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Nucleotide and amino acid sequences for cytochrome caa_3-type oxidase of *Bacillus stearothermophilus* K1041 and non-Michaelis-type kinetics with cytochrome *c*

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Abstract

A pseudo-sigmoidal cytochrome *c*-dependence curve of oxidase activity was observed with cytochrome oxidase from the *Bacillus stearothermophilus* strain K1041, while the other thermophilic *Bacillus* PS3 which has been extensively studied possessed normal Michaelis-Menten type kinetics. The genes coding for four subunits of cytochrome caa_3-type oxidase and for heme O synthase were isolated from a genomic DNA library of K1041 by using a PS3 DNA fragment containing the highly-conserved region of the largest subunit as a probe, and sequenced. Most residues in subunits I (COI/*caaB* product), III (COIII/*caaC* product), and IV (COIV/*caaD* product) of K1041 were highly conserved when compared with those of PS3. However, the sequence of K1041 subunit II (COII/*caaA* product) was distinctly different from that of the PS3 subunit II. These *Bacillus* COIIs have an additional sequence for cytochrome *c* after the Cu_A binding protein portion with two transmembrane segments which is homologous to the mitochondrial counterpart, and represents the site of electron ingress. Several charged residues in the vicinity of cytochrome *c* moiety are replaced by oppositely charged residues. It is likely that these amino acid replacements in subunit II are the cause of the abnormal sigmoidal saturation curve for extrinsic cytochromes *c* of the K1041 enzyme.

Keywords: Cytochrome *c* oxidase; *ctaBCDEF* operon; DNA sequence; Protein sequence; Heme O synthesis; (*B. stearothermophilus*)

1. Introduction

Several bacterial cytochrome oxidases such as those from thermophilic *Bacillus PS3* [1,2] and *Thermus thermophilus* HB8 [3,4] contain covalently-bound cytochrome *c* in their COII. Although these features were once interpreted as characteristics of the oxidases from thermophiles, DNA analyses revealed that mesophilic *Bacilli* such as *B. subtilis* [5] and *B. firmus* [6] as well as thermophilic *Bacillus* PS3 [7,8] and *T. thermophilus* [9] share the feature that a sequence coding for cytochrome *c* moiety is found just 3′-downstream of the sequence for the Cu_A-binding site of COII. Since these oxidases have cytochrome *a* and a binuclear center composed of cytochrome a_3 and Cu_B in addition to the cytochrome *c* moiety, these enzymes are often called cytochrome caa_3 [10,11]. The intrinsic cytochrome *c* moiety is shown to mediate electron flow from extrinsic cytochrome *c* with the PS3 [2] and *Thermus* enzymes [4], and is supposed to correspond not to cytochrome c_1 [3,12], but to tightly-bound cytochrome *c* [13,14]. In fact, these enzymes displayed simple Michaelis-type kinetics against the concentration of externally added cytochrome *c*, while mitochondrial cytochrome aa_3-type oxidase gave 'two K_m values' [13,14]. It is also noteworthy that cytochrome *c* of Gram-positive bacteria must be membrane-bound, since they lack periplasmic or intermembranous space in which cytochromes *c* are generally confined to work efficiently [15–17].

Bacillus stearothermophilus strain K1041 (*Bst* K1041), isolated from soil in Japan, was reported to transform with plasmid *p*UB110 at the highest efficiency by electropora-

Abbreviations: COI–IV, subunit I–IV of cytochrome *c* oxidase; HPLC, high pressure liquid chromatography; *Bst*, *Bacillus stearothermophilus*; SDS, sodium dodecyl sulfate; SDS-PAGE, polyacrylamide gel electrophoresis with SDS; PCR, polymerase chain reaction; TMPD, *N,N,N′,N′*-tetramethyl *p*-phenylenediamine; Mops, 4-morpholinepropanesulfonic acid; kDa, kilodalton; ORF, open reading frame.

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tion among 67 strains tested [18]. This strain grows at up to 65°C, and is easily cultured and lysed with lysozyme as the thermophilic *Bacillus* PS3 [1,19]. Its membrane fraction also displays similar respiratory activity and absorption spectra. However, the cytochrome *c*-dependence of the oxidase activity was apparently sigmoidal, quite different from those of the PS3 and mitochondrial enzymes. Using DNA encoding a conserved part of COI of PS3 cytochrome *c* oxidase, we have cloned the genes encoding subunits of K1041 oxidase. Analyses of the deduced amino acid sequences of the K1041 enzyme revealed several interesting amino acid replacements in COII. From *B. stearothermophilus* strain ATCC 7954, De Vrij et al. purified a cytochrome caa_3-type oxidase whose general properties were very similar to those of the thermophilic *Bacillus* PS3, but they did not report cytochrome *c* concentration dependency of its oxidase activity [20].

Here, we report the cytochrome *c* dependency of the thermophilic *Bacillus* oxidases, and DNA sequence coding for the four subunits of the K1041 cytochrome aa_3-type oxidase and heme O synthase. We also highlight several characteristics of the deduced amino acid sequences.

2. Materials and methods

2.1. Materials

Membrane fractions were prepared from PS3 and *Bst* K1041 as described previously [1,19]. Oligonucleotides were synthesized with an Applied Biosystems DNA Synthesizer Model 381A using beta-cyanoethyl phosphoamidites as monomers. The large fragment of *Escherichia coli* DNA polymerase, restriction enzymes, M13 vectors, sequencing primers, and a deletion kit were purchased from Takara Shuzo (Kyoto). ^{32}P-labelled nucleotide triphosphates and DEAE-Toyopearl (DEAE-Fractogel) 650S were purchased from Amersham and Toso (Tokyo), respectively.

2.2. Purification of cytochrome c oxidases

PS3 cytochrome *c* oxidase was prepared as described previously [19]. *Bst* K1041 oxidase was purified similarly as PS3 cytochrome *c* oxidase [19]. However, the purification was carried out only to step 5, and the DEAE-cellulose (step 4) was replaced by DEAE-Fractogel. Since the affinity of the *Bst* enzyme was higher than that of the PS3 enzyme, the elution buffer contained 20 mM NaCl instead of 10 mM to elute the *Bst* enzyme from the DEAE-Fractogel columns.

2.3. Isolation of the caa / cta operon and sequencing

Purified genomic DNA of *Bst* K1041 was partially digested with *Sau*3A, fractionated with agarose gel electrophoresis, and ligated to the *Bam*HI site of the M13-mp19 phage vector. This library was screened with a ^{32}P-labelled probe, which was prepared by PCR using cloned DNA (*Pst*I-*Eco*RI fragment, 2059–3430 of *caa*B) from PS3 [8] as a template and the oligonucleotides 5′-dTGGATCGTTTGTTTGGC (2432–2448) and 5′-dCTTGGTTTGACAGG (3088–3101) targetting the most conserved region of COI (cf. Fig. 6) as the set of primers [21]. The plaque-hybrydization was carried out at 60°C in 5 × SSC (0.45 M NaCl, 0.045 M sodium citrate buffer, pH 7.0) containing the blocking reagent (0.5%, Boehringer Mannheim), 0.1% sodium lauroyl sarcosinate and 0.02% SDS. *E. coli* XL1-Blue and *p*UC118 were used as the sequencing vector and its host, and deletion was carried out by exonuclease III digestion as described in the manufacturer's protocol (Takara). Nucleotide sequences were determined by the chain termination method [22] using [α-^{32}P]dCTP. The sequence data were analyzed with a software program (Genentyx, Tokyo) adapted for a Macintosh II-CX computer.

2.4. Assay of cytochrome c oxidase activity and other methods

This was carried out at 37°C in a 2 ml cuvette containing 10 mM KCl, 2.5 mM $MgSO_4$,10 mM sodium ascorbate and 2 mM K-Mops buffer (pH 6.9–7.0) according to Eq. (1) through measurements of pH changes using ascorbate as a final electron donor [2].

$$\text{ascorbate} - H^- + H^+ + 1/2O_2 \rightarrow \text{dehydroascorbate} + H_2O \quad (1)$$

The net alkalization was determined by back-titration with 50 mM HCl. The activity was also followed with an oxygen electrode (Yellow Spring Instrument). These pH changes and polarographic currents were recorded with a strip-chart recorder.

Protein determination, SDS-PAGE, and absorption spectrum measurement were carried out as described previously [1].

3. Results

3.1. Cytochrome c oxidase from Bst K1041

Cytochrome *c* oxidase was prepared from membrane fraction of *Bst* K1041, and partially purified. Fig. 1 shows a typical electrophoregram with sodium dodecyl sulfate of the preparation, in comparison with the PS3 enzyme. Three main bands in the *Bst* sample were observed at the same respective positions as those of PS3, indicating that the *Bst* enzyme has a subunit composition similar to that of the PS3 enzyme, and is almost purified. The fourth band of the K1041 enzyme, however, was not clear, indicating that a part of this subunit was lost during the purification.

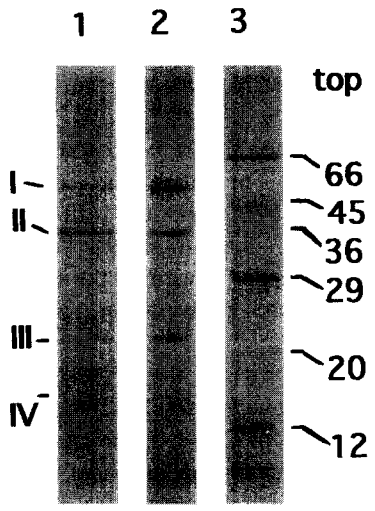

Fig. 1. SDS-PAGE pattern of the partiallly purified cytochrome *c* oxidase from *Bst* K1041. The concentration of the gel was 13.5% and it was stained with Coomassie brilliant blue R-250. Lane 1, the oxidase preparation from *Bst* K1041 (3 μg); lane 2, PS3 cytochrome *c* oxidase (5 μg); lane 3, molecular weight marker proteins.

Fig. 2 shows the difference spectrum, indicating that the enzyme from *Bst* K1041 contains cytochromes *c* and aa_3, as does the PS3 enzyme. The contents of cytochrome *c* and cytochrome aa_3 were 7.7 and 6.9 nmol/mg protein, respectively, if the extinction coefficients of the PS3 enzyme [1] are applied. Accordingly, the *Bst* enzyme is a cytochrome caa_3-type oxidase, as is the PS3 enzyme, and the preparation is about 90% pure, assuming the protein molecular mass of the *Bst* enzyme to be 130 kDa.

3.2. Activity of the Bst K1041 enzyme

The PS3 enzyme showed Michaelis-Menten type saturation curves using cytochromes *c* of horse heart, two yeasts (*S. cerevisiae* and *Candida krusei*) and PS3 cytochrome *c*-551 as substrate [2]. In contrast, the *Bst* K1041 enzyme showed sigmoidal-like saturation curves with yeast (*S. cerevisiae*) and cytochrome *c*-551 at low ionic strength, as shown in Fig. 3A. At high ionic strength, however, the saturation curves almost followed the usual regular hyperbolic curves (Fig. 3B), suggesting that the apparent sigmoidal relationship is caused by some ionic interaction involved in the oxidase reaction. On the other hand, it is at the same time noteworthy that the V_{max} values at high ionic strength are much smaller than those at low strengths, suggesting that the interaction of cytochrome *c* with the catalytic site of the oxidases is also ionic.

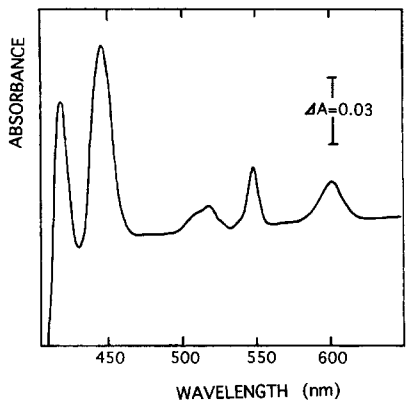

Fig. 2. Redox difference spectrum of cytochrome *c* oxidase from *Bst* K1041. $Na_2S_2O_4$-reduced *minus* oxidized as prepared.

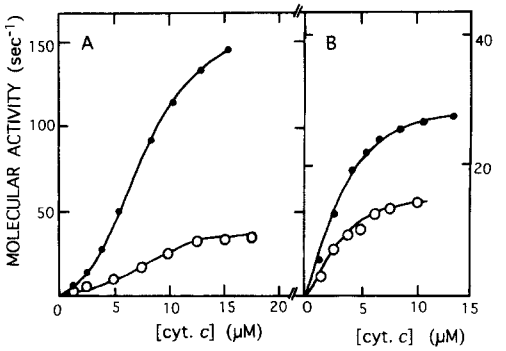

Fig. 3. Effect of cytochrome *c* concentration on the oxidase activity of the *Bst* enzyme. (A) The oxidation rate was followed with a glass electrode in a reaction medium (2.0 ml) containing 10 mM sodium ascorbate, 10 mM KCl, 2.5 mM $MgSO_4$, 0.1 mg/ml soybean P-lipids, and 2 mM K-Mops buffer (pH 6.8–6.9) at 37°C; (B) the reaction medium also contained 100 mM NaCl. The *Bst* caa_3-type oxidase used was 23 pmol (A) and 46 pmol (B). ●, PS3 cytochrome *c*-551; ○, *S. cerevisiae* cytochrome *c*.

The concentration dependency of the TMPD oxidation of the *Bst* enzyme followed the Michaelis-Menten equation (not shown), as did that of the PS3 enzyme [1,2]. Table 1 summarizes V_{max} values of the K1041 and PS3 cytochrome oxidases with different substrates. The V_{max} and K_m values of both oxidases with TMPD, a small artificial and effective electron donor, are almost the same. It is also noteworthy that the rate of TMPD oxidation was not affected by salt concentration, as reported with the PS3 enzymes [2]. On the contrary, V_{max} values with different cytochromes *c* of the two oxidases were different, although the tendency that cytochrome *c*-551 was the best

Table 1
Comparison of kinetic parameters for the oxidation of several cytochromes *c* and TMPD

Electron donor	PS3 enzyme		K1041 enzyme	
	K_m (μM)	TN_{max} (s^{-1})	K_m (μM)	TN_{max} (s^{-1})
Equine cytochrome *c*	17	27	–	31
Yeast cytochrome *c*	7.0	128	–	41
PS3 cytochrome *c*-551	3.9	163	–	183
TMPD	275	464	290	336

The caa_3-type cytochrome *c* oxidases from PS3 (25 pmol) and *Bst* K1041 (23 pmol) were used. The conditions for the activity measurement were the same as in Fig. 3A. The kinetic parameters of the PS3 enzyme were obtained using a Lineweaver-Burk plot.

substrate, yeast *c* the midle, and equine *c* the worst one among cytochromes *c* tested was similar. Especially yeast cytochrome *c* was a good substrate as cytochrome *c*-551 with the PS3 enzyme, while the *Bst* enzyme did not oxidize yeast cytochrome *c* well. The V_{max} values of the PS3 enzyme were caluculated on Lineweaver-Burk plots as before [2]. They followed the Michaelis-Menten equation nicely (not shown). The saturation curve of the *Bst* enzyme with cytochromes *c* appears sigmoidal (Fig. 3A), but they also follow the Michaelis-Menten equation if a certain amount (2.1 μM) of cytochrome *c* is assumed to be absent due to absorption. In fact, this amount was estimated to be 1.0 μM when the concentration of the *Bst* enzyme was reduced to one half. In order to determine the structural background of this phenomenon and the different cytochrome *c* specificity, the protein sequences of the oxidase subunits of K1041 were determined from DNA. The sigmoidal dose response was also observed with the membrane fraction of *Bst* K1041. The oxidase activities of the K1041 membrane fraction measured with an oxygen electrode showed 4, 56 and 102 nmol/min of O_2 uptake with 2, 4 and 6 μM yeast cytochrome *c* in the presence of 10 mM ascorbate at 37°C, while the values with the PS3 membrane fraction were 56, 120 and 180 nmol/min. Thus, sigmoidal response also occurred when O_2 uptake measurement was carried out such that much more substrate oxidation takes place than in the case of pH meter measurement.

3.3. Cloning, DNA sequence, ORF and operon structure

PCR was performed using a set of oligonucleotides as the primers (see Materials and Methods) to obtain a highly-conserved region of the PS3 *caa*B. The resulting product, about 700 bp DNA as expected, was used as a probe to select the oxidase genes from the *Bst* K1041 library in M13 phage. About 4000 recombinants were screened with the probe at 60°C in 5 × SSC. Three plaques were positive and really contained the genes for the cytochrome oxidase.

Fig. 4 shows a map of the *caa* operon containing one ORF and four subunits of cytochrome *c* oxidase. The same gene structure has been reported with several *Bacillus* DNA such as of PS3 [7,8], *B. subtilis* [5] and alkaliphilic *B. firmus* [6]. The genes cloned in the three recombinant M13 vectors, and the corresponding probe site is also shown in Fig. 4.

Fig. 5 shows two parts of the DNA sequences, one (A) 5′-upstream region (nucleotides 1–1200) with the deduced amino acid residues for ORF1 (heme O synthase or farnesyl tranferase for heme O synthesis) and COII, and another (B) 3′-downstream region (nucleotides 6501–6700) showing the presence of a terminator-like structure after the COIV stop codon. One putative promotor structure (−10 region), TAATAT, is found in the 5′-upstream of *caaE/ctaB* gene, and another, TTCTTT and TATATA (−10 and −35 region), between *caaE/ctaB* and *caaA/ctaC* genes as TTCTTT and TATATA as boxed. The putative terminator structure showing inverted repeats with stems is marked with arrows. Since there is no terminator structure between *caaE* and *caaA* as in the PS3 gene, and *caaABCD* genes encoding COI-IV array in a tandem fashion with very short spacing non-coding regions, these struture genes may form an operon which may be transcribed into two kinds of mRNA: one for the structural genes of all four cytochrome oxidase subunits (COII, COI, COIII and COIV), and another for heme O synthase (*caaE/ctaB*) plus COI-IV, as pointed out by Saraste et al. [5]. The presence of two mRNAs with and without *ctaB* for the oxidase genes has been confirmed in alcalophilic *B. firmus* [6]. The *Bst caaE/ctaB* gene product is composed of 307 amino acid residues (34597 Da). We show no complete nucleotide

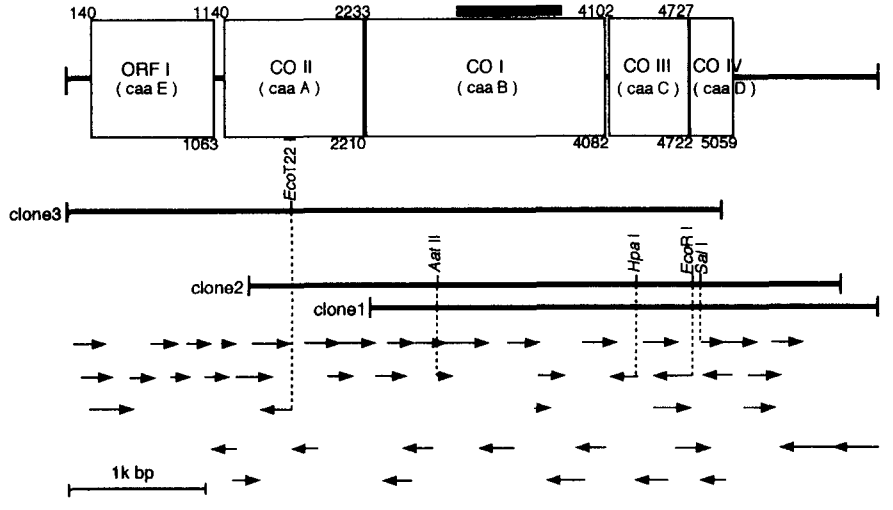

Fig. 4. A map of *Bst* K1041 genes coding for subunits of cytochrome *c* oxidase and heme O synthase (ORF 1). The restriction sites used for subcloning for sequencing are shown, but most parts are sequenced with series of deletion series. The numberings at the top and at the bottom of the genes are the initial base of the initiation codon and the last base of the stop codon, respectively. The thick bar at the top denotes the probe for cloning.

sequence covering *caaABCD / ctaCDEF* region, but nucleotide numbers of the initiation and stop codons for respective ORFs are shown in Fig. 4 and the complete nucleotide sequence is in the data bank (DDBJ/EMBL/GenBank, D70843).

3.4. Deduced protein sequences

Fig. 6 shows alignment of the *caaE / ctaB* gene with the corresponding genes reported [5,6,33]. Only a few gaps are necessary for alignment, and several residues conserved in other *Bacillus*, *E. coli* and purple bacterial sequences are also conserved in *Bst* ORF1 (heme O synthase). Hydropathy analysis of the deduced *Bst* protein indicated that there are 7 hydrophobic segments just as other counterparts. Moreover, *E. coli cyoE* [23,24] and PS3 *caaE* [25] were recently over-expressed in an *E. coli* expression system, and the genes were elucidated to encode heme O synthase (protoheme-transfarnesylase). Heme O is the precursor of heme A, and these hemes play roles as the prosthetic groups in the terminal oxidases almost exclusively [10,26]. It is of interest that the *caa / cta* operon also contains the gene for heme A synthesis as well as the structural genes of the oxidase subunits.

The amino acid sequences of the oxidase subunits from K1041 were compared with those from PS3 as illustrated in Fig. 7, in order to find structural differences which could be relevant to the kinetic difference in the cytochrome *c* oxidase activity. There are 43 differences in COI composed of 615 amino acid residues (68 101 Da).

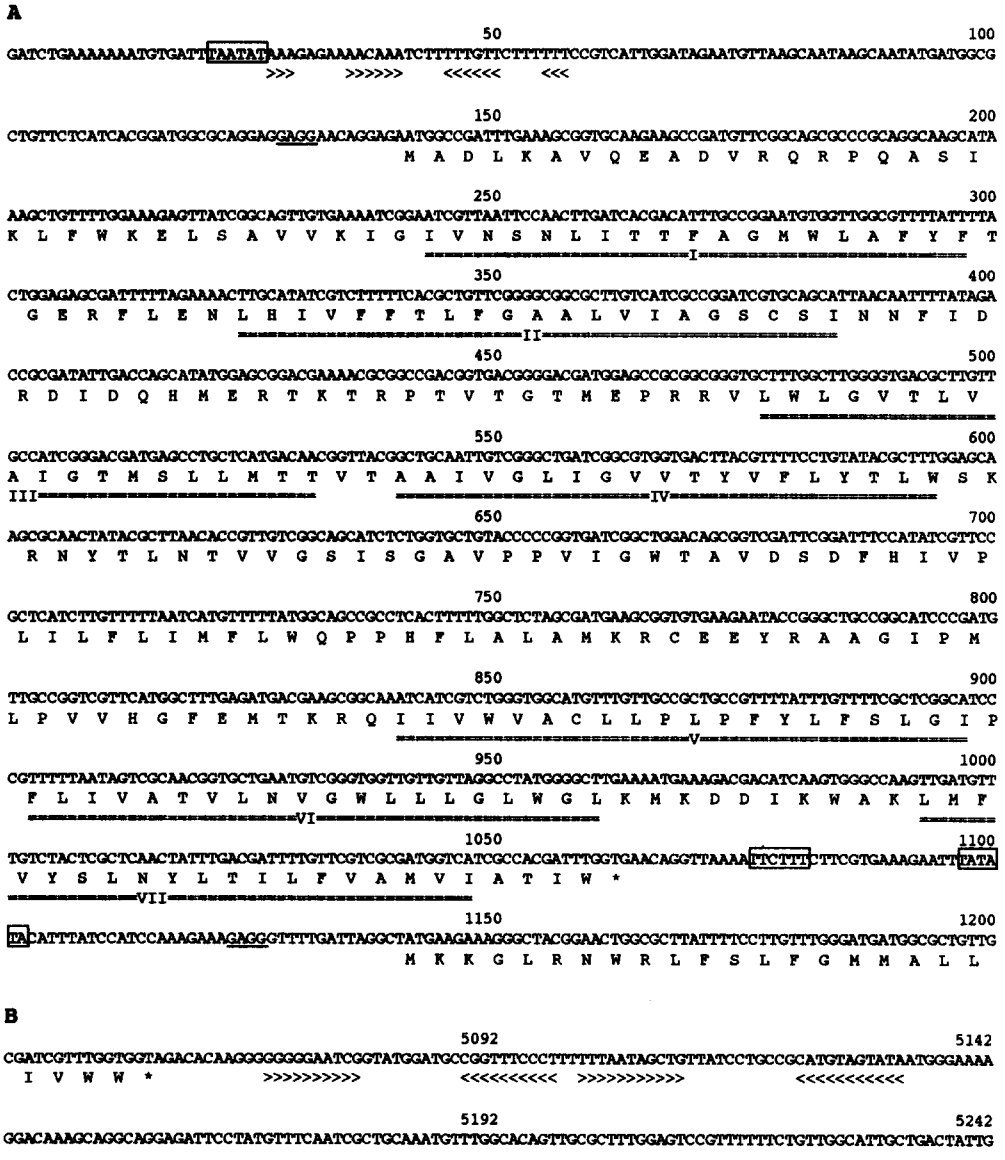

Fig. 5. Partial nucleotide and deduced amino acid sequences of *caa / cta* operon. (A) The 5′-upstream region showing two putative promotors and protein sequence of the ORF for heme O synthase. (B) 3′-downstream region showing the terminator of the operon. Putative ribosomal-binding sites (boxed), possible promotor elements (underlined), and inverted repeats (> > < <) are marked.

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                                                    ==========I==========        ==========II==
Bst K1041          MADLKAVQEADVRQRPQASIKLFWKELSAVVKIGIVNSNLITTFAGMWLAFYFTGERFLENLHIVFFTLFGAAL  74
B.subtilis           MANSRILNDTAIDGQIEETTAWKDFLSLIKIGIVNSNLITTFTGMSVALHISGLSFLGNINTVLLTLIGSSL  72
B.firmus       MNKSNTAIDPTNVIEAGPDSSVADVQQKSWKDYLVLAKQGIVTSNLITTFAGIYLAIVYTGTVFTMHLDTMIFALLGAAL  80
E.coli                                      MMFKQYLQVTKPGIIFGNLISVIGGF-----LIASKGSIDYPLFIYTLVGVSL  49
P.denit.                                          MSLVVFTAFVGL-----WIAPQPVNPFVAFCAVLF-IAL  33

               ========                                ========III========  ==========IV=========
Bst K1041      VIAGSCSINNFIDRDIDQHMERTKTRPTVTGTMEPRRVLWLGVTLVAIGTMSLLMTTVTAAI-VGLIGVVTYVF--LYTL 151
B.subtilis     IIAGSCAINNWYDRDIDHLMERTKVRPTVTGKIQPSQALWSGILLVALGLIMLLMTTVMAAV-IGFIGVFTYVV--LYEM 149
B.firmus       VMAGGCTLNNYIDRDIDHLMERTKERPTVTGRFSAKHVLLVGLAQAALGIIFLALTTPTAAV-IGLIGLFIYVV--LYTM 157
E.coli         VVASGCVFNNYIDRDIDRKMERTKNRVLVKGLISPAVSLVYATLLGIAGFMLLWFGANPLACWLGVMGFVVYVG--VYSL 126
P.denit.       GGGASGALNMWYDADIDAVMRRTAGRPVPSGRVTSQEPLAVGIALSGLSVM-MLGAGGNWFA-AGFLAFTIFFYAVVYTI 112

               =
Bst K1041      WSKRNYTLNTVVGSISGAVPPVIGWTAVDSDFHIVPLILFLIMFLWQPPHFLALAMKRCEEYRAAGIPMLPVVHGFEMTK 231
B.subtilis     WTKRRYTINTVVGSVSGAVPPLIGWTAVEGNIGVVAWVLFMILFIWQIPHFLALAIKKTEDYRAANIPMLPDVYGFEVTK 229
B.firmus       WTKRTTTLNTIVGSFSGAVPPLIGWAAIDGGLHLYAWLLFFIMFLWQPPHFLALAMKRVEEYRAAGIPMLPVVAGFEMTK 237
E.coli         YMKRHSVYGTLIGSLSGAAPPVIGYCAVTGEFDSGAAILLAIFSLWQMPHSYAIAIFRFKDYQAANIPVLPVVKGISVAK 206
P.denit.       WLKRSTPQNIVIGGAAGAFPPMIGWALPTGGIGIESLLMFALIFFWTPPHFWALALFMKDDYSKAGVPMLTVTHGRKVTR 191

                 ==========V==========          ==========VI========           ========VII=======
Bst K1041      RQIIVWVACLLPLPFYLFSLGIP--------FLIVATVLNVGWLLLGLWGLKMK-DDIKWAKLMFVYSLNYLTILFVAMV 302
B.subtilis     RQIIVWVACLMPLPFFLGSLGLP--------IVILGLLLNIGWLILGLMGFRSK-NIMKWATQMFVYSLNYMTIYFVAMV 300
B.firmus       RQMVVYVAALLPVSLMLYPFGLV--------YTIVAAVLGVGWLALGIAGFKMK-DDIKWARLMFVYSLNYLTILFVLMV 308
E.coli         NHITLYIIAFAVATLMLSLGGYAG-----YKYLVVAAAVSVWWLGMALRGYKVA-DDRIWARKLFGFSIIAITALSVMMS 280
P.denit.       CHIFAYTLVLAPFALWLGFTSVGGPLYLAVSVVLNALFIAGGWQILRRSEDQAQADGYRVEKRYFRLSLYYTFLHFLALL 271

               =
Bst K1041      IATIW                    307
B.subtilis     VLTLF                    305
B.firmus       IVHF                     312
E.coli         VDFMVPDSHTLLAAVWX        297
P.denit.       VQHWVGGW                 279
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Fig. 6. An alignment of Heme O synthase (*caaE / ctaB*) protein with homologous sequences. The deduce protein sequences compared were encoded by *ctaB* in *B. subtilis* [5], *ctaB* in *B. firmus* [6], *cyoE* in *E. coli* [33], ORF1 in *P. denitrificans* [34]. The transmembrane segments are marked with = . The residues which are conserved among four sequences are shadowed.

Most amino acid changes are replacement by the similar residues. Only one replacement (Ala-508 to Arg) may have resulted in the addition of a positive charge not found in PS3 to K1041 COI in the cytoplasmic loop. In COIII, one residue (Asn-19) is deleted in *Bst* K1041 composed of 206 residues (23 367 kDa). Lys-98 is replaced by Gln, but the next residue, Asn-99, is replaced by Lys. Other changes take place between similar amino acid residues. In COIV, composed of 110 residues (12 902 Da), all ten replacements are between similar amino acid residues.

On the contrary, rather drastic replacements are observed in COII, which is composed of 355 amino acid residues (40 098 Da). There are 35 conversions (and an insertion) in the nascent K1041 COII composed of 334 ($= 357 - 23$) amino acid residues after processing [8]. Several changes affecting positive and negative charges occur: D131N and N40D may be compensated, but K247E, N251K, K253E and K254N occur between the Cu_A-binding site and the cytochrome *c*-domain. The adjacent replacements, E264Q and A267E, in this region may be compensated. There are two positive charge appearances (N284K, and A289R) and compensatable changes (D297G and N323E) in the cytochrome *c*-domain. As the result of these changes, appearence of five negative charges and one positive charge, and disappearence of three negative and six positive charges at least, the p*I* value of COII should be affected. The calculation from peptide sequences produces a p*I* of 8.9 for K1041 COII, while that of PS3 is 9.3.

3.5. Comparison of Bst K1041 gene and Bacillus PS3 gene and codon usage

The percentage identity of the region of *Bst* K1041 DNA (nucleotides 461–5195) with the corresponding PS3 DNA (nucleotides 1–4736 in Ref. [8]) is 84.6%. Comparison of the deduced amino acid sequences of COI-IV gives 93.0%, 89.6%, 94.2% and 90.9%, respectively. The order of these values between PS3 and K1041 is very similar to that of those between four different enzymes from human,

Fig. 7. Deduced amino acid sequences of four subunits of the *Bst* oxidase and comparison with the corresponding sequences of *Bacillus* PS3. The names of amino acid residues different from those of the *Bst* enzyme are shown in the column of PS3. Hydrophobic transmembrane segments are underlined and numbered with Roman figures. Important and invariant residues, such as ligands for hemes and coppers of COI and COII, and Glu in COIII, are shadowed.

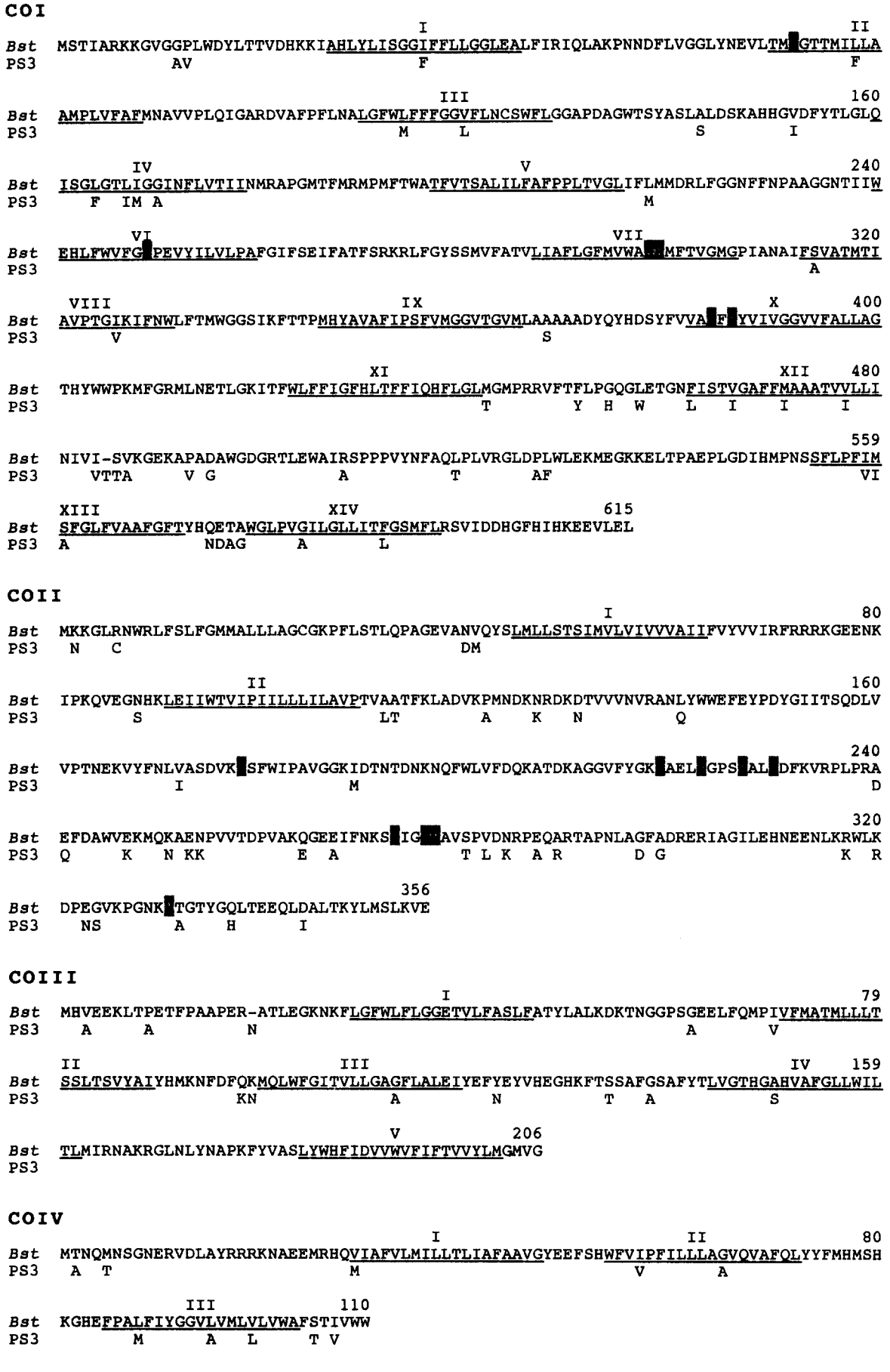
COI
COII
COIII
COIV
Bst
PS3

Table 2
Effect of base differences between *Bst* and PS3 genes in a codon on replacement of amino acid residues in the resultant oxidase subunits

Subunit	Numbers of replaced amino acid residue/total codon change occurring					
	Due to 1 base change at			Due to	Due to	Sum
	1st lett.	2nd lett.	3rd lett.	2 base change	3 base change [a]	
COI	13/21	3/3	6/197	17/17	3/3	42/241
COII	7/14	3/3	7/108	17/18	2/2	36/145
COIII	4/7	2/2	1/52	3/4	2/2	12/67
COIV	5/6	1/1	0/35	5/6	1/1	12/49
COI–IV	29/48	9/9	14/392	42/45	8/8	103/502

[a] Including three-letter deletion.

P. denitrificans, PS3 and *E. coli* (cytochrome *bo*), as reported previously [8]. The order probably reflects the constancy of residues due to functional demands.

Table 2 summarizes the kinds of base change which induced amino acid changes in the respective subunits. A two-base change in a codon took care of about half of total amino acid changes, while a one-base change in the first and second letters and three-base change in a codon shared the rest. Very few changes at the third letter induced an amino acid change, although most of the base changes occurred at the third position of the codon. Especially, none of the 35 changes of the third letter resulted in an amino acid change in COIV, and only one out of 52 changes in COIII. Table 3 gives the codon usage in the four genes for each subunit of K1041 cytochrome aa_3-type oxidase. This codon usage is very similar to that of PS3 [8]. We found the tendency for G or C to be preferentially chosen at the third position. But two codons using amino acids such as Tyr, His, Glu, Gln and Lys rather use A or T. Similar codon usage was also reported for the PS3 F_1-ATPase [27,28]. The G + C contents of DNA of PS3 and *Bst* K1041 in the *caa/cta* operon are 50.9% and 48.5%, respectively, probably reflecting the fact that PS3 cells, originating in a Japanese hot spring, grow upto 75°C, while *Bst* K1041 is a normal thermophilic *Bacillus* in soil.

4. Discussion

The thermophilic bacterium PS3, isolated from Mine spa in Japan has been used for research on oxidative phosphorylation as the source of enzymes and their genes [1,7,8,27,28]. In this work, we used a similar thermophilic *Bacillus*, Bst K1041 for the sake of its transformation ability by electroporation [18], and the apparent similarity of the enzymes in catalyzing oxidative phosphorylation. In fact, cytochrome *c* oxidase (caa_3-type) purified from *Bst* K1041 possessed an absorption spectrum (Fig. 3), subunit composition (Fig. 2) and TMPD oxidase activity very similar to those of the PS3 enzyme. Sequencing of the K1041 genes encoding the oxidase indicates that the sequences are also very similar to those of PS3. The hydropathy plots of the four subunits of the K1041 oxidase gave the same patterns as those of the PS3 oxidase,

Table 3
Comparison of codon usage of the structural genes for cytochrome *c* oxidase of *B. stearothermophilus* K1041 with that of PS3

	Bst/PS3		*Bst*/PS3		*Bst*/PS3		*Bst*/PS3
TTT-Phe	63/23	TTC-Ser	7/2	TAT-Tyr	18/24	TGT-Cys	4/5
TTC-Phe	52/62	TCC-Ser	4/5	TAC-Tyr	22/16	TGC-Cys	2/2
TTA-Leu	30/9	TCA-Ser	7/5	TAA-***	1/0	TGA-***	2/4
TTG-Leu	44/53	TCG-Ser	17/20	TAG-***	1/0	TGG-Trp	34/34
CTT-Leu	31/24	CCT-Pro	7/3	CAT-His	25/28	CGT-Arg	10/8
CTC-Leu	18/27	CCC-Pro	2/1	CAC-His	13/12	CGC-Arg	18/22
CTA-Leu	3/3	CCA-Pro	8/4	CAA-Gln	17/16	CGA-Arg	2/2
CTG-Leu	20/27	CCG-Pro	41/44	CAG-Gln	9/8	CGG-Arg	7/8
ATT-Ile	51/44	ACT-Thr	3/4	AAT-Asn	19/19	AGT-Ser	4/3
ATC-Ile	25/32	ACC-Thr	7/8	AAC-Asn	29/31	AGC-Ser	10/14
ATA-Ile	2/1	ACA-Thr	15/13	AAA-Lys	45/43	AGA-Arg	1/1
ATG-Met	56/58	ACG-Thr	49/55	AAG-Lys	13/19	AGG-Arg	0/0
GTT-Val	31/24	GCT-Ala	18/14	GAT-Asp	18/21	GGT-Gly	31/18
GTC-Val	23/40	GCC-Ala	26/39	GAC-Asp	20/21	GGC-Gly	44/58
GTA-Val	16/6	GCA-Ala	17/8	GAA-Glu	40/39	GGA-Gly	14/58
GTG-Val	37/32	GCG-Ala	47/54	GAG-Glu	18/13	GGG-Gly	23/19

The total codon usage numbers of all four subunits were compared.

indicating that there are 14, 2, 5 and 3 hydrophobic helices in COI, COII, COIII and COIV, at the same respective positions. Important residues such as His residues for heme A and Cu_B bindings in COI, His and Cys residues for Cu_A binding in COII, and the DCCD-binding Glu residue in COIII, are all conserved at just the same positions (Fig. 6). On the other hand, distinct amino acid changes were found in the COII sequence: many replacements accompanying redistribution of charged groups, especially negative charges.

Although most characteristics of K1041 cytochrome *c* oxidase are very similar to those of the PS3 enzyme, the apparent sigmoidal dose response (Fig. 3A) has, to the best of our knowledge, not been reported in the PS3 cytochrome oxidase or in the case of other bacterial and mitochondrial enzymes. The COII in cytochrome oxidases is thought to mediate the electron transfer via Cu_A from cytochrome *c* to cytochrome *a* and the binuclear center where O_2 is reduced [10]. The *Bacillus* caa_3-type cytochrome oxidases contain cytochromes *c* covalently bound to the C-terminal region of COII, and oxidize extrinsic cytochromes *c* via the intrinsic cytochrome *c* as reported previously [2]. It is thus likely that some replacement of charge group(s) such as addition of negative charge(s) to COII of the K1041 enzyme may be responsible for the apparent sigmoidal curve of the K1041 cytochrome *c* oxidase. The fact that addition of salt to the reaction medium changed the apparent sigmoidal curve into an almost regular Michaelis-Menten type saturation curve (Fig. 3B), suggests the importance of ionic interaction in the binding of cytochromes *c* to the oxidases, which has already been pointed out with the mitochondrial enzyme [29]. The two acidic residues (Glu and Asp) of COII which are conserved are supposed to be important for the interaction with lysine-rich heme C domain of cytochrome *c* [10,30]. The fact that the substrate specificity of the *Bst* enzyme is different from that of the PS3 enzyme (Table 3) may also suggest the importance of ionic interaction between cytochrome *c* and the oxidase.

On the contrary, another difference between the enzymes from K1041 and PS3 is a partial loss of COIV in the former. However, the loss of COIV may not be responsible for the abnormal dose-response of the K1041 enzyme, since almost all bacterial cytochrome *c* oxidases, including the enzyme from *B. stearothermophilus* ATCC 7954 [20], have been prepared without COIV, and no enzyme has been reported to show sigmoidal dose-response.

From the viewpoint that the concentration of cytochrome *c* is also of the order of cytochrome oxidase itself, the apparent sigmiodal dose response due to the extra binding of cytochrome *c* may cause serious inhibition of electron transfer to the terminal oxidase. However, the effect of this extra binding on the bacterial physiology seems difficult to evaluate, since *Bacillus* cytochrome oxidase (caa_3-type) may form a super-complex with the bc_1-complex to form a quinol-oxidizing super-complex in situ [31].

The identity of bases between *Bst* K1041 DNA and PS3 is 83.5%, while the identities of amino acids of COI-IV are 89.6–94.2%. Although the base changes are rather frequent, deduced protein sequences for COI and COIII and COIV, and sequences for the promotors, the ribosome-binding sites and the terminator are almost conserved. Codon usage is also almost the same in PS3 and *Bst* K1041. As was reported previously, there is a tendency to choose G and C for the third letter, except for the case of amino acids using two codons [7,8]. As to codon usage of the sodium/proton/glutamate symport protein from *Bst* and *B. caldotenax*, Tolner et al. reported that strong preference for A or T over C or G at the third position [32]. They also reported that the GC contents of these *gltT*Bs and *gltT*Bc genes are 40.3 and 40.7%, respectively. On the contrary, the GC contents of the vicinity of the *caa / cta* operon of PS3 and *Bst* K1041 are 50.9% and 48.5%, respectively. It is thus likely that the different codon usage between *Bst* K1041 *caaEABCD / ctaBCDEF* and *Bst gltT* is due to the character of individual gene, rather than strain difference.

The present work demonstrated an abnormal cytochrome *c* (substrate) dependence in the *Bst* K1041 cytochrome caa_3-type oxidase, and suggested formation of a cytochrome *c*-binding site other than catalytic site. We have also presented a distinctive sequence of COII of the enzyme, which may be responsible for this characteristic. This could be a good example of how accumulated base changes induce kinetic difference between homologous enzymes in closely related species. Furthermore, we showed the complete sequence of the *caa / cta* operon encoding cytochrome *c* oxidase and heme O synthase. We are currently trying to disrupt the genes for these subunits and express mutant genes in transformable *Bst* K1041 cells. Present cloning and sequencing of the *Bst* K1041 genes will open a way to manipulate the gene of thermophilic cytochrome *c* oxidase.

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