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Nucleotide Sequence and Characterization of the Gene Coding for a Thermostable Heme O Synthase from the Thermophilic *Bacillus* PS3

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Abstract

The whole *caaF-caaE-caaABCD* gene cluster, contains two open reading frames (*caaE* and *caaF* seem to be coding for the proteins related to heme biosynthesis pathway) on the 5'-upstream of the caa_3-type cytochrome *c* oxidase structural genes (*caaA, caaB, caaC, and caaD)*, was isolated from a genomic DNA library of the thermophilic bacterium *Bacillus* PS3. The *caaE* gene and its flanking regions were identified. The deduced amino acid sequence of the *caaE* product is composed of 309 amino acid residues, and is homologous to that of the *cyoE* product (heme O synthase) of *bo*-type ubiquinol oxidase operon from *Escherichia coli*. The *caaE* is located on the 5'-upstream of the *caaA* (corresponds to subunit II of PS3 cytochrome *c* oxidase) apart from 77 base pairs, and there are a large stem-loop structure and promoter-like structures in this region. This promoter-like structure was needed for the *in vivo* expression of *caaA* from PS3 in *E. coli*. The *caaF* is located on the 5'-upstream of the *caaE* apart from 175 base pairs in the reverse direction. A recombinant *E. coli* cell harboring the *caaF-caaE-caaABCD* gene cluster of PS3 synthesized heme A. The product of *caaE* (CaaE) was expressed in *E. coli*, and had a thermostable heme O synthase (protoheme IX farnesyltransferase) activity in cytoplasmic membrane [Saiki, K., Mogi, T., Ishizuka, M., and Anraku, Y., FEBS Lett. 351(2), 385-388, 1994]. Furthermore, PS3 CaaE amino acid residues as candidates for taking part in prenyl binding, transfer, or structure preserving, and for participating heme B binding or structure preserving by the comparison among CaaE homologues are discussed.

1. Introduction

Cytochrome, heme-containing protein, plays an oxidoreduction. Cytochrome *c* contains heme C added two cystein residues of polypeptide chain to two vinyl groups of ferrous protoheme IX (heme B) at pyrrole rings A and B. Both heme O and A are derivatives of heme B in which the vinyl residue at pyrrole ring A is substituted by a 17-carbon hydroxyethylfarnesyl side chain, in addition, heme A contains a formyl residue replacing the methyl group at pyrrole ring D [1-3].

The thermophilic *Bacillus* PS3, one of gram-positive spore-forming bacilli, contains caa_3-type cytochrome *c* oxidase as the respiratory terminal oxidase (complex IV) in aerobic condition, and shows clear proton pump activity in addition to electron transfer across the membrane as shown in Fig. 1A. We have already reported DNA sequence and several characteristics of the structural

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Key words: *Bacillus* PS3, Heme O synthase, Protoheme IX farnesyltransferase, DNA sequence, Amino acid sequence, Heme A biosynthesis
The sequence has been submitted to the DDBJ Data library under the accession number D37961.
Abbreviations: bp, base pair; DNA, deoxy ribo nucleic acid; HPLC, high performance liquid chromatography; IPTG, isopropyl-1-thio-β-D-galactoside; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

genes (*caaA, caaB, caaC,* and *caaD*) coding for the four subunits {subunit II (COII), subunit I (COI), subunit III (COIII), and subunit IV (COIV), respectively} of PS3 caa_3-type cytochrome *c* oxidase [4], and showed several characteristics based on the deduced amino acid sequences of four subunits [4,5]. Sequencing analysis for both directions have shown that at least four genes for COII, COI, COIII, and COIV are located following this order in an operon (*caa*) coding for caa_3-type cytochrome *c* oxidase that is different from the structural genes (*ctaC, ctaD, and ctaE*) for three subunit (COII, COI, and COIII, respectively) of aa_3-type cytochrome *c* oxidase from *Synechococcus valcanus* [6,7]. The caa_3-type cytochrome *c* oxidase belongs to the heme-copper respiratory oxidase family based on its prosthetic groups. Compared with aa_3-type cytochrome *c* oxidases from mitochondria and *Paracoccus denitrificans*, the *Bacillus* PS3 caa_3-type oxidase contains *c*-type cytochrome as an extra domain covalently fused to C-terminus of subunit II (COII), and also the gene encode cytochrome *c* polypeptide is fused to 3'-downstream of COII gene. This *c*-type cytochrome domain of COII is suggested to a functional equivalent to cytochrome *c* mediating electron transfer from complex III to complex IV.

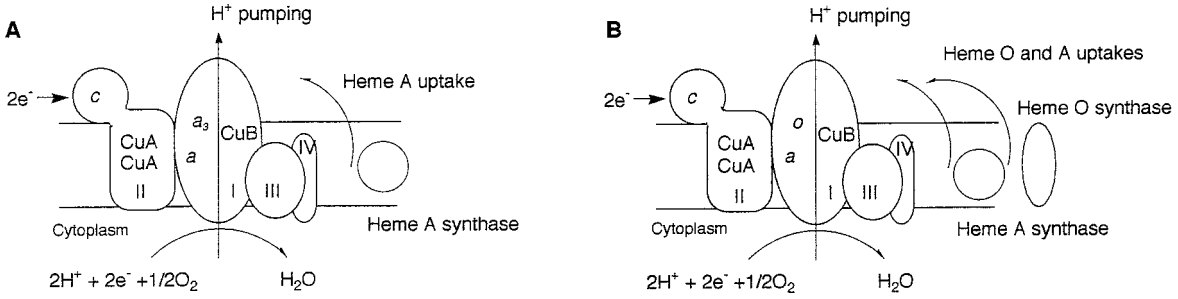

Fig. 1. PS3 cytochrome *c* oxidase models. A: *caa*$_3$-type oxidase in the case of oxygen-saturated culture condition, B: *cao*-type oxidase in the case of slightly oxygen-limited culture condition.

On the other hand, under slightly air-limited growth conditions, the conversion of the caa_3-type cytochrome *c* oxidase to the *cao*-type cytochrome *c* oxidase occurred in the thermophilic *Bacillus* PS3 [8]. Although the turnover number of the purified *cao*-type cytochrome *c* oxidase was higher than that of the caa_3-type cytochrome *c* oxidase, the Km's of for cytochrome *c* and O_2, pattern of four subunits as a result of gel electrophoresis in the presence of sodium dodecyl sulfate (SDS), and heme A and C composition of the two enzymes were almost the same except the replacement of heme A to heme O as shown in Fig. 1B. Similar phenomenon was reported that the cytochrome *ba*-type ubiquinol oxidase changed to the cytochrome *bo*-type oxidase in *Acetobactor aceli* [9]. Properties of the cytochrome *cao*-type oxidase from alkalophilic *Bacillus* YN-2000 were reported [10].

We have cloned a gene cluster (*named caaF-caaE-caaABCD* gene cluster), which contains two open reading frames (named *caaE* and *caaF* gene) at 5'-upstream of a promoter-like structure of *caaABCD* operon from PS3 that was able to work in *Escherichia coli* cell to produce PS3 COII [11,12]. The *caaE* gene product (CaaE) was homologous to *cyoE* gene product (CyoE) of *E. coli* ubiquinol oxidase operon [13] and *ctaB* gene product (CtaB) of *Bacillus subtilis* aa_3-type cytochrome *c* oxidase gene cluster [14]. The *caaF* gene product (CaaF) was similar to *ctaA* gene product (CtaA) from *Bacillus subtilis* [15,16]. However, the molecular mechanism of the heme O and heme A biosynthesis and heme replacement mechanism of cytochrome *c oxidase* were unknown.

Recently, it has been reported that the *E. coli cyoE* gene of the *bo*-type quinol oxidase operon (*cyoABCDE* [13]) encodes the heme O synthase, protoheme IX farnesyl transferase (EC:2.5.1.-),

which CyoE-overproduced membranes can catalyze a transfer of the polyprenyl moiety of farnesyl diphosphate (FPP) to the vinyl group of ferrous protoheme IX in the presence of divalent cations such as Mg^{2+} [17-19].

We have examined the functional role of the *caaE* gene in the *caaF-caaE-caaABCD* gene cluster for caa_3-type cytochrome *c* oxidase in thermophilic *Bacillus* PS3 [11,12,20]. From genetic complementation test in *E. coli* and the heme O synthase assay using the CyoE-CaaE-chimera overproduced cytoplasmic membranes, we found that the CyoE-CaaE-chimera protein expressed in *E. coli* functions as a thermostable heme O synthase *in vivo* and *in vitro,* and suggested that the CaaE protein supplies heme O as an intermediate for heme A biosynthesis in thermophilic *Bacillus* PS3 [20].

Here, we report nucleotide sequence and characterization of the *caaE* gene coding for the thermostable heme O synthase and its flanking regions from the thermophilic *Bacillus* PS3, which locates on 5'-upstream of the caa_3-type cytochrome *c* oxidase operon, and show several characteristics based on the deduced amino acid sequence of *caaE* gene product (CaaE).

2. Materials and Methods

Oligonucleotides were synthesized with a MilliGen Biosearch Cyclone Plus DNA Synthesizer using β-cyanoethyl phosphoamidites as monomers and purified by Oligo-pack column (MilliGen Biosearch). M13mp18 (19), pUC118 (119) vectors, nick translation kit, DNA sequencing kits (7-DEAZA and *Bca*BEST), sequencing primers, T4 DNA polymerase, T4 polynucleotide kinase, T4 DNA ligase, and restriction enzymes, were purchased from Takara Shuzo Co. LTD., and labeled Nucleotide triphosphates and L-[^{35}S]Methionine were purchased from ICN corporation. Both lambda vector EMBL-3 arms and *in vitro* packaging kit (GIGAPACK GOLD) were obtained from Stratagene Cloning Systems Co. LTD. *In vitro* translation kit was purchased from Amersham Co. LTD. Both shuttle vector pHP13 between *E. coli and B. subtilis*, and anti-serum for PS3 cytochrome *c* oxidase were gifts from Dr. M. Saraste.

Isolation of *caaF-caaE-caaABCD* Gene Cluster and analysis: PS3 genomic DNA library was prepared as described previously [4]; purified genomic DNA of the thermophilic bacterium PS3 was partially digested with *Sau*3A I, 15-20 kbp fragments were isolated by agarose gel electrophoresis and collected from the gel slice, ligated to the *Bam*H I-site of the lambda vector EMBL-3 arms, and subjected to *in vitro* packaging using GIGAPACK GOLD, and this library was screened by plaque hybridization at 65°C in 4 × SET (0.15 M NaCl, 2 mM M disodium ethylene diamine tetraacetate, 0.03 M Tris-HCl buffer, pH 8.0) containing 0.5% SDS and 5 × Denhardlt's solution (0.02% Ficol, 0.02% polyvinylpyrrolidone, 0.02% Bovine serum albumin) using *Bam*H I-*Sal* I 1.8 kbp DNA fragment encode both the carboxyl terminal region of *caaE* gene product and the subunit II (COII) of PS3 cytochrome *c* oxidase as a probe (the DNA fragment was labeled with nick translation using [α-^{32}P]dCTP) The filters were washed at the same temperature in 2 × SSC (0.15 M NaCl, 0.015 M sodium citrate buffer, pH 7.0) containing 0.1% SDS and autoradiography. A clone containing the whole *caaF-caaE-caaABCD* gene cluster from six positive clones capable of hybridizing the probe was detected. This clone, named λE2, was digested with *Sal* I, *Bam*H I, or partially digested with *Sal* I and subcloned into pUC118, pUC119 or pHP13 and amplified: *Sal* I partial digested 8 kbp fragment was subcloned into the *Sal* I site of pHP13 (named pCI-8000). *Bam*H I-*Sal* I 1.8 kbp fragment was subcloned into the *Bam*H I-*Sal* I site of pUC119 (pCI-1800). *Sal* I-*Sal* I 3.5 kbp fragment was subcloned into the *Sal* I site of pUC119 (pCI-3500). *Sal* I-*Bam*H I 1.7 kbp fragment was subcloned into the *Sal* I-*Bam*H I site of pUC119 (pCI-1700).

DNA sequencing was carried out by the chain termination method using [α-^{32}P]dCTP and M13 mp18 (mp19), or PCR method. The sequencing strategy is indicated in Fig. 2. The sequence data were analyzed with BLAST2 protein database search web program (http://kr.expasy.org/cgi-bin/BLASTEMBnet-CH.pl), ClustalW multiple sequence analyzer (http://www.ch.embnet.org/software/ClustalW.html) or a software program (SDC Genetyx, Tokyo) adapted for a personal computer. For *in vitro* translation, *Sal* I-*Sal* I 3.5 kbp DNA fragment contain *caaF, caaE* and *caaA* were incubated at 37°C in the cell free crude extract of *E. coli* using *in vitro* translation kit and L-[^{35}S]Methionine. This sample was loaded on SDS-polyacrylamide gel. After electrophoresis, autoradiography was carried out. PS3 cytochrome *c* oxidase was prepared as described previously [5]. Western blotting analysis of PS3 *caaA* gene product was carried out by an enzyme-linked immuno sorbent assay using anti-serum for PS3 cytochrome *c* oxidase with 12.5% SDS polyacrylamide gel electrophoresis and PVDF transfer membrane. For analysis of heme composition, cytoplasmic membrane vesicles were isolated by gradient centrifugation and the hemes were extracted from the membranes by acid acetone and were separated by reverse phase HPLC (Gilson Co. Ltd.). The flow rate was 0.5 ml/min and the elution profile was monitored by the absorbance at 406 nm.

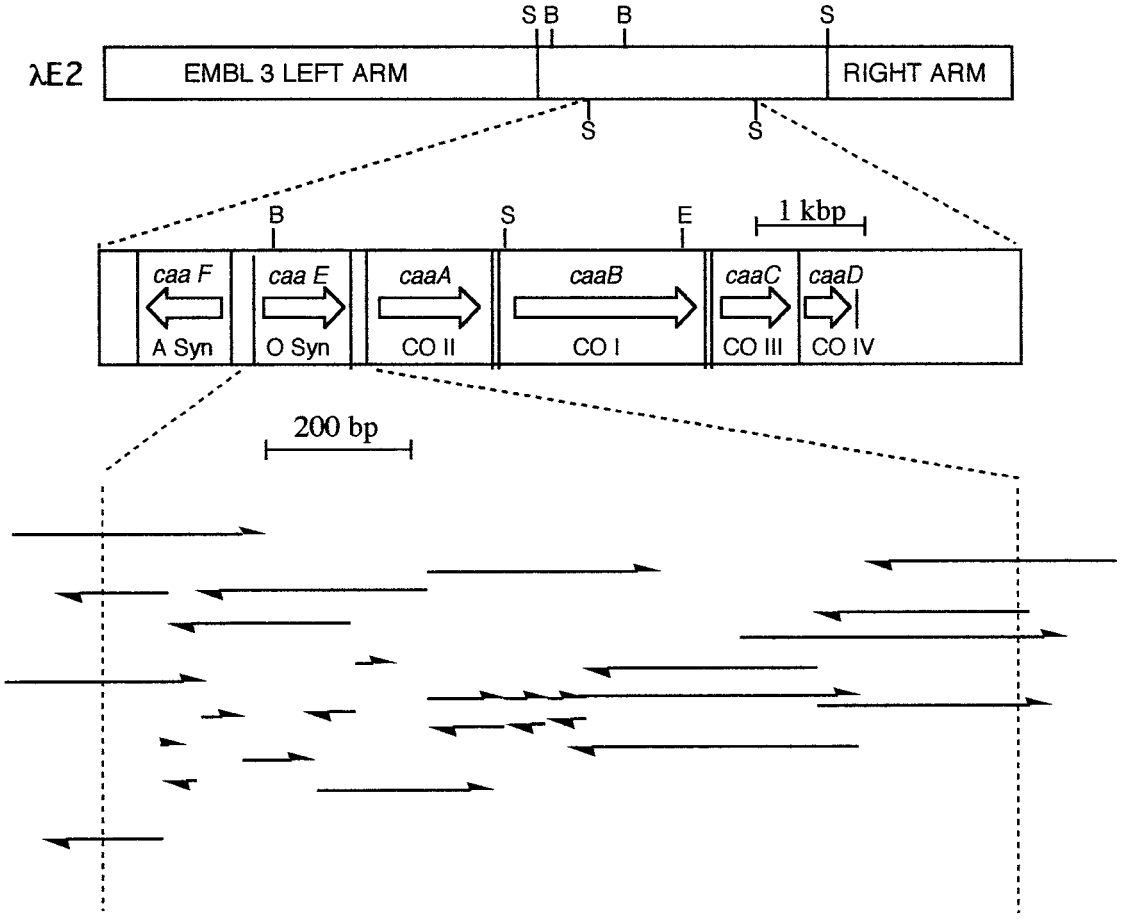

Fig. 2. A map of the *caaF-caaE-caaABCD* gene cluster in the PS3 chromosome and sequencing strategy of *caaE* gene encoding heme O synthase. The location of six genes and some restriction sites (B: *Bam*H I, E: *Eco*R I, S: *Sal* I) are shown. λE2 clone isolated is ligated the 15kbp partially digested *Sau*3A I fragment of PS3 chromosome to the *Bam*H I sites of λEMBL-3 arms (*Sal* I sites of both left and right arms are adjacent to the *Bam*H I sites). The sequencing strategy is indicated in the figure. The sequence was determined by the use of M13 single strand DNA sequencing system with *Alu* I, *Hae* III, and *Sau*3A I, or using specific primers; the sequencing direction is shown by the arrows.

3. Results and Discussion

3-1. PS3 *caaF-caaE-caaABCD* gene cluster and heme A biosynthesis

About 3,000 recombinants from the thermophilic bacterium *Bacillus* PS3 genomic DNA library were screened with *Bam*H I-*Sal* I 1.7 kbp DNA fragment encode both the carboxyl terminal region of *caaE* gene product and the subunit II of PS3 cytochrome *c* oxidase as a probe by plaque hybridization at 65°C in 4 × SET. Six clones that obtained were tested for this probe. Only one clone, named λE2, containing a whole *caaF-caaE-caaABCD* gene cluster from six positive clones capable of hybridizing the probe was detected. Further Southern blot analysis (data not shown) and sequence analysis showed λE2 clone indeed contained the whole *caaF-caaE-caaABCD* gene cluster (Fig. 2). This gene cluster that contains two open reading frames (*caaE* and *caaF* which seem to be coding for the enzymes related to heme A biosynthesis pathway) on the 5'-upstream of the caa_3-type cytochrome *c* oxidase structural genes (*caaA, caaB, caaC,* and *caaD)* shown in Fig. 2. Structure of this gene cluster is same as them both from *Bacillus subtilis (ctaA-ctaB-ctaCDEF)* [14-16] and *Bacillus firmus* (*ctaA-ctaB-ctaCDEF*) [21], although different from *Escherichia coli bo*-type ubiquinol oxidase operon (*cyoABCD-cyoE*) [13]. The *caaE* gene and the flanking regions of the cluster were subcloned and sequenced as shown in Fig. 2. Fig. 2 shows a restriction map in this region of the chromosome and sequencing strategy of the *caaE* gene and the flanking regions of the gene cluster. Fig. 3 shows the nucleotide sequence and the deduced amino acid sequence of the *caaE* gene product (CaaE). The *caaE* gene is separated from the *caaABCD* operon. The *caaE* is located on the 5'-upstream of the *caaA* (corresponds to COII) apart from 77 base pairs, and there are a large stem-loop structure and a promoter-like structure in this region shown in Fig. 4 and Fig. 5. The *caaE* seems to have an individual promoter (P2)-terminator (T2) structure shown in Fig. 4. Furthermore, the *caaF* gene, homologous to that of the *ctaA* product of aa_3-type cytochrome *c* oxidase gene cluster from *Bacillus subtilis* [15,16] (*ctaA* is needed to heme A biosynthesis [22]) and *B. firmus* [21], is located on the 5'-upstream of the *caaE* apart from about 175 base pairs in the reverse direction. We have tried gene expression of the *caaF-caaE-caaABCD* gene cluster in order to search of function of *caaE and caaF* products [11,12]. There are promoter-terminator-like structures in *caaF, caaE,* and cytochrome *c* oxidase structural genes (*caaABCD)* unit, individually described above. Fig. 6 shows the Western blotting analysis of the expression level of the CaaA protein (COII) in *E. coli* cell harboring pCI-1800 plasmid using the anti-PS3 cytochrome c oxidase antiserum. CaaA protein (COII) was detected. pCI-1800 contains the *Bam*H I-*Sal* I 1.8 kbp DNA fragment encode both the carboxyl terminal region of *caaE* gene product and the subunit II (COII) of PS3 cytochrome *c* oxidase. The promoter-like structure (TTTAGT; -35 region, ATTTAT; -10 region) located on the 5'-upstream of *caaA* seems to be needed for the *in vivo* expression of *caaA* from PS3 in *E. coli* shown in Fig. 4 and Fig. 5, as CaaA not detectable in the case of lacking this region. But, this structure did not work in *Bacillus subtilis* (*ΔctaCDEF*) as a host. As a result of *in vitro* translation test with the fragment of *caaF-caaE-caaA* gene cluster, two expressed proteins were detected [11,12], but could not identified the proteins.

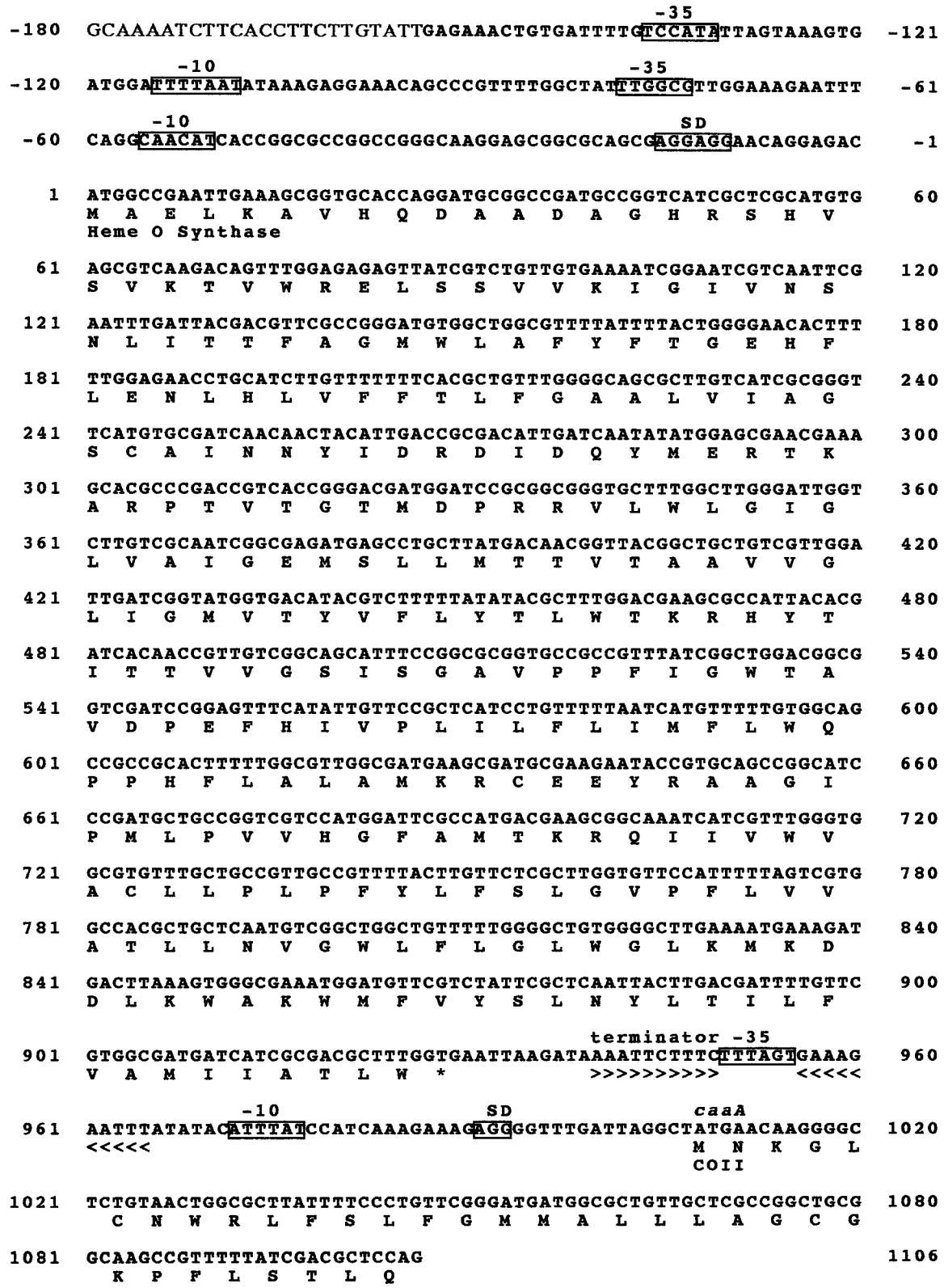

Fig. 3. DNA and deduced amino acid sequence of the gene for the thermostable heme O synthase and its flanking regions from PS3. Putative Shine-Dalgarno (SD) portions, a putative stem for the terminator of the *caaE* gene, and putative promoter regions are shown in the figure.

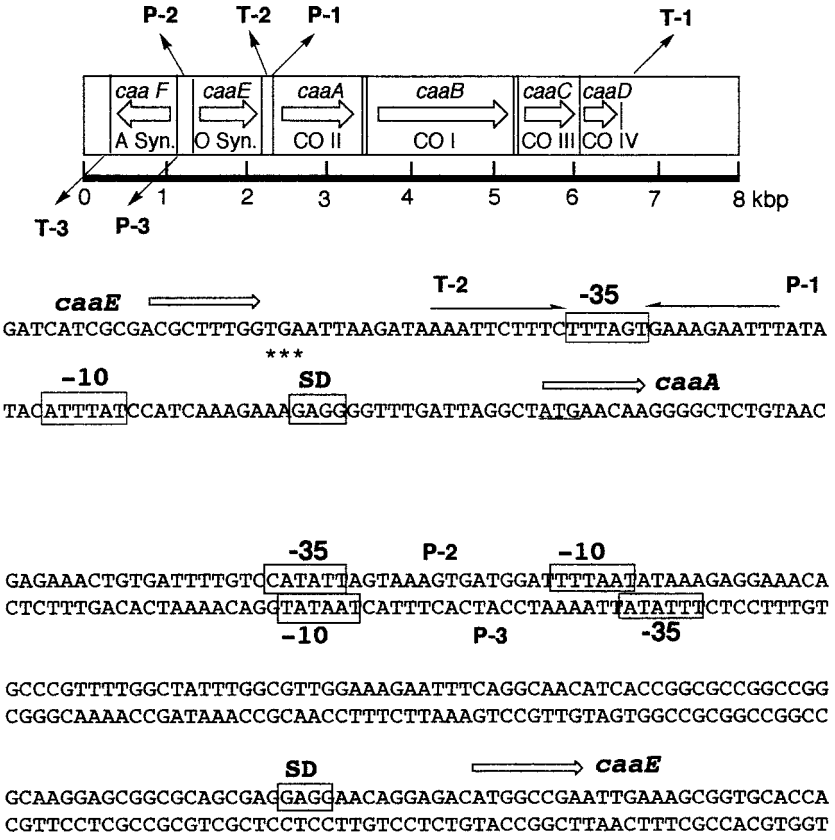

Fig. 4. Structure of deduced overlapped promoters and terminators in the flanking regions of PS3 *caaE* gene. P1, P2, and P3 are putative promoter regions for *caaABCD, caaE, and caaF,* respectively. T1, T2, and T3 are putative terminator regions for *caaABCD, caaE, and caaF,* respectively. Putative Shine-Dalgarno (SD) portions and deduced promoter (-35 and –10) regions are boxed. A putative stem for the terminator of *caaE* is shown with arrows in the figure.

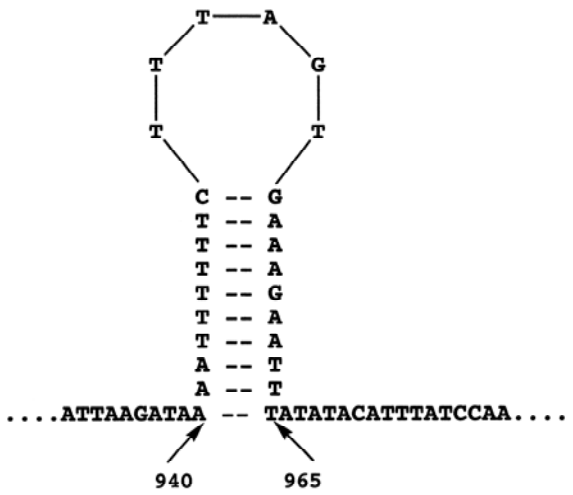

Fig. 5. Stem-loop structure that seems to be needed to the expression of PS3 subunit II (COII) in *E. coli* as a host. A940 and T965 (see Fig. 3) are shown in the figure. The solid lines show diester bonds of DNA, and the broken (or dashed) lines indicate hydrogen bonds between bases.

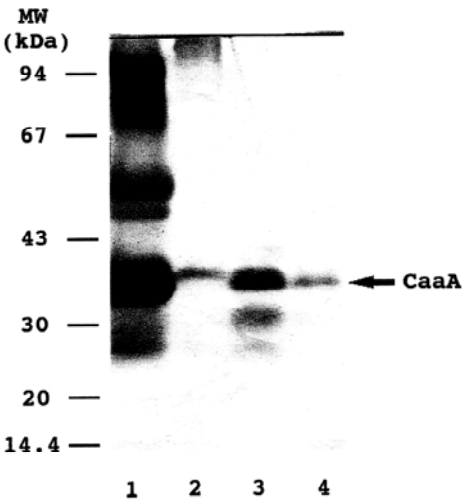

Fig. 6. Western blotting analysis of PS3 caaA gene product in *E. coli.* Lane 1; PS3 cytoplasmic membrane, lane 2; *E. coli* JM109 cytoplasmic membrane, lane 3; *E. coli* JM109 cytoplasmic membrane harboring the *Bam*H I-*Eco*R I fragment of PS3, lane 4; Purified subunit II of PS3 *caa*$_3$-type cytochrome *c* oxidase.

Fig. 7 shows the reverse phase HPLC analysis of the heme composition of cytoplasmic membranes isolated from *E. coli* JM109 harboring pCI-8000 (contains *caaF-caaE-caaABCD*), recombinant plasmid with the two step acetonitrile gradients (0-5 min.;50-70%, 5-25min.;70-100%) The elution profile was monitored by the absorbance at 406 nm. For heme A synthesis, PS3 *caaF-caaE-caaABCD* gene cluster is needed, as *E. coli* JM109 can not synthesize heme A. We also found that the order of heme A synthesis ability was pCI-8000 > pCI-3500 (contains *caaF-caaE-caaA*), pCI-1700 (contains *caaF*) and pCI-1800 (contains part of *caaE* and *caaA*), could not synthesize heme A by further analysis (data not shown). Although P1/T1 and P2/T2 transcription units work in *E. coli* cells, P3/T3 transcription unit may not seem to work by oneself. Fig. 8 shows a proposed heme A biosynthetic path way based on the expression test. There are promoter-terminator-like structures in *caaF, caaE,* and cytochrome *c oxidase* structural gene (*caaA, caaB, caaC, and caaD)* unit, individually shown in Fig. 4 and Fig. 5. Both *caaE* and *caaF* products can synthesize heme A *in vivo*. P1/T1 and P2/T2 transcription units work in *E. coli* cells, and the product of *caaE* was expressed in *E. coli* individually and had a thermostable heme O synthase (protoheme IX farnesyltransferase) activity in cytoplasmic membrane [20]. The *caaF* product of PS3 seems to take part in heme A biosynthesis from heme O (exchange of methyl group at position 18th of heme O to formyl group). Furthermore, the promoter-like structure on the 5'-upstream of

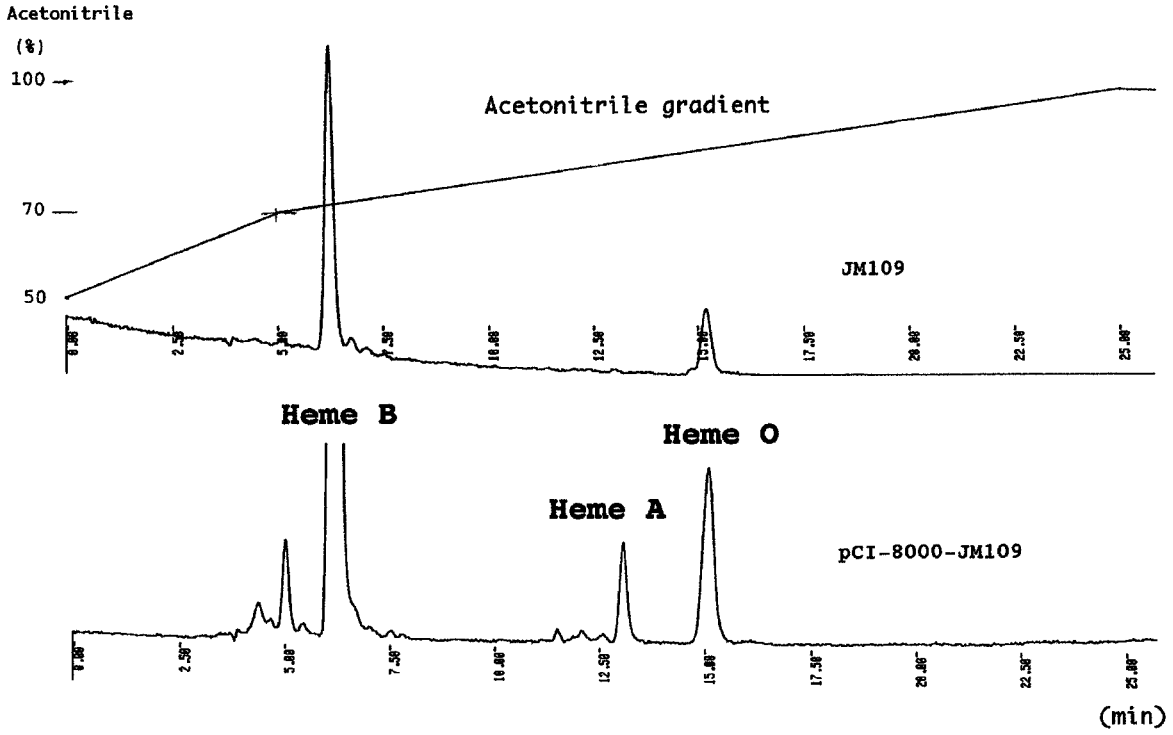

Fig. 7. Reverse phase HPLC analysis of the heme composition of cytoplasmic membranes isolated from *E. coli* JM109 harboring pCI-8000 recombinant plasmid.

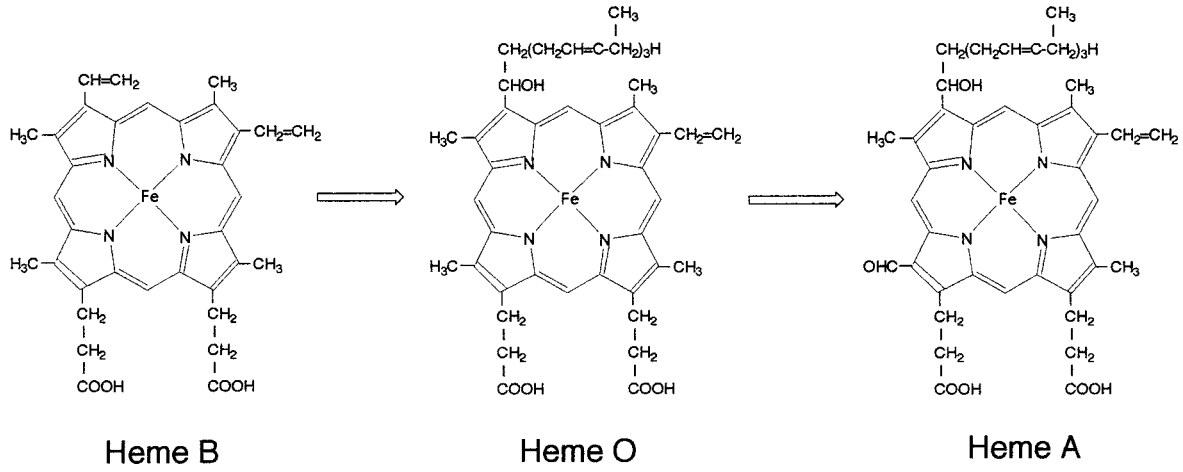

Fig. 8. Proposed pathway of heme A synthesis.

caaA was needed for the *in vivo* expression of *caaA* from PS3 in *Escherichia coli*. Recently, it was reported that heme A is not essential for assembly of the subunits of aa_3-type cytochrome *c* oxidase of *Rhodobacter sphaeroides,* and co-purification of subunits II and III with aposubunit I isolated from *cox*10 deletion strains indicated that assembly of the core oxidase complex occurred without the binding of heme A[23]. On the other hand, in *Bacillus subtilis ctaA-ctaB-ctaCDEF* gene cluster, *ctaCDEF* was not expressed independently to *ctaB*, different from PS3, in spite of the inability to detect *ctaC*-specific transcripts the absence of a promoter in the *ctaB-ctaC* 240 bp intercistronic space [24]. Further regulational analysis seems to be needed in order to realize the function of the gene cluster.

3-2. PS3 *caaE* gene product as heme O synthase (protoheme IX farnesyltransferase)

The deduced amino acid sequence of the *caaE* gene product (CaaE) is composed of 309 amino acid residues (molecular weight estimated from the sequence is 34,809) shown in Fig. 3, and is homologous to that of the *cyoE* gene product (CyoE: heme O synthase or protoheme IX farnesyltransferase) of *bo*-type ubiquinol oxidase operon (*cyoABCDE*) from *E. coli* [13] and of the *ctaB* gene product (CtaB) of aa_3-type cytochrome *c* oxidase gene cluster (*ctaA-ctaB-ctaCDEF*) from both *Bacillus subtilis* [14] and *Bacillus firmus* [21] (Fig. 2). We have tried gene expression of *caaE and caaF* in order to search of function of *caaE and caaF* products [11,12]. As described above, both *caaE and caaF* products take part in the heme A biosynthesis pathway. Our second effort to search the role of CaaE was a preparation of *cyoE-caaE* chimera gene and over-expression of the gene in *E. coli* [20]. A summary of the results is shown below. We examined the functional role of the *caaE* gene product in the *caaF-caaE-caaABCD* gene cluster for caa_3-type cytochrome *c* oxidase in the thermophilic *Bacillus* PS3. For efficient translation of a heterogeneous gene in *E. coli*, we took an advantage of the over-expression system established for the *E. coli cyoE* gene [17,18]. Thus, the *caaE* gene corresponding to Val32 to Trp309 (C-terminus) of thermophilic *Bacillus* PS3 was placed behind the 5'-terminal sequence corresponding to Gln8 of the CyoE (nucleotide sequence of 5'...(SD)...ATGATGTTTAAGCAATACCTGCA(A/G) **GTAACGAAA**....**TGG**...3' corresponds to N-term -MMFKQYLQ**VVK**....**W**-C-term). The junction site was chosen as a putative end of the N-terminal protruding region from membrane [25]. When the of *cyoE-caaE* chimera gene was expressed in ST4676 (Δ*cyo cyd*$^{+}$)/pTTQ18-*caaE* by induction with IPTG, a 24.5 kDa polypeptide was specifically over-expressed in the cytoplasmic membrane [20]. The apparent molecular weight of the CyoE-CaaE chimera protein in 12.5% SDS polyacrylamide gel electrophoresis was smaller than

that deduced from the DNA sequence (32.3 kDa), probably due to aberrant electrophoretic mobility of hydrophobic membrane proteins. The expression level of the CyoE-CaaE chimera protein was estimated to be about 5% of membrane proteins by densitometric measurement. From genetic complementation analysis in *E. coli* using the chimeric operon *cyoABCD-caaE* and the heme O synthase assay using the CyoE-CaaE chimera over-produced *E. coli* cytoplasmic membranes, we found that the CyoE-CaaE chimera protein expressed that in *E. coli* functions as a thermostable heme O synthase *in vivo* and *in vitro* and suggested that the CyoE-CaaE chimera protein supplies heme O as an intermediate for heme A biosynthesis in thermophilic *Bacillus* PS3 [20]. Fig. 9 shows hydropathy profiles of the *caaE, cyoE-caaE* chimera, and *cyoE* gene products (CaaE, CyoE-CaaE chimera, and CyoE proteins, respectively). The Kite and doolittle index [26] was used to calculate the profile with a window length of 10. The profile of CyoE-CaaE chimera protein is resembled to those of Both PS3 CaaE and *E. coli* CyoE. Although the optimum temperature of the reaction with the CyoE-CaaE chimera membranes was found to be about 60°C and retained the activity even at 70°C at a level comparable to that at 37°C, At higher temperature, both membranes lost completely the heme O synthase activity, may be due to either the thermo-instability of *E. coli* membranes or the deletion of N-terminal 31 amino acid residues of PS3 CaaE. Furthermore, a recombinant *E. coli* cell harboring the *caaF-caaE* gene cluster of PS3 synthesized heme A [11,12]. As *B. subtilis* mutant strain lacked *ctaA* gene cannot synthesize heme A, CaaF, *caaF* gene product of PS3, seems to take part in heme A biosynthesis from heme O (exchange of methyl group at position 18 of heme O to formyl group).

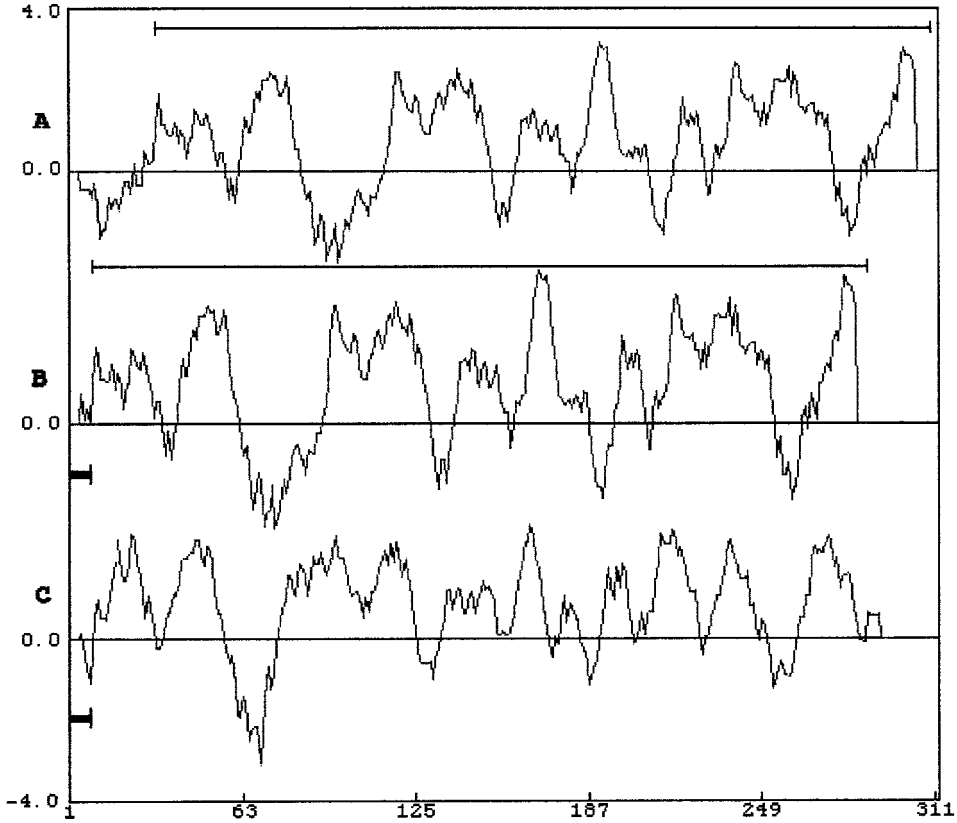

Fig. 9. Hydropathy profiles of the *caaE, cyoE-caaE chimera, cyoE* gene products (CaaE, CyoE-CaaE chimera, CyoE, respectively). The Kite and Doolittle index [26] was used to calculate the profile with a window length of 10. A; *Bacillus* PS3 CaaE, B; CyoE-CaaE chimera, C; *E. coli* CyoE.

Protoheme IX farnesyltransferase (heme O synthase) from yeast and mammals (gene *coxX*), and from bacteria (genes *cyoE, ctaB, and caaE*) is also called a cytochrome *c* oxidase assembly protein due to the requirement of its assistance in building the complex of active cytochrome *c* oxidase. Fig. 10 shows an alignment of the *caaE* gene product of Bacillus PS3 with homologous sequences. The predicted *caaE* gene product (B.PS3 CaaE) is aligned with other known sequences (An alignment of deduced amino acid sequences of the *caaE/ctaB/cyoE/coxX/coxD/coxE/yjdK* gene products: B.ste CtaB in *Bacillus stearothermophilus* K1041 [27], B.fir CtaB in *Bacillus firmus* [21], B.sub CtaB in *Bacillus subtilis* [14], slightly modified by [28], B.sub YjdK in *Bacillus subtilis* [28,29], E.col CyoE in *E. coli* [13], P.den CtaB(ORF1) in *Paracoccus denitrificans* [30], P.aer CtaB in *Pseudomonas aeruginosa* [31], Sy.sp CoxD in *Synechocystis* sp. [32], B.jap CtaB in *Bradyrhizobium japonicum* [Direct submission by Rossmann, R. (20, May, 1999)], Yeast CoxX in *Saccharomyces cerevisiae* [33], Human CoxX in Human [34]). Seven probable transmembrane segments are marked with double-underlining and invariant residues with asterisk. Sharp sign (#) means Asp or Glu, both have resemble side chains. The conservation clusters to two regions: one between helices II and III, and another between IV and V. Fig. 11 shows comparison of hydropathy of *caaE* products from PS3 with *ctaB* (*cyoE*) gene products from other species. The *caaE* gene product seems not only to be very hydrophobic but also to have high structural homology among all *ctaB (cyoE)* products from other species. High correlation was also seen among *caaE (cyoE, ctaB)* products by Harr plots analysis (data not shown). Fig. 12 shows a topological model of PS3 heme O synthase based on three points of view shown below. First, an evidence for the topology of the *cyoE* gene product in the cytoplasmic membrane was obtained by using the technique of gene fusions [25]. There are seven probable transmembrane segments and the conservation clusters to two regions, one between helices II and III, and another between IV and V. Both are possibly on the cytoplasmic side of the bacterial membrane [25]. Second, an evidence for the amino acid residues needed for the activity of heme O synthase from *E. coli* by the use of the technique of site-deleted mutagenesis [19]. Third, Invariant residues were obtained from the multiple alignment of deduced amino acid sequences of the another putative *caaE/ctaB/cyoE/CoxX* gene products from DNA data bank of Japan (DDBJ) including various complete genome sequences and Swiss-plot protein data bank. Among the CaaE homologues, a total of 12 amino acid residues have been shown to be strictly conserved and mainly localized in the putative cytoplasmic domains: Lys-34, Asp-93, Met-96, Arg-102, Tyr-151, Lys-156, Gly-166, Gly-170, Trp-199, His-203, Tyr-215, and Ser-292 of PS3 CaaE are conserved that correspond to Lys-11, Asp-65, Met-68, Arg-74, Tyr-124, Lys-129, Gly-143, Gly-150, Trp-172, His-131, Tyr-188 and Ser-268 of *E. coli* CyoE, respectively. Asp-93 and Gul214 of PS3 CaaE are conserved or substituted Glu or Asp. Met-68, Gly-150, and His-131 of PS3 CaaE were reported to be unnecessary for active CyoE (Heme O synthesis) as a result of site-deleted mutagenesis [19]. Other amino acid residues shown in Fig. 12 are not strictly conserved. In addition to these observations, a local hydrophobic region, widely conserved, was found between Trp178 and Trp199 (Fig. 10 and Fig. 12). PS3 CaaE also seems to have seven potential transmembrane domains (I-VII) and three cytoplasmic domains (1, 2a, and 2b) from hydropathy profile (Fig. 11) and Harr-plot analysis (data not shown).

```
Yeast CoxX      1 --------MSYFPRTYAHLMRNVLAHNKGNIYLQIGTQLHDTQIKIRFNGVRYISRNHGG   52
Human CoxX      1 MAASPHTLSSRLLTGCVGGSVWYLERRTIQDSPHKFLHLLRNVNKQWITFQHFSFLKRMY   60

Yeast CoxX     53 KQQHINTAPIEFTPNFGYGDRTSNCNKKVESTAMKTLRCTDDISTSSGSEATTDASTQLP  120
Human CoxX     61 VTQLNRSHNQQVRPKPEPVASPFLEKTSSGQAKAEIYEMRPLSPPSLSLSRKPNEKELIE  120

                                                  ========= I ========
                                                   *                  *
B.PS3 CaaE      1 --------MAELKAVHQDAADAGHRSHVSVKTVWRELSSVVKIGIVNSNLITTFAGMWLA   52
B.ste CtaB      1 -----------MADLKAVQEDVRQRPQASIKLFWKELSAVVKIGIVNSNLITTFAGMWLA   49
B.fir CtaB      1 ----MNKSNTAIDPTNVIEAGPDSSVADVQQKSWKDYLVLAKQGIVTSNLITTFAGIYLA   56
B.sub CtaB      1 ------------MANSRILNDTAIDGQIEETTAWKDFLSLIKIGIVNSNLITTFTGMWLA   48
B.sub YjdK      1 -MQFWGRRLFLENTRDSAAISETKYIKASNRVTIYDFIKLAKPGIIISNSIATFAGFWIA   59
E.col CyoE      1 --------------------------------MMFKQYLQVTKPGIIFGNLISVIGGFLLA   29
P.den CtaB      1 --------------------------MGPAEAGFGDYVALLKPRVMSLVVFTAFVGLWIA   34
P.aer PAO       1 ----------------------MATVIDRHSQPTWRDFLELTKPKVVVLMLITSLIGMLLA   39
Sy.sp CoxD      1 ----------------MVTSTKIHRQHDSMGAVCKSYYQLTKPRIIPLLLITTAASMWIA   44
B.jap CoxE      1 --------------MSVLDQNAVDINPRISEAEVGDYIALLKPRVMSLVIFTALVGMAMA   46
Yeast CoxX    113 FNVKLVDPMVRKSKRPSHAISEGLNMKTLKKKVIMPYLQLTKPRLTILVMLSAICSY--A  170
Human CoxX    121 LEPDSVIEDSIDVGKETKEEKRWKEMKLQVYDLPGILAQLSKIKLTALVVSTTAAGF--A  178

                              ======== II =======                    ====
                                        *     *   #   *  * **  *     *
B.PS3 CaaE     53 --FYFTGEHFLENLHLVFFTLFGAALVIAGSCAINNYIDRDIDQYMERTKARPTVTGTMD  110
B.ste CtaB     50 --FYFTGERFLENLHIVFFTLFGAALVIAGSCSINNFIDRDIDQHMERTKTRPTVTGTME  107
B.fir CtaB     57 --IVYTGTVFTMHLDTMIFALLGAALVMAGGCTLNNYIDRDIDHLMERTKERPTVTGRFS  114
B.sub CtaB     49 --LHISGLSFLGNINTVLLTLIGSSLIIAGSCAINNWYDRDIDHLMERTKVRPTVTGKIQ  106
B.sub YjdK     60 FASAEKTLTGLAFLMTMVTAMLGTAFVMASGTVYNNYFDRHMDAKMARTRSRASVTGKMP  119
E.col CyoE     30 --SK-----GSIDYPLFIYTLVGVSLVVASGCVFNNYIDRDIDRKMERTKNRVLVKGLIS   82
P.den CtaB     35 --PQPVN-----PFVAFCAVLFIALGGGASG-ALNMWYDADIDAVMRRTAGRPVPSGRVT   86
P.aer PAO      40 --TKAPLDGFVPWQVLIFGNLGIGLCAGA-AAAVNHVVDRRIDSIMARTHKRPLAEGRVS   96
Sy.sp CoxD     45 --SEGRVD--LPKL-LI--TLLGGTLAAASAQTLNCIYDQDIDYEMLRTRARPIPAGKVQ   97
B.jap CoxE     47 --PGHFH-----PVLAITS-LLCIAVGAGASG-ALNMALEGDIDAKMSRTANRPIPRGRIT   98
Yeast CoxX    171 L---SPYPASVNE-LLCLTVGTTLCSGSAN--AINMGREPEFDRQMVRTQARPVVRGDVT  224
Human CoxX    179 L---APGPFDWPCFLLTSVGTGLA-SCAAN--SINQFFEVPFDSNMNRTKNRPLVRGQIS  232

                 ====III========          = ========IV========
                                *                    *   *    **        *
B.PS3 CaaE    111 PRRVLWLGIGLVAIGEMSLLMTTVT-AAVVGLIGMVTYVFLYTLWTKRHYTITTVVGSIS  169
B.ste CtaB    108 PRRVLWLGVTLVAIGTMSLLMTTVT-AAIVGLIGVVTYVFLYTLWSKRNYTLNTVVGSIS  166
B.fir CtaB    115 AKHVLLVGLAQAALGIIFLALTTPT-AAVIGLIGLFIYVVLYTMWTKRTTTLNTIVGSFS  173
B.sub CtaB    107 PSQALWSGILLVALGLIMLLMTTVM-AAVIGFIGVFTYVVLYTMWTKRRYTINTVVGSVS  165
B.sub YjdK    120 PAMILTYGSVLGIAGLAMLYSLNPLTA-FLGLAAFIFYAIIYTVWVKRTSVWSTFVGSFP  178
E.col CyoE     83 PAVSLVYATLLGIAGFMLLWFGANPLACWLGVMGFVVYVGVYSLYMKRHSVYGTLIGSLS  142
P.den CtaB     87 SQEPLAVKIALSGLSVMMLGAGGNWFAAGFLAFTIFFYAVVYTIWLKRSTPQNIVIGGAA  146
P.aer PAO      97 PSMALGFALLLALAGMAVLLAFTNPLTAWLTLASLLGYAALYTGFLKRATPQNIVIGGLA  156
Sy.sp CoxD     98 PRHALIFALALGVLSFALLATFVNVLSGCLALSGIVFYMLVYTHWLKRHTAQNIVIGGAA  157
B.jap CoxE     99 RPEAMTFGMTLAFFSVMTLGILVNWI-AGALLAFTIFYVVIYTMWLKRWTAQNIVIGGAA  157
Yeast CoxX    225 PTQAFEFAALIGTLGVSILYFGVNPTVAILGASNIALYGWAYTSM-KRKHIINTWLGALV  283
Human CoxX    233 PLLAVSFATCCAVPGVAILTLGVNPLTGALGLFNIFLYTCCYTPL-KRISIANTWVGAVV  291
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                                                                                ====
                     *  **     *           *       *  **          #*
B.PS3 CaaE    170 GAVPPFIGWTAVDPEFHIVPLILFLIMFL-WQPPHFLALAMKRCEEYRAAGIPMLPVVHG  228
B.ste CtaB    167 GAVPPVIGWTAVDSDFHIVPLILFLIMFL-WQPPHFLALAMKRCEEYRAAGIPMLPVVHG  225
B.fir CtaB    174 GAVPPLIGWAAIDGGLHLYAWLLFFIMFL-WQPPHFLALAMKRVEEYRAAGIPMLPVVAG  232
B.sub CtaB    166 GAVPPLIGWTAVEGNIGVVAWVLFMILFI-WQIPHFLALAIKKTEDYRAANIPMLPVVYG  224
B.sub YjdK    179 GAAPPLMGYCAVTGDFSMTAVLLYTIMFL-WQPPHFWAIGIRRKEEYRAAGVPLLPVVKG  237
E.col CyoE    143 GAAPPVIGYCAVTGEFDSGAAILLAI-FSLWQMPHSYAIAIFRFKDYQAANIPVLPVVKG  201
P.den CtaB    147 GAFPPMIGW-ALPTGGIGIESLLMGALIFFWTPPHFWALALFMKDDYSKAGVPMLTVTHG  205
P.aer PAO     157 GAAPPLLGWVAITGHLSAEPLLLVLIIFA-WTPPHFWALCIHRKDEYAKADIPMLPVTHG  215
Sy.sp CoxD    158 GSIPPLVGWAAVTGDLSWTPWVLFALIFL-WTPPHFWALALMIKDDYAQVNVPMLPVIAG  216
B.jap CoxE    158 GALPPVVAWAAVTGTVDVEPLLLFAIIFF-WTPPHFWALALFRSDDYARAGIPMLPNVAG  216
Yeast CoxX    284 GMVPPLMGWAAASPLSHPGSWCLAGLLFA-WQFPHFNTLSHNIRNEYKNAGYVMTAWKNP  342
Human CoxX    292 GAIPPVMGWTATTGSLDAGAFLLGGILYS-WQFPHFNALSWGLREDYSRDGYCMMSVTHP  350

                  ===V==========                 ========VI=======
B.PS3 CaaE    229 FAMTKRQIIVWVACLLPLPFYL-FSLG----VPFLVVATLLNVG-WLFLGLWGLKMKDDL  282
B.ste CtaB    226 FEMTKRQIIVWVACLLPLPFYL-FSLG----IPFLIVATVLNVG-WLLLGLWGLKMKDDI  279
B.fir CtaB    233 FEMTKRQMVVYVAALLPVSLML-YPFG----LVYTIVAAVLGVG-WLALGIAGFKMKDDI  286
B.sub CtaB    225 FEVTKRQIIVWVACLMPLPFFL-GSLG----LPIVILGLLLNIG-WLILGLMGFRSKNIM  278
B.sub YjdK    238 NHVTKIKMMQYIAVLVPVTLLFPFSLGTGHISPFYFLAALVLGGIWIKKSIKGFKTDDDV  297
E.col CyoE    202 ISVAKNHITLYIIAFAVATLML--SLGGYAGYKYLVVAAAVSVW-WLGMALRGYKVADDR  258
P.den CtaB    206 RKVTRHIFAYTL-VLAPFALWL----GFTSVGGPLYLAVSVVLNALFIAGGWQILRRSED  260
P.aer PAO     216 ERYTKLHILLYTLVLFAVSLMP----FVIHMSGLVYLLCALALGARFLDWAWALYCDSRP  271
Sy.sp CoxD    217 EEKTVSQIWYYS-LLVVPFSLL-LVYPLHQLGILYLAIAIILGGQFLVKAWQLKQAPGDR  274
B.jap CoxE    217 PDATRLQILLYTIVLIAVAAAP-WALGYFDAVYGVVSLILGAGMLVLAINVYMRRERSQS  275
Yeast CoxX    343 LLNARVSLRYSILMFPLCFGLSYFNITDWYYQIDSGLINAWLTFWAFKFYWQQRINYSAK  402
Human CoxX    351 GLCRRVALRHCLALLVLSAAAPVLDITTWTFPIMALPINAYISHLGFRFYVDADRRSS--  408

                                        =======VII========
                                        *   *
B.PS3 CaaE    283 KWA----------------KWMFVYSLNYLTILFVAMIIATLW------------------  309
B.ste CtaB    280 KWA----------------KLMFVYSLNYLTILFVAMVIATIW------------------  306
B.fir CtaB    287 KWA----------------RLMFVYSLNYLTILFVLMVIVHF-------------------  312
B.sub CtaB    279 KWA----------------TQMFVYSLNYMTIYFVAMVVLTLF-----------------  305
B.sub YjdK    298 KWA----------------KDMFVYSLIYFCLLFFIMMIDSFMMFLIR------------  329
E.col CyoE    259 IWA----------------RKLFGFSIIAITALSVMMSVDFMVPDSHTLLAAVW------  296
P.den CtaB    261 QAQADGYRVE----------KRYFRLSLYYTFLHFLALLVQHWVGGW--------------  297
P.aer PAO     272 HAA----------------IKTFKYSIVYLFLLFMALLVDHYLPLKLLL------------  304
Sy.sp CoxD    275 DLA----------------RGLFKFSIFYLMLLCLAMVIDSLPVTHQLVAQMGTLLLG--  316
B.jap CoxE    276 LRAT---------------RKLFAFSILYLFALFATLLAEVVFRALAPMAGGA--------  313
Yeast CoxX    403 TLKDNVKFNKGLSVANIYARKTFMASVLHLPAILILAIIHKKGRWDWIYPGEAKRPQERF  462
Human CoxX    409 --------------------RRLFFCSLWHLPLLLLLMLTCKRPSGGGDAGPPPS------  443
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Fig. 10. An alignment of *caaE* gene product with homologous sequences. The predicted *caaE* gene product (CaaE) of *Bacillus* PS3 is aligned with other known sequences (An alignment of deduced amino acid sequences of the *caaE/ctaB/cyoE/coxX* gene products: B.ste CtaB in *Bacillus stearothermophilus* K1041 [27], B.fir CtaB in *Bacillus firmus* [21], B.sub CtaB in *Bacillus subtilis* [14], slightly modified by [28], B.sub YjdK in *Bacillus subtilis* [28,29], E.col CyoE in *E. coli* [13], P.den CtaB (ORF1) in *Paracoccus denitrificans* [30], P.aer CtaB in *Pseudomonas aeruginosa* [31], Sy.sp CoxD in *Synechocystis* sp. [32], B.jap CtaB in *Bradyrhizobium japonicum* [Direct submission by Rossmann, R. (20, May, 1999)], Yeast CoxX in *Saccharomyces cerevisiae* [33], Human CoxX in Human [34]). Seven probable transmembrane segments are marked with double-underlining and invariant residues with asterisk. Sharp sign (#) means Asp or Glu, both have resemble side chains.

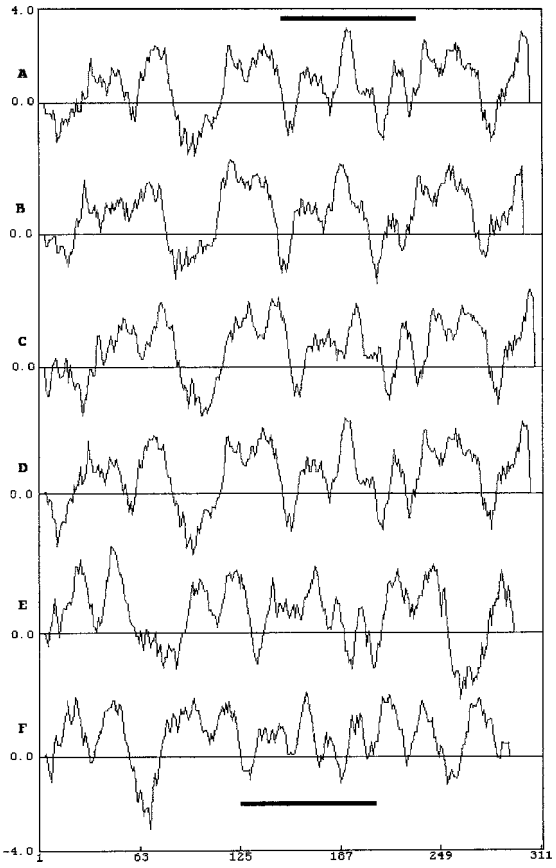

Fig. 11. Hydropathy profiles of the *caaE/ctaB/cyoE* gene products. The Kite and Doolittle index [26] was used to calculate the profile with a window length of 10. The numbers above the peaks indicate potential membrane-spanning α-helical regions. A; B.PS3 CaaE, B; B.sub CtaB, C; B.fir CtaB, D; B.ste CtaB, E; P.den CtaB, F; E.col CyoE.

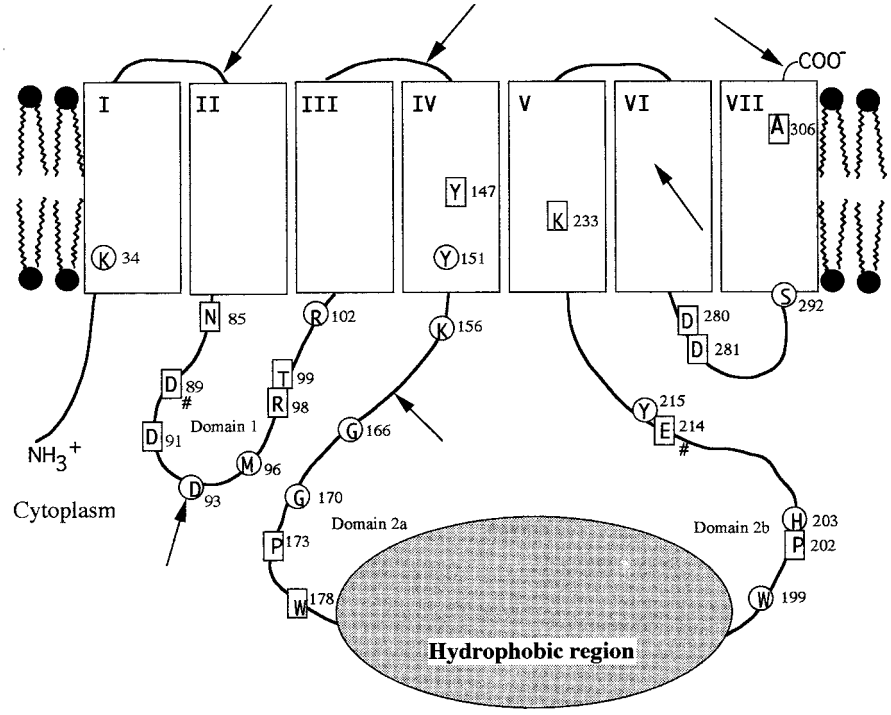

Fig. 12. Topological model for PS3 heme O synthase. This model was based on the Kyte-Doolittle hydropathy profile (Fig. 9 and Fig. 11), the gene fusion experiments of *E. coli cyoE* [25] and the site-directed mutagenesis approach of *E. coli cyoE* [19]. The putative membrane-spanning regions are indicated by rectangles. Conserved amino acid residues among the CaaE homologues are enclosed with circles, but not strictly conserved residues with quadrangles. Residues in the case of a choice between Asp and Glu are marked by shape signs.

Table I shows comparison of conservative amino acid residues of 57 putative heme O synthases and 36 putative 4-hydroxy benzoate polyprenyl transferases. Invariant residues were obtained from the multiple alignment of deduced amino acid sequences of the another putative gene products from DNA data bank of Japan (DDBJ) including various complete genome sequences and Swiss-plot protein data bank. The following prenyltransferases are known to be evolutionary related [35,36]: Bacterial 4-hydroxybenzoate octaprenyltransferase (gene *ubiA*), Yeast mitochondrial para-hydroxybenzoate polyprenyltransferase (gene *coq2*), Protoheme IX farnesyltransfe rase (heme O synthase) from bacteria (genes *caaE, cyoE or ctaB*) and from yeast and mammals (gene *coxX*).

Table I. Comparison of conservative amino acid residues of heme O synthases and para-hydroxybenzoate polyprenyl transferases. Amino acid residues with underline correspond to the residues that seem to be essential for heme O synthase activity from *E. coli* as a result of site-deleted mutagenesis [19]. Residues in bold face type indicate the conservative residues only among heme O synthases.

Conservative residues among heme O synthases	Charged	K34, N85, D(or E)89, D93, R98, R102, K156, **H203**, E(or D)214,
	Polar	T99, Y151, **T**(or S)**152**, **Y215**, **S292**
	Nonpolar	**M96**, **G166**, **G170**, W199, **L**(or I)**207**
Conservative residues among para-hydroxybenzoate polyprenyl transferases	Charged	K(or R)34, N85, D89, D93, R98, R102, K156, R157, D204, D(correspond to E214 of PS3)
	Polar	T(or S)99, Y151,
	Nonpolar	W199,

N-XXX-[DEH]-XX-[LIMF]-D-XX-[VMN]-X-R-[ST]-XX-R-XXXX-G (X; not strictly conserved), known as prenyltransferase family signature, is well conserved in PS3 CaaE (N-XXX-D-XX-I-D-XX-M-X-R-T-XX-R-XXXX-G). In addition to this region, Lys (or Arg)-34, Tyr-151, Lys-156, Trp-199 of PS3 CaaE is conserved among prenyltransferase family. Glu-214 of PS3 CaaE is replaced with Asp in the case of all 4-hydroxybenzoate polyprenyltransferases and several heme O synthases. Therefore, Lys-34, Asn-85, Asp-89, Asp-93, Arg-98, T-99, Arg-102, Tyr-151, Lys-156, Trp-199, Glu-214 of PS3 CaaE seem to be candidates for taking part in prenyl binding, transfer, or structure preserving. Met-96, Gly-166, Gly-170, His-203, Tyr-215, Phe-289 and Ser-292 of PS3 CaaE seem to be candidates for participating heme B binding or structure preserving. Consequently, Gly-170, His-203,Tyr-215, and Ser292 of PS3 CaaE may be indispensable for the binding by the comparison with CyoE [13,19]. In addition to biochemical and genetic studies containing the regulational mechanism of the *ctaA-ctaB-ctaCDEF gene* cluster, crystallographic studies on the CaaE and CaaF proteins are indispensable.

Acknowledgments

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