Cloning of Bacillus stearothermophilus ctaA and Heme A Synthesis with the CtaA Protein Produced in Escherichia coli

Junshi Sakamoto,† Akiko Hayakawa, Tomoko Uehara, Shunsuke Noguchi, and Nobuhito Sone

Department of Biochemical Engineering and Science, Kyushu Institute of Technology, Kawanabe 680-4, Iizuka, Fukuoka 820-8502, Japan

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The Bacillus stearothermophilus ctaA gene, which is required for heme A synthesis, was found upstream of the ctabCDEF/ caaEABCD gene cluster as in B. subtilis and B. firmus. The deduced protein sequence indicates that CtaA is a 35-kDa intrinsic membrane protein with seven hydrophobic segments. Alignment of CtaA sequences showed conserved residues including histidines that may be involved in heme B binding and substrate binding. Expression of ctaA in E. coli resulted in increased formation of a membrane-bound b-type cytochrome, heme A production, and severe growth inhibition. Furthermore, B. stearothermophilus CtaA produced in E. coli was found to catalyze the conversion of heme O to heme A in vitro.

Key words: heme O; over-expression; protoheme; respiratory chain; therophilic bacteria

CtaA was first found as a gene required for the expression of the heme A-containing terminal oxidases, cytochromes aa₃ and caa₃, in Bacillus subtilis. Ishizuka et al. cloned the Bacillus PS3 caaEABCD gene cluster and then Saraste et al. cloned a similar cluster, B. subtilis ctabCDEF, both of which code for four subunits of the cytochrome caa₃-type terminal oxidase in addition to CtaB/CaaE. CtaB/CaaE is similar in sequence to Escherichia coli CyoE. Saiki et al. have shown that CyoE is heme O synthase or heme B: farnesyl-PP, trans-farnesylase, and that ctab/CaaE of Bacillus PS3 is able to complement a cyoE mutation in E. coli.

The ctaA gene in B. subtilis is upstream of the ctabCDEF gene cluster but is transcribed in the opposite direction. Recently, Svensson et al. showed that both ctaA and ctaB are necessary for heme A biosynthesis from heme B (protoheme IX), and suggested that the CtaA product may catalyze monoxygenation and oxidation of a methyl side group to form a formyl group at the 8th position in the porphyrin ring of heme O. They also purified the CtaA product from membranes and showed that the protein is a novel b-type cytochrome and that its over-production in E. coli needs the presence of both ctaA and ctaB genes. A similar modification step from a methyl group to formyl also occurs in the biosynthesis of chlorophyll b from chlorophyll a. It is known that the formyl oxygen atom originates from dioxygen molecules, however, nothing is known about the enzymes for that reaction.

We have been involved in a series of studies on the cytochrome caa₃-type terminal oxidase of the therophilic Bacillus PS3 including purification of the oxidase and cloning of ctabCDEF/ caaEABCD. The strain is, however, difficult to transform. Therefore, we have switched to using a closely related therophilic strain B. stearothermophilus K1041, which is transformable by electroporation. Recently, we reported the sequence of the ctabCDEF/ caaEABCD gene cluster of B. stearothermophilus K1041. In this work, we have found that B. stearothermophilus K1041 ctaA is upstream of ctabCDEF but in the opposite, strand just as in the mesophiles B. subtilis and B. firmus. Furthermore, we detected in vitro conversion of heme O into heme A with the CtaA protein produced in E. coli membranes.

Materials and Methods

Materials. Membrane fractions from PS3 and B. stearothermophilus K1041 were prepared as described previously. Oligonucleotides were custom synthesized with an Applied Biosystems DNA Synthesizer Model 381A using β-cyanoethyl phosphoamidites as monomers. The large fragment of Escherichia coli DNA polymerase, restriction enzymes, M13 vectors, sequencing primers, and a Kilo-Sequence Deletion Kit were purchased from Takara Shuzo (Kyoto). 32P-labelled nucleotide triphosphates and DEAE-Toyopearl (DEAE-Fractogel) 650S were purchased from Amersham and Toyo (Tokyo), respectively. For cloning of B. stearothermophilus ctaA, a DNA fragment containing the proximal end of ctaA/ ctab (nucleotide 1-276 of ref. 19) was used as a probe. Digoxigenin labeling followed the manufacturer’s protocol (Boehringer). E. coli XL-1 blue and pUC118 were used for cloning and sequencing. BamHI-cut pUC118 was ligated with FbaI-BamHI fragments of genomic DNA of B. stearothermophilus K1041. The DNA fragments were prepared by digesting

1 To whom correspondence should be addressed. J. Sakamoto, Department of Biochemical Engineering and Science, Kyushu Institute of Technology, Kawanabe 680-4, Iizuka, Fukuoka 820-8502, Japan; Tel: +81-948-29-7823; Fax: +81-948-29-7801; E-mail: sakamoto@bs.e.kyutech.ac.jp

Abbreviations: IPTG, isopropyl-1-thio-β-D-galactoside; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

The nucleotide sequence data of the ctaA gene has been deposited with the DDBJ/EMBL/GenBank databases under the accession number D70843
genomic DNA with FbaI, fractionation by agarose gel electrophoresis to recover a 3-kb fragment, and then cut with BamHI. Hybrydization was done at 50°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 lauryl sarcosinate and 0.02% sodium dodecyl sulfate (SDS). Deletions were obtained by exonuclease III-digestion as described in the manufacturer's protocol (Takara), and the derived plasmids were used for sequencing. Nucleotide sequences were determined by the chain termination method\(^\text{21}\) using [\(\alpha-\text{\[^{32}\text{P]dCTP, The sequence data were analyzed with a software program (Genentex, Tokyo) adopted for a Macintosh II-CX computer. Heme O was extracted from cytochrome \(b\) of \(E. coli\), which was a kind gift from Dr. T. Mogi and Prof. Y. Anarak of Tokyo University, and purified essentially as described previously\(^\text{22}\) using a reversed phase column, Resource RPC (Pharma- cia Biotech). DEAE-Toyopearl anion exchange gel and hydroxyapatite were obtained from Tosoh (Tokyo) and Bio-Rad (Hercules, respectively.

Construction of expression vector for ctaA. The two expression vectors named pUCctaA and pTrcctaA were constructed as follows: pUCcE1, the original pUC cloning vector containing the ctaA region of \(B. stearothermophilus\) DNA (see Fig. 1), was digested by XbaI and Smal, and the resulting 1.8 kb XbaI(vector)/Smal(vector) fragment, containing ctaA, was isolated. This fragment was ligated with XbaI/Smal-treated pUC119, generating pUCctaA. The ctaA gene (bp 247-1210, Fig. 2) was amplified by polymerase chain reaction using pUCctaA as the template and primers, 5'-AT-CCATGGTAAGATTTGCAACG-3' and 5'-TAG- GTACCTTATTTAATGGCGGCCG-3'. The resulting PCR product contained introduced Ncol and KpnI sites. The product was then digested by these two enzymes, and ligated with Ncol/KpnI treated pTrc99A (Pharmacia) to generate pTrcctaA, which has the ctaA gene downstream from the tac promoter.

Transformation and expression of ctaA. Competent cells of XL-1 blue were prepared by the method of Inoue et al.\(^\text{23}\) Cells transformed with pUCctaA and pTrcctaA are referred to as \(E. coli/pUCctaA\) and \(E. coli/pTrcctaA\), respectively. The transformants were grown on 2XTY agar plates containing 50 \(\mu g/\text{ml}\) ampicillin. Precultures were grown in liquid medium at 37°C in about 6 ml of 2XTY containing 50 \(\mu g/\text{ml}\) ampicillin in 15 mm diameter test tube with a tight screw cap. Reciprocal shaking was at about 100 rpm. For the preparation of membranes containing CtaA, the cells were cultured in 1-liter baffled flasks with 500 ml of 2XTY containing ampicillin. About 5 ml of the overnight preculture was used for inoculation. The cells were harvested 3–5 hours after induction with 0.1 mg/ml isopropyl-\(\beta\)-thiogalactoside (IPTG). \(E. coli\) cells were suspended in buffer containing 0.1–0.2 mg/ml egg white lysozyme, 20% (w/v) sucrose, 10 mM EDTA, and 30 mM Tris-HCl, pH 8.0. After 30-min incubation on ice, spheroplasts were recovered by centrifugation and resuspended in solution containing 20% (w/v) sucrose and 3 mM EDTA, pH 7.4. The spheroplasts were disrupted by sonication, and the membranes were recovered by ultracentrifugation at 144,000 \(\times g\) for 30 min.

Purification of CtaA product. Membranes from \(E. coli/pUCctaA\) were suspended to a protein concentration of 10 mg/ml in buffer containing 2% (w/v) sodium cholate, 100 mM NaCl, and 20 mM Tris-HCl, pH 8.0. The mixture was sonicated for 2 min on ice and centrifuged at 100,000 \(\times g\) for 40 min. After another wash with 2% cholate solution, the pellet was suspended in buffer containing 5% (w/v) Triton X-100, 100 mM NaCl, and 20 mM Tris-HCl, pH 8.0, followed by sonication and centrifugation as above. The extract was dialyzed twice against 50 volumes of 20 mM Tris-HCl buffer, pH 8.0, and put on a DEAE-Toyopearl column (1.0 cm \(\times\) 3.0 cm) equilibrated with buffer A (0.5% Triton X-100, 20 mM Tris-HCl, pH 8.0). After the column was washed with buffer A, proteins were eluted with a 60-ml linear 0- to 300-mM gradient of NaCl in buffer A. The peak fraction was concentrated using Centricon-30 (Amicon) and injected into a TSKgel G3000SW Gel glass gel filtration column (Tosoh Co., Tokyo) with a TSK guard column SW Glass (Tosoh) through a liquid chromatography pump CCPM (Tosoh). The column had been equilibrated with buffer containing 0.1% Triton X-100, 100 mM NaCl, 1 mM EDTA, 20 mM Hepes-NaOH, pH 7.0. The peak fractions were pooled and put onto a hydroxyapatite column (0.5 cm \(\times\) 0.9 cm) equilibrated with the same buffer. The CtaA protein flowed through the column, while most of the other proteins were absorbed to the column and eluted with the same buffer except that Hepes-NaOH was replaced with an indicated concentration of sodium phosphate.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done as described by Laemmli\(^\text{24}\) without boiling samples. For sequencing, proteins were separated by SDS-PAGE, electro-transferred to a polyvinylidene difluoride membrane as described by Towbin et al.,\(^\text{25}\) and put on a pulse-liquid peptide sequencer (Applied Biosystems, model 477A).

Heme analysis. Redox difference spectra at room temperature were recorded using a Beckman DU-70 spectrophotometer. An oxidized sample as prepared was taken as the baseline and a few grains of solid sodium dithionite were added to reduce the sample, followed by spectral measurement. Spectra of pyridine ferrohemochromes were measured in the presence of 10% (v/v) pyridine, 0.05 N NaOH, and 1% (w/v) SDS. Hemes were extracted with HCl-acetone from \(E. coli\) membranes and identified essentially as described previously\(^\text{22}\) using a reversed phase Hikarisil-C18 column (Asahi Chemical Industry Co., Ltd., Kawasaki). For detection of in vitro conversion of heme O to heme A, membrane fractions from \(E. coli\) transformants at 2.4 mg/ml protein were incubated with 8.7 \(\mu\) M heme O in the presence of 1 mM sodium dithiothreitol, 1 mM MgSO\(_4\), and 0.1 M HEPES-NaOH, pH 7.0, at 37°C for 1 hour. Spectra of pyridine ferrohemochrome of the
total reaction mixture were measured as described above.

Results

Cloning of the ctaA gene

Probing with the proximal end of ctaB, five colonies were positive among approximately 1500 recombinants containing Fbal-BamHI fragments of the B. stearothermophilus genomic DNA. At least one plasmid clone, named pUCue1, contained the entire ctaA gene, as shown in Fig. 1. The gene is adjacent to the ctaBCDEF/ caeaEABCD gene cluster,19 and transcribed in the opposite direction, as in B. subtilis9 and alkalophilic B. firmus OF4.20

DNA and deduced amino acid sequence

Figure 2 shows the DNA and deduced amino acid sequences for ctaA (D70843). Base pairs 1 to 4, GATC (Sau3AI site) is the end of the previously cloned ctaB- CDEF/ caeaEABCD region of this bacterium.19 The open reading frame was of 954 bases, and the deduced CtaA protein is composed of 317 amino acid residues with a molecular mass of 35,101.5 Da, if not processed. A hydrophy plot of B. stearothermophilus CtaA indicates 7 hydrophobic helices like those in B. subtilis and B. firmus CtaA (data not shown).

Expression of CtaA

Two constructions were prepared for expression of the ctaA gene in E. coli, pUCctaA and pTrctaA (see Materials and Methods). E. coli cells were transformed with these two plasmids. The transformation efficiencies were about $5 \times 10^4$ cells for 1 µg of each type of plasmids, which were about one half of that with the vectors pUC119 and pTrc99A. Figure 3 shows growth curves of E. coli cells with plasmids containing ctaA compared with the plasmids without ctaA. E. coli/pUC119 grew slightly more slowly than E. coli/pUC119 even before addition of IPTG. After its addition, E. coli/pUC119 grew almost as well as before, while growth of E. coli/ pUCtauA was inhibited by the induction (Fig. 3(A)). With some cultures, growth was not inhibited, but this seemed due to growth of mutants that did not produce CtaA. E. coli/pTrctaA grew well in the absence of IPTG (Fig. 3(B)). Cells containing plasmids with ctaA showed a reddish color when harvested 3-5 hours after induction.

Cytochrome contents of membranes

Figure 4 shows redox difference spectra of Triton X-100-solubilized E. coli membranes. E. coli/pUCctaA (trace B) and E. coli/pTrctaA (trace C) showed higher absorption at about 560 nm than control cells or E. coli/pUC118 (trace A). The absorption peaks in this region were at 559 nm for both traces B and C, and 561 nm for trace A. Besides these peaks due to b/o-type cytochromes, an additional peak was observed at around 583 nm in traces B and C. Analysis of the membranes for hemes were done by pyridine ferrohemochrome formation and by HPLC on a reversed phase C18 column after heme extraction with HCl-acetone. The results showed that heme O and heme A are present in addition to heme B (data not shown). Table 1 shows the amounts of cytochromes b plus o and heme A.

Identification of the CtaA product

Membranes from E. coli/pUCctaA were solubilized with Triton X-100, put on a DEAE anion-exchange column, and eluted with a linear gradient of NaCl. Absorbance at 410 nm due to the $\gamma$-band of cytochromes was monitored. There were two major peaks. The first, eluting at 90 mM NaCl, showed a characteristic redox difference spectrum with two absorbance maxima at 557 and 583 nm (Fig. 5(A)). The second, eluting at 140 mM NaCl, showed a spectrum of b-type cytochrome with a single absorbance maximum at 560 nm (Fig. 5(B)). Pyridine ferrohemochrome analysis of the first peak fraction showed maxima at 557 and 586 nm, indicating that the fraction contained protoheme IX and heme A. Similar chromatography with solubilized membranes from E. coli/pUC118 showed only the peak at 140 mM NaCl and the redox difference spectrum was similar to that of the corresponding peak of E. coli/pUCctaA. Therefore, cytochrome in the 90-mM NaCl peak was further purified using a gel filtration and then a hydroxylapatite column. The cytochrome flowed through the last column (Fig. 5(C)). The final sample contained one major protein with apparent molecular mass of 28 kDa (Fig. 6). N-terminal sequence analysis of this protein gave MQRSLK, which correspond to that deduced from the nucleotide sequence of the ctaA gene (Fig. 2). This protein was also found in the membranes from E. coli/pTrctaA, but was not observed in those from control

<table>
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<th>Membranes</th>
<th>cytochromes b plus o</th>
<th>heme A</th>
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<tr>
<td>pUCctaA</td>
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<td>0.17</td>
</tr>
<tr>
<td>pTrctaA</td>
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<td>0.06</td>
</tr>
<tr>
<td>wild</td>
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(nmol/mg protein)
Fig. 2. DNA and Deduced Amino Acid Sequence of *B. stearothermophilus* ctaA.

Putative seven hydrophobic membrane-spanning regions predicted by hydropathy plot are marked with =. Nucleotides that may constitute the promoter region are underlined, a putative ribosome-binding (Shine-Dalgarno) sequence is boxed, and an inverted repeat as a stem in a putative terminator structure showing are marked with arrow heads.

cells. These results confirmed that the 28-kDa protein is the CtaA product and suggested that it might be a *b*-type cytochrome holding heme A. The apparent molecular mass estimated by SDS-PAGE is lower than that calculated from the deduced amino acid sequence, as many other hydrophobic membrane proteins are.
Fig. 3. Growth Curves of *E. coli* transformed with Plasmids for Over-expression of *citaA*.
IPTG was added at the times indicated by arrows. A, pUCctaA (∆) and pUC119 (●); B, pTrctaA (○) and pTrc99A (▲).

CitaA purified from *E. coli*/pTrctaA contained less heme A than CitaA isolated from *E. coli*/pUCctaA. This difference in heme A content is also seen in crude membrane preparations (Table 1). CitaA almost comigrated with an intrinsic *E. coli* protein in 12% acrylamide gels of SDS-PAGE (Fig. 6(A)), while CitaA migrated a little faster than that protein in 10% gels (Fig. 6(B)). That protein was also abundant in the control host cells and its N-terminal sequence was APKDN, indicating that it is OmpA, a major outer membrane protein of *E. coli* cells. CitaA flowed through the hydroxyapatite column, but most of OmpA was absorbed to it and eluted with sodium phosphate buffer (Fig. 6(B)).

**In vitro conversion of heme O to heme A**
To analyze whether CitaA can catalyze the conversion of heme O to heme A, membranes from *E. coli*/pTrctaA was incubated with heme O in the presence of dithiothreitol. The amount of heme A increased when the membranes were incubated in the presence of heme O, but not in its absence (Fig. 7(A) and (B)). Such heme A synthesis was not observed with membranes from *E.
**Fig. 6.** SDS-PAGE of CtaA Isolated from Membranes of *E. coli/pUCctaa*.  
A: The acrylamide concentration was 12%. The samples are membrane fractions of *E. coli/pUCctaa* (20 μg protein, lane 1), Triton X-100 extract (10 μg, lane 2), peak fraction from DEAE-Toyopearl chromatography (10 μg, lane 3), peak fraction from gel filtration chromatography (5 μg, lane 4) and fraction that flowed through a hydroxylapatite column (3 μg, lane 5). B: The acrylamide concentration was 10%. The samples are peak fraction from gel filtration chromatography (10 μg, lane 6), fractions that flowed through (5 μg, lane 7) and eluted with 50 mM sodium phosphate from a hydroxylapatite column (10 μg, lane 8). Molecular mass standards are bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), and trypsin inhibitor (20 kDa).

coli/pUCctaa, which from the start contained an appreciable amount of heme A, or with membranes from *E. coli* without ctaA on plasmids. Heme A production was not detected using the purified CtaA instead of the membranes (data not shown).

**Discussion**

These data showed that *ctaA* of *B. stearothermophilus* K1041 has close similarity to those of other *Bacillus* species such as *B. subtilis* and *B. firmus* not only in the location relative to the structural gene cluster of cytochrome *caaa*-type oxidase but also in the deduced amino acid sequence (Fig. 8). The N-terminal sequence (MQRSLK) of the expressed CtaA suggested that the initiation codon of the gene is TTG (Fig. 2). This assignment is also supported by the presence of a ribosome-binding motif and high correspondence between the N-terminal sequences of the three *Bacillus* CtaA (Fig. 8). TTG is not a very rare initiation codon in *Bacillus* species. For example, complete genome sequencing of *B. subtilis* indicates that 13% of the genes start with TTG, 78% with ATG and 9% with GTG in this species. The amino acid sequence of *B. stearothermophilus* CtaA is very similar to that of *B. subtilis* (54.4% in identity) and *B. firmus* CtaA (37.2%). CtaA is a very hydrophobic protein, probably containing seven hydrophobic membrane-spanning helices, and has five conserved His residues. Four of the five His may function as axial ligands for the protoheme IX and the heme A, since both heme have been found to be low-spin in isolated *B. subtilis* CtaA. If both hemes have a perpendicular orientation relative to the membrane plane and the elec-
Fig. 8. CtaA Sequence Comparison.

Data of *B. subtilis* and *B. firmus* are compared with that of *B. stearothermophilus* K1041. Transmembrane segments are marked with *, conserved residues are boxed, and conserved histidines are also indicated by stars. The N-terminal of *B. stearothermophilus* CtaA is presented as MQRS.LK in accordance with sequencing data.

<table>
<thead>
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<th>Species</th>
<th>Sequence Information</th>
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<td><em>B. stearo</em></td>
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</tr>
<tr>
<td><em>B. subtil</em></td>
<td>PFLAVLSPFLVLNVLSGFSVRLPSLQPSGKSP--CSSDPSLVPLPLPLPSGLPFSKPSYGLVQNKSLVISGGLQH</td>
</tr>
<tr>
<td><em>B. firmu</em></td>
<td>MFLFAAIVLIIQLKSPILKLFAKLQPSGKSP--CSSDPSLVPLPLPLPSGLPFSKPSYGLVQNKSLVISGGLQH</td>
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<td><em>B. stearo</em></td>
<td>PSLGVSQFVQVYFYQKVKVQVYFYQKVKVQVYFYQKVKVQVYFYQKVKVQVYFYQKVKVQVYFYQKVKVQVYFY</td>
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<tr>
<td><em>B. subtil</em></td>
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<td><em>B. firmu</em></td>
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The electron transfer site is more peripheral, His61 and His122 may ligate the protoheme while His216 and His278 may ligate the hydrophobic substrate, heme O or its product heme A.

*E. coli* cells bearing ctaA in plasmids produced the CtaA protein and it contained heme A, suggesting that the protein is involved in heme A synthesis through oxygenating/oxidizing the methyl side chain of the porphyrin ring of heme O. Heme A production was observed when membranes of *E. coli* pTrcctaa were incubated with heme O (Fig. 7). The final amount of heme A was comparable to that of the protoheme in CtaA. On the other hand, *in vitro* formation of heme A was not observed with membranes of *E. coli* pUCctaa, which usually had heme A per se, and its amount was comparable to that of the protoheme in CtaA. These findings suggest that CtaA have a protoheme-binding site for electron donation and a substrate/product-binding site, and that heme A is produced from heme O only by a single turnover and stays firmly at the substrate/product site. The latter may be because of the absence of a heme A acceptor such as an apo-enzyme of cytochrome aa-type oxidase and/or the lack of other factors essential for repeated turnover of CtaA.

The presence of a protoheme-binding site reminds us of cytochromes P-450, which catalyzes monoxygenations. However, the low-spin protoheme in CtaA by itself should not be able to constitute an active center of a monoxygenase in contrast to the high-spin protoheme in P-450. Consequently, another factor may be necessary for an efficient monoxygenase reaction. Since a monoxygenase generally yields hydroxymethyl groups from methyl groups, a dehydrogenase is additionally needed to convert a hydroxymethyl group to a formyl group. In fact, heme A production was not observed using the purified CtaA instead of the membranes (data not shown). It is noteworthy that the *Paracoccus denitrificans* ctaA gene and its yeast homolog *cox11* are required for cytochrome aa synthesis. Therefore, it may be interesting to find out if a counterpart of CtaG, in addition to CtaA, is required for efficient heme A synthesis, although ctaG homologs have not yet been identified in *Bacillus* species.

In conclusion, this is the first report of *in vitro* synthesis of heme A from exogenously added heme O in the presence of CtaA. It might be more advantageous to use enzymes of thermophilic bacteria since they are often more stable than their counterparts of mesophilic organisms. Thus, further experiments such as site-directed mutagenesis and enzyme kinetics would be important to verify molecular mechanism of heme A synthesis using the gene cloned from the thermophile in this study.

**Acknowledgments**

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