Structural analyses of the deduced amino acid sequences of a novel type heme-copper terminal oxidase, cytochrome aco3, from alkalophilic Bacillus YN-2000

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journal or publication title	Canadian Journal of Microbiology
volume	47
number	12
page range	1075-1081
year	2001-01-01
URL	http://hdl.handle.net/2297/9565

doi: 10.1139/cjm-47-12-1075

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

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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 *aco*I (subunit I) gene revealed that the *aco*II (subunit II) and *aco*III (subunit III) genes were concomitantly clustered upstream and downstream of the *aco*I 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 *aco*I (sous-unité I) ont révélé que les gènes *aco*II (sous-unité II) et *aco*III (sous-unité III) étaient regroupés de façon concominante respectivement en amont et en aval du gène *aco*I, 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 ferrocytochrome *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 (Orii 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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 DOI: 10.1139/cjm-47-12-1075

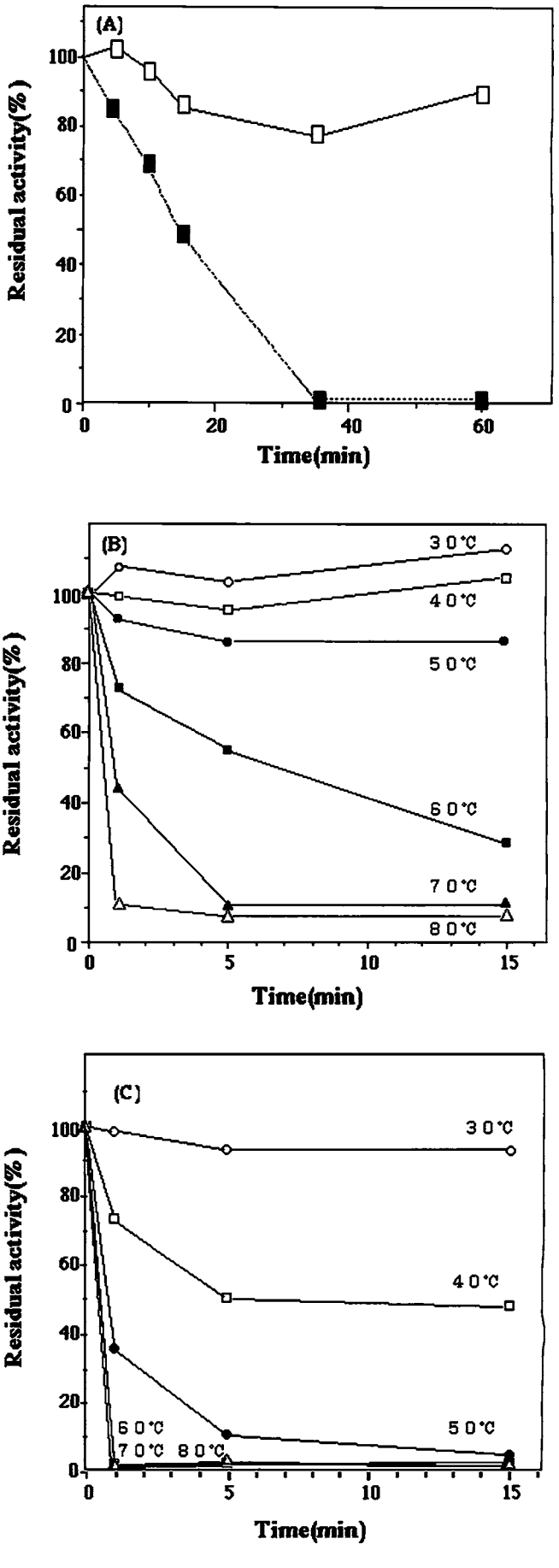


Fig. 1. Enzymatic stabilities of *Bacillus* YN-2000 cytochrome aco_3 and mitochondrial cytochrome aa_3 after exposure to alkaline pH and high temperature. (A) Alkaline tolerance of *Bacillus* YN-2000 cytochrome aco_3 (□) and mitochondrial cytochrome aa_3 (■) at pH 12. (B) Heat tolerance of *Bacillus* YN-2000 cytochrome aco_3. (C) Heat stability of mitochondrial cytochrome aa_3. The incubation temperature is indicated in the figure.

Raman spectra indicated that the complex contained Cu_A, and that the environment around heme *o* is similar to that observed with heme *a* in mitochondrial cytochrome aa_3 (Yumoto et al. 1993). These results suggest that the *Bacillus* YN-2000 complex, provisionally designed as cytochrome aco_3, is closely related to mitochondrial cytochrome aa_3. 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_3. To investigate the structural and functional features of cytochrome aco_3, we also report cloning of the genes for this cytochrome aco_3 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_3 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* ferrocytochrome *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_3 or 66 nM mitochondrial cytochrome aa_3, and the oxidation of ferrocytochrome *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_3, *Paracoccus denitrificans* cytochrome aa_3, *Bacillus subtilis* cytochrome aa_3, and mitochondrial cytochrome aa_3 (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 FWFFGHP 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 μM deoxynucleoside triphosphate (Amersham-Pharmacia, U.K.), 67 mM Tris-HCl buffer (pH 8.8), 6.7 mM $MgCl_2$, 0.25 μM mixed oligonucleotide primers, and 2 U of DNA polymerase (TOYOBO, Japan) in a total volume of 100 μL. 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 *aco*II–*aco*I–*aco*III 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_3

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_3. Figure 1A shows the effects of alkaline treatment on the cytochrome *c* oxidase activities of *Bacillus* YN-2000 cytochrome aco_3 and bovine heart mitochondrial cytochrome aa_3. The mitochondrial cytochrome aa_3 was completely denatured after 40 min of incubation at pH 12, while *Bacillus* YN-2000 cytochrome aco_3 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_3 and mitochondrial cytochrome aa_3, respectively. Although the mitochondrial cytochrome aa_3 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_3 retained about 85% of the original activity under the same experimental conditions. *Bacillus* YN-2000 cytochrome aco_3 was more stable at a higher temperature than was mitochondrial cytochrome aa_3. Therefore, *Bacillus* YN-2000 cytochrome aco_3 has unique structural features that are significantly different from mitochondrial cytochrome aa_3. 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_3 to directly compare its amino acid sequence with that of other terminal oxidases.

Table 1. Amino acid composition of *Bacillus* YN-2000 cytochrome aco_3.

Amino acids	Moles percent deduced from DNA		
	YN-2000 aco_3[†]	PS3 caa_3	*B. subtilis* caa_3
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_3, PS3 caa_3, and *B. subtilis* caa_3 denote *Bacillus* YN-2000 cytochrome aco_3, *Bacillus* PS3 cytochrome caa_3, and *B. subtilis* cytochrome caa_3, respectively.
[†]Yumoto et al. 1993.
*Amount of cysteine and tryptophan was not determined.

Cloning and sequencing analysis of the *Bacillus* YN-2000 cytochrome aco_3 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'ACOI(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_3 oxidase are identical.

It is possible that the structural genes may be within a single operon and in the order *aco*II–*aco*I–*aco*III, with very short intergenic sequences separating them. Based on the organization of genes coding for cytochrome aco_3 from *Bacillus* YN-2000, cytochrome aco_3 oxidase resembled that of the caa_3-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 *aco*I 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_3 (B.YN) (accession No. D85547), *Bacillus* PS3 cytochrome caa_3 (B.PS3) (accession No. D13955), *Bacillus subtilis* cytochrome caa_3 (B.sub) (accession No. X54140), and *Bacillus firmus* cytochrome caa_3 (B.firmus) (accession No. M94110). The histidine residues ligated for heme a (*o*) and Cu_B 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.

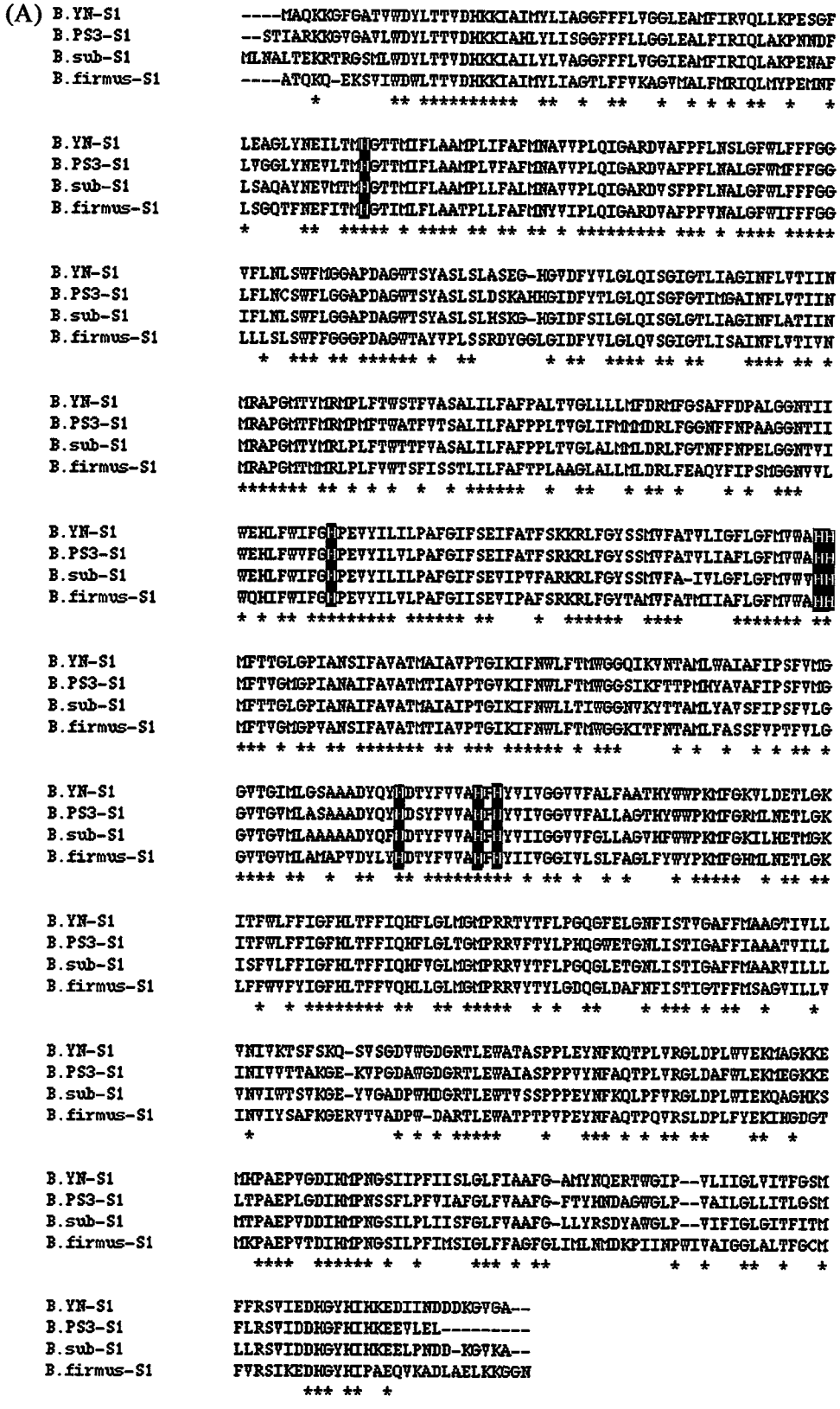
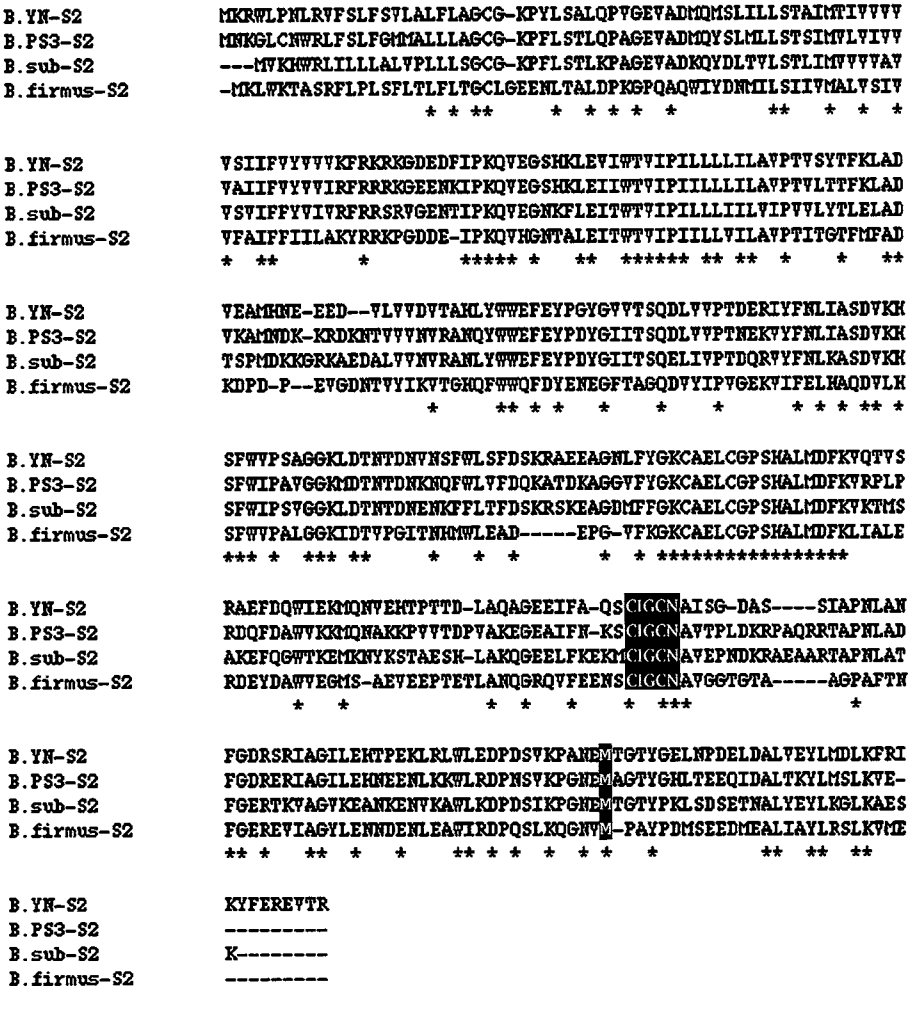
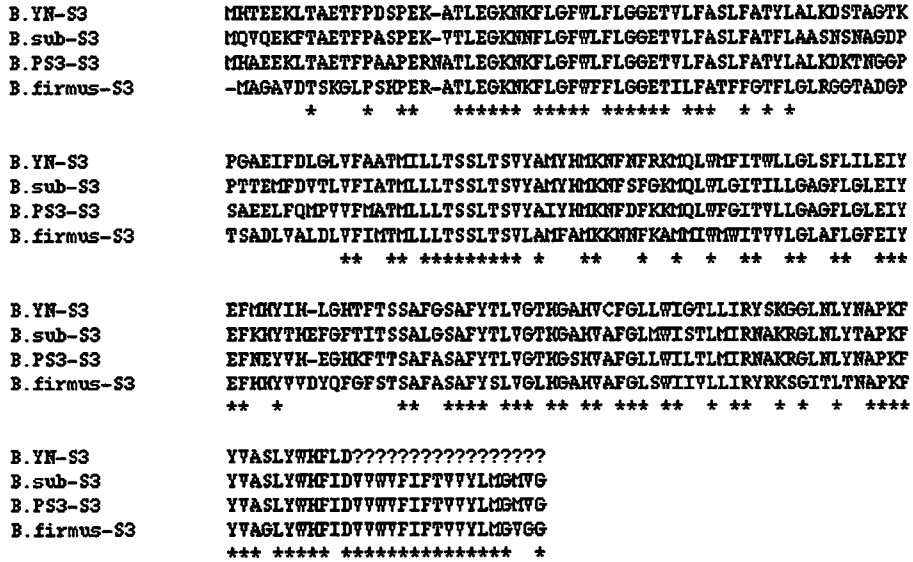


Fig. 2 (*concluded*).

(B)



(C)



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_3, 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_3, 64%; *B. subtilis* cytochrome caa_3, 76%; *Bacillus* PS3 cytochrome caa_3, 77%; and *E. coli* cytochrome *bo*, 56%. The hydropathy profile showed that it had 14 transmembrane helices, just like other caa_3-type oxidases. It should be noted that there is little significant structural difference between cytochrome aa_3 and cytochrome aco_3, 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 *aco*II 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_3 was identical to that of *B. firmus* cytochrome caa_3, 41%; *B. subtilis* cytochrome caa_3, 56%; and *Bacillus* PS3 cytochrome caa_3, 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_3. 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_3 has five putative transmembrane helices just like those of caa_3-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_3 and that of *Bacillus* PS3 cytochrome caa_3. Therefore, we tentatively assume that the C-terminal sequence of subunit III is identical. In this case, subunit III of cytochrome aco_3 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_3, 64%; *B. subtilis* cytochrome caa_3, 77%; *Bacillus* PS3 cytochrome caa_3, 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_3 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_3. It should be noted that under conditions of slight oxygen limitation, *Bacillus* PS3 synthesizes an alternative isozyme of cytochrome caa_3 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_3 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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