science, Sports, and Culture of Japan. We are also grateful to Dr. F. Arisaka and Dr. T. Suzuki, Tokyo Institute of Technology, for their helpful discussions.

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