ELSEVIER

Biochimica et Biophysica Acta 1188 (1994) 302–310

BBA Biochimica et Biophysica Acta

Over-expression of membrane-bound cytochrome *c*-551 from thermophilic *Bacillus* PS3 in *Bacillus stearothermophilus* K1041

Shunsuke Noguchi, Tamayo Yamazaki, Atsuo Yaginuma, Junshi Sakamoto, Nobuhito Sone *

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

Received 18 April 1994; revised 4 August 1994

Abstract

Cytochrome *c*-551 is a lipoprotein of about 10500 Da, found in thermophilic *Bacillus* PS3 grown under air-limited conditions. An expression vector was constructed from a structural gene of PS3 cytochrome *c*-551, syththetic oligonucleotide as a promoter for *Bacillus stearothermophilus* and a shuttle vector for *Escherichia coli* and *B. stearothermophilus*. The transformed cells of *B. stearothermophilus* K1041 expressed cytochrome *c*-551 as much as 5 nmol/mg membrane protein. The effects of over-expression on the host cells are analyzed; a slightly slower growth rate and an increased synthesis of cytochrome oxidase (about twofold) occurred. Over-expressed (4–10-fold) cytochrome *c*-551 were purified, and its properties were examined to know whether the protein is processed as in PS3 cells grown under air-limited conditions. The molecular mass determination and treatment with *Rhizopus* lipase suggested that the same processes, cleavage of signal peptidase, blocking of the N-terminal group and acylation of glycerol residue by two fatty acids, took place in the over-expression system. Fatty acylation seems useful for the cytochrome *c* to be effectively oxidized.

Keywords: Cytochrome *c*; Over-expression; *cccA*; Post-translational modification; (*B. stearothermophilus*)

1. Introduction

The thermophilic *Bacillus* PS3 isolated from Japanese hot spring uses cytochrome caa_3-type oxidase when cultured under highly aerobic conditions [1–3]. The cells also synthesize cytochrome $bc_1(b_6f)$ complexes [4], and these two respiratory complexes form a super-complex without requiring another cytochrome *c* to oxidize menaquinol for yielding chemiosmotic energy for ATP synthesis [5]. In contrast, PS3 cells grown under air-limited conditions synthesized higher amounts of *b*-, *c*- and *o*-type cytochromes, suggesting that a different respiratory chain is operating [2,6]. One of them, synthesized profoundly under air-limited conditions, is cytochrome *c*-551, which is cholate-extractable membrane-bound small-sized (M_r of about 10500) *c*-type cytochrome [7]. These characteristics of respiratory chain seems to be general among thermophilic *Bacilli*, since *Bacillus stearothermophilus* showed the similar response of respiratory chain variation [8,9].

We purified cytochrome *c*-551 from PS3 cells grown under air-limited conditions [7], and cloned its structural gene from genomic DNA [10]. Comparison of the deduced amino acid sequence from DNA with characteristics of cytochrome *c*-551 allowed us to speculate that nascent cytochrome*c*-551 containing 111 amino acids is processed to release the signal peptide of 17 or 18 amino acids, and the new N-terminus is blocked. *Rhizopus* lipase treatment also suggests that the cysteine residue at the new N-terminus was modified with two fatty acids probably via glycerol, as in the case of *c*-type cytochrome of photosynthetic *Rhodopseudomonas viridis* reaction center [11].

We have been trying to over-express PS3 cytochrome *c*-551 in order to get large amounts of the cytochrome for determination of its structure and for seeking an interesting application as a thermo-stable and hydrophobic small-sized cytochrome *c*. Here we report a method to over-express cytochrome *c*-551 in a

Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tc, tetracycline; HPLC, high performance liquid chromatography; GC, gas chromatography; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

* Corresponding author. Fax: +81 948 297801.

SSDI 0005-2728(94)00137-5

transformable *Bacillus stearothermophilus* strain K1041, the effect of the expression vector on K1014 cells, and several properties of the expressed cytochrome, suggesting that the same processing observed in *Bacillus* PS3 takes place in *B. stearothermophilus* K1041.

2. Materials and methods

2.1. Materials

Bacillus stearothermophilus K1041 [12], pSTE12 [13] and pJN531 [14] were kindly donated by Dr. Narumi and Prof. Kihara of Kansai Medical University (Jichi Medical School at that time). An expression vector pTrc99A was purchased from Pharmacia (Uppsala). The low molecular weight protein standards (bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme) were purchased from Bio-Rad, Richmond. Horse heart cytochrome *c* and MOPS were purchased from Sigma Chemicals, St Louis. Lipase from *Rhizopus delemer*, fine grade were products of Seikagaku Kogyo (Tokyo). DEAE-Toyopearl HW-60 was a product of Tosoh (Tokyo). Other reagents were obtained as described previously [10].

2.2. Analytical methods

Oxidase activity was measured polarographically with an oxygen electrode (YSI #4001) in a semiclosed cell, or followed with a pH meter (Beckman 4500) with ascorbate (10 mM) in the medium (1.6 ml) composed of 25 mM K_2SO_4, 2.5 mM $MgSO_4$ and 1 mM MOPS-KOH buffer (pH 6.4) at 40°C as described previously [15].

Absorption spectra were measured with a recording spectrophotometer (Beckman DU70) at room temperature. The amount of cytochrome *c*-551 was determined from the $Na_2S_2O_4$-reduced minus oxidized difference spectrum using a millimolar extinction coefficient of 20.9 at 551 [6]. The following millimolar extinction coefficients were used for the determination of other cytochromes; 23.2 at 604 to 630 nm for cytochrome aa_3 [1], and 17.2 at 551 to 538 nm [16] for total cytochrome *c* contents.

Hydrophobicity of cytochrome *c*-551 was monitored by HPLC with a C_4 column (Waters microbondasphare N10036) as described previously.

Fatty acyl groups of cytochrome *c*-551 were methylated with 5% BF_3 in methanol at 70°C for 30 min, and analyzed with a Shimadzu GC6BM gas chromatograph or a GC-mass system composed of HP5QQGC and JOEL DX-303 with DA-500 Data System.

Ion spray mass spectra were measured in a Sciex API III instrument (Sciex, MDS Health Group, Thornhill, Canada). The samples of cytochrome *c*-551 from the revcerse phase HPLC were applied to the capillary by a syringe pump at a flow rate of 3 μl/min. The spray potential was 5 kV, and the orifice potential was 100 V.

SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli with 15% gel [17]. Staining for hemes was carried out with *o*-toluidine [18].

Other methods were the same as described previously [6,11].

2.3. Construction of plasmids for over-expression of cytochrome c-551

The construction of the expressing plasmid, pSTEc551, is summarized Fig. 1. We used three kinds of plasmids; pTrcc551 is a source of cytochrome*c*-551 structure gene with the initiation codon, which is derived from commercially-available pTrc99A, while pJN531 is the source of promoter which has been used to express *Escherichia coli* aspartate transcarbamylase by Narumi et al. [14], and pSTE12 is a shuttle vector of *E. coli* and *B. stearothermophilus* used by them [13]. In order to construct pTrcc551 we used 676 bp DNA cloned in pUC19 [10]. Treatment of plasmids and several methods for molecular cloning followed the methods of Maniatis et al. [19].

2.4. Transformation of B. stearothermophilus

A strain K1041 was transformed by electroporation using A Bio-Rad Gene Pulser apparatus with a pulse controller by the method of Narumi et al. [12].

2.5. Culture of cells

The small scale culture (200 ml) of *B. stearothermophilus* K1041 was carried out at 55°C in a 1-L flask with baffle by shaking vigorously (220 rpm). The medium contained 0.8% polypeptone, 0.2% yeast extract, 0.3% NaCl and 0.05% K_2HPO_4 (pH 7.2–7.8). The concentration of tetracycline, if present, was 1–2 mg/l. The large scale culture (6 l) was carried out at 55°C in a jar fermentor (Tokyo Rika, MBF801) with vigorous aeration (1 l/min) and stirring (360 rpm). The cells were harvested at an early stationary phase when A_{650} was about 1.4. In order to follow the growth curve of cells more correctly the turbidity (light scattering at 90) was monitored at 575 nm. The turbidity of 10 was about 1.0 A_{650}. The membrane fragments were prepared as reported previously [20].

2.6. Purification of over-expressed cytochrome c-551

The following procedure is simplified and modified from the previous one which was used to purified

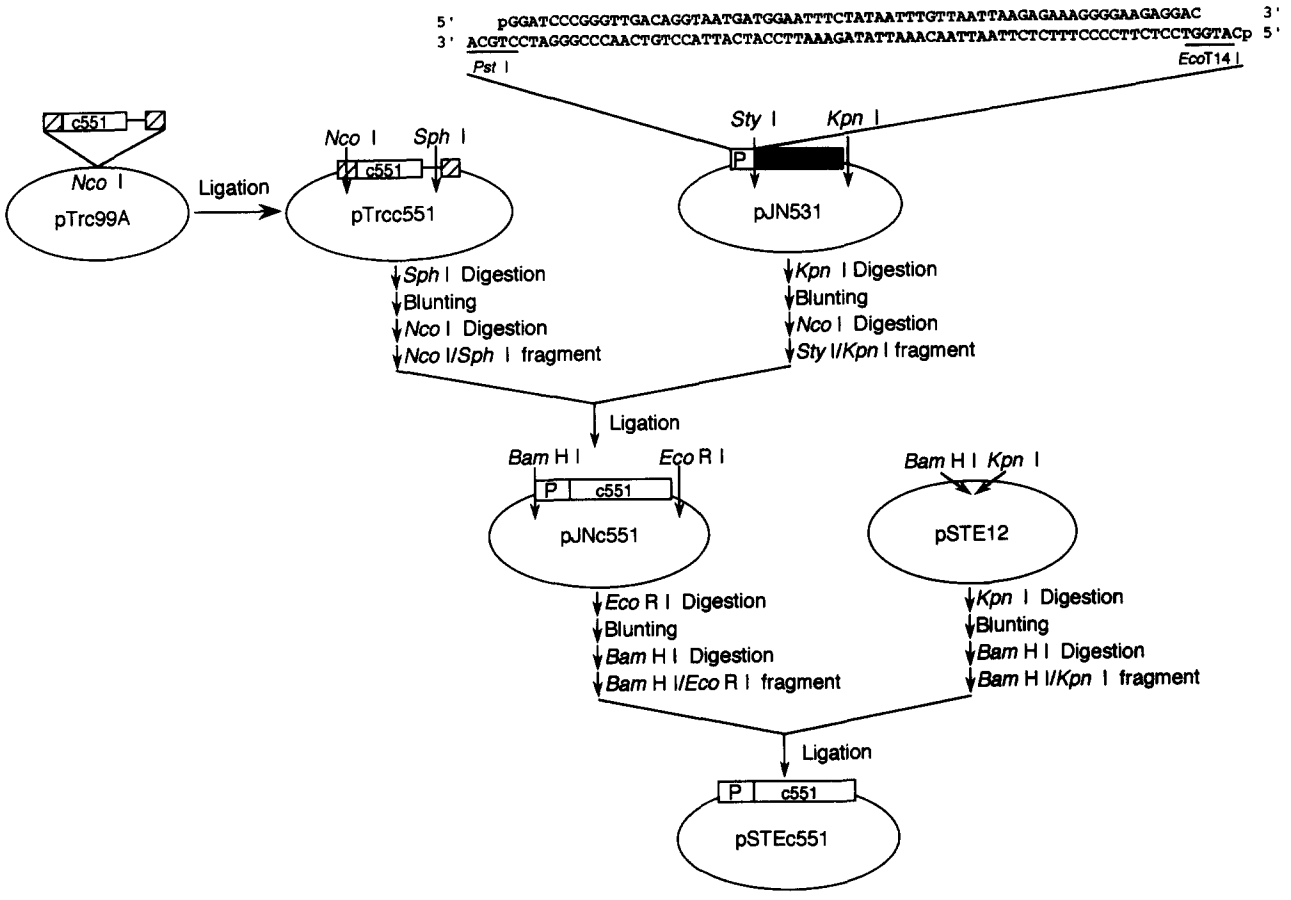

Fig. 1. Construction of pSTEc551 for expression in *B. stearothermophilus* K1041. The 676 bp *Pfl*MI-*Hin*dIII fragment of *cccA* gene coding for cytochrome *c*-551, after being ligated with *Nco*I linker (pGCCATGGC) was introduced into *Nco*I site of pTrc99A to construct pTrcc551. The 666 bp *Nco*I-*Sph*I (blunt) fragment of pTrcc551 was introduced into pJN531 containing the promoter segment to construct pJNc551, and the 745 bp *Bam*HI-*Eco*RI (blunt) fragment of pJNc551 containing *cccA* gene after the promoter and Shine-Dalgarno sequence was introduced into the multicloning site of pSTE12 to construct pSTEc551, which can be amplified in *E. coli* and be expressed in *B. stearothermophilus* K1041.

cytochrome *c*-551 from PS3 cells grown under air-limited conditions [7,10].

Step 1. The washed membrane fraction from K1041(pSTEc551) [10] was suspended in a solution containing 2% cholate, 0.5 M Na_2SO_4, 30 mM Tris-H_2SO_4 buffer (pH 8.0) and 1 mM EDTA in a final volume of 100 ml. The mixture was sonicated in an ice bath for 10 min at an output of 5 with a Sonicator (Tomy UD 201), and centrifuged at 140 000 × *g* for 40 min after 30 min stirring.

Step 2. Polyethylene glycol #6000 was added to the supernatant fraction of Step 1 at a final concentration of 8%. The slightly turbid solution was stirred for 30 min at room temperature then centrifuged for 20 min at 32 000 × *g*. The pink supernatant was mixed with polyethylene glycol and $MgSO_4$ at a final concentration of 30% and 5 mM, respectively, and stirred and centrifuged as before and the red precipitate was solubilized in 5–10 ml of 10 mM Tris-HCl buffer (the buffer) containing 1% Triton X-100, and dialyzed against 10 mM Tris-HCl buffer for several hours.

Step 3. The sample from Step 2 was applied to a DEAE-Toyopearl column (2 × 5 cm) equilibrated with distilled water. The column was then washed with the buffer containing 1% Triton X-100 and 5 mM NaCl (200 ml). Upon raising NaCl concentration to 10 mM the red band moved slowly, and this band due to cytochrome *c*-551 was eluted by raising the NaCl concentration to 20 mM, when the red band reached 1 cm above the botton. The red peak fraction (about 25 ml) was collected.

Step 4. This fraction, after dialyzing against 10 mM Tris-HCl (pH 8.0), was applied to a DEAE-Toyopearl column (1.5 × 6 cm). The column was treated as in Step 3, and the red eluate with the buffer containing 20 mM NaCl was dialysed against water and absorbed on a small sized DEAE Toyopearl column to concentrate. The column was eluted with 10 mM Tris-HCl buffer containing 0.1% Triton X-100 and 0.1 M NaCl. The cytochrome *c*-551 preparation (usually 1–2 ml) was kept frozen at −80°C until use.

2.7. Preparation of de-acylated cytochrome c-551

Purified cytochrome *c*-551 (20 nmol) was treated with *Rhizopus* lipase (20 μg) in 1.5 ml of 20 mM

Tris-HCl buffer (pH 8.0) at 37°C for 2–5 h. The two-step deacylation was monitored by reverse-phase HPLC on a C4 column by HPLC as described previously [10]. The deacylated cytochrome was recovered using a small DEAE-Toyopearl column as described in Step 4.

3. Results

3.1. Construction of expression vector and transformation

The procedure for construction of an expression vector for cytochrome *c*-551 is shown in Fig. 1. The constructed plasmid pSTEc551 was first introduced into competent *E. coli* XL-1B for amplification, and then the plasmid DNA from *E. coli* was used to transform *B. stearothermophilus* K1041 by an electroporation method [12]. Out of 35 colonies grown from 100 ng plasmid DNA, 10 transformants were examined for the plasmid. All colonies were slightly reddish and contained the plasmid, and the most reddish one was chosen for the following experiment under the name of K1041(pSTEc551).

3.2. Growth of transformed cells

Fig. 2 shows growth curves of K1041 (pSTEc551) in comparison with wild-type K1041 (A), and K1041

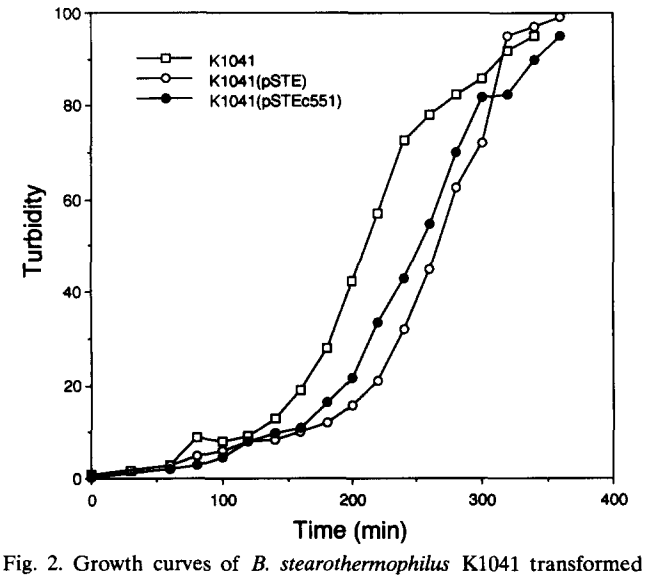

Fig. 2. Growth curves of *B. stearothermophilus* K1041 transformed with a plasmid for over-express of cytochrome *c*-551 (pSTEc551) or with a vacant plasmid (pSTE). The culture of cells, carried out in a jar fermentor containing 1 l of 0.8% polypeptone, 0.2% yeast extract, 0.3% NaCl and 0.05% K_2HPO_4, pH 7.2–7.8 with vigorous aeration (360 rpm), was started by the addition of 20 ml of grown preculture medium which showed an absorbance of 1.0 at 650 nm. The growth was followed, and the turbidity was measured at the indicated time. The culture medium for the transformed cells also contained 2 mg/l Tc. Curve A, K1041 (wild-type). Curve B, K1041(pSTE). Curve C, K1041(pSTEc551).

Table 1
A comparison of respiration rate of the transformed and the wild-type cells

Cell	Oxidase activity (μg atom/min)	
	endogenous	TMPD
Wild-type K1041	25	50
K1041(pSTE)	19	51
K1041(pSTEc551)	18	69

An aliquot (200 μl) of the culture medium which contained cells showing 1.0 absorbance at 650 nm was added to the reaction medium (2.1 ml) composed of 0.5% NaCl, 2 mM EDTA and 10 mM Hepes-NaOH buffer (pH 7.0), and endogenous respiration rate was measured. TMPD oxidation rate was measured in the presence of ascorbate (10 mM) and TMPD (0.3 mM). The rate due to endogenous substrate was not subtracted.

(pSTE12) containing the plasmid without *cccA* gene. The cytochrome*c*-551-expressing transformant, K1041 (pSTEc551), showed a slightly slower growth rate than the wild-type. This decreased rate of growth seems not due to the effect of over-expression of cytochrome *c*-551, because K1041(pSTE12) also showed a similar slow growth to K1041(pSTEc551). The presence of tetracycline (Tc) in the growth medium of the strains with plasmids (pSTE12/pSTEc551) may not be the reason for the slower growth rate, because K1041 (pSTEc551) in the Tc-free medium showed a similar growth rate to the cells grown with Tc (not shown).

Table 1 summarizes the respiration rates of these three strains, which have been harvested at a log phase when A_{650} was about 1. The endogenous respiration rate of wild K1041 was faster than others, while the respiration induced by ascorbate plus TMPD was the fastest with K1041(pSTEc551).

3.3. Cytochrome contents and oxidase activities of membrane fractions

Membrane fractions were prepared from cells at early stationary phase. Fig. 3 shows that the membrane fraction of K1041(pSTEc551) contains *c*-type cytochrome having the α-peak at 551 nm abundantly (A), and most parts can be solubilized by 0.4% cholate (C). Thus PS3 *cccA* gene in the plasmids over-expresses cytochrome *c*-551 as expected. The spectrum of the membrane fraction from the wild-type is also shown for comparison (B).

Table 2 shows a typical result on cytochrome contents and oxidase activities. The cytochrome *c*-551 content of K1041(pSTE551) membrane was about 4 nmol/mg protein, which was about 6-times as much than the content of the wild-type and of K1041(pSTE 12). The cytochrome aa_3 content of K1041(pSTE551) was about 2-fold higher than those of others. This tendency was reproducible. Cytochrome aa_3 and *c*-551 contents of K1041(pSTEc551) in different batches were

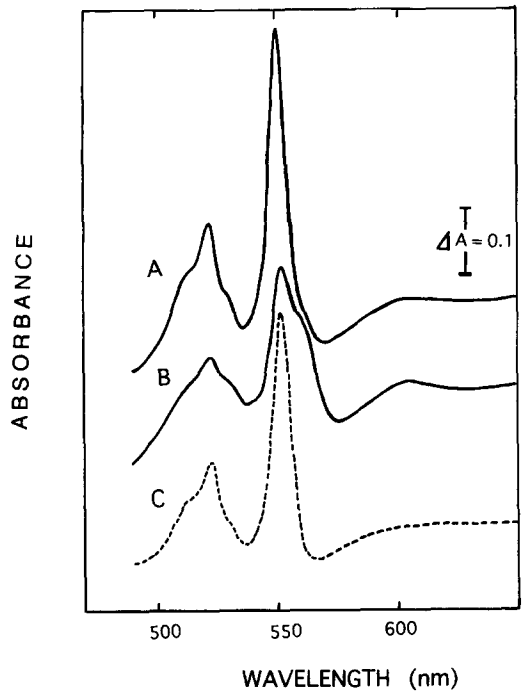

Fig. 3. Redox difference spectra of membrane fraction and cholate-extract. The spectrum of membrane fraction was suspended in 4% Triton X-100 containing 50 mM Tris-HCl (pH 8.0) and briefly sonicated. Reduced (by $Na_2S_2O_4$ addition) minus oxidized (as prepared) difference spectra are shown. (A) Membrane fraction from K1041 (pSTEc551), 3.7 mg protein/ml. (B) Membrane fraction from K1041, 4.2 mg protein/ml. (C) Cholate-extract of membrane fraction from K10411(pSTEc551).

0.33 ± 0.12 nmol/mg protein ($n = 13$) and 5.3 ± 1.6 nmol/mg protein ($n = 13$) in mean ± S.D., while those of the wild-type were 0.18 ± 0.05 ($n = 8$) and 0.46 ± 0.17 ($n = 8$) nmol/mg protein. The data with TMPD-oxidase activities coincide with the data of cytochrome aa_3 contents. On the contrary, NADH oxidase activity of K1041(pSTEc551) was a little slower than those of K1041 and K1041(pSTE12).

Table 2
A comparison of cytochrome contents and oxidation rates of membrane fractions from wild and transformed cells

Cells	Cytochrome content (nmol/mg protein)			Oxidase activity (μg atom/min per mg protein)	
	aa_3	total *c*	*c*-551	TMPD	NADH
Wild-type K1041	0.20	1.3	0.63	1.00	0.46
K1041(pSTE)	0.17	1.2	0.62	1.10	0.44
K1041(pSTEc551)	0.35	4.9	4.0	2.40	0.22

The membrane fraction was prepared from vigorously aerated cells as described in Section 2. NADH and TMPD oxidase activities were measured polarographically and started by the addition 0.22 mM (final conc.) of NADH and 0.3 mM TMPD plus 10 mM ascorbate, respectively. The membranes used were 0.15–0.3 mg protein. Cytochrome *c*-551 content was the amount of cholate extractable cytochrome *c* per mg protein of the membrane fraction used for the extraction, while the total cytochrome *c* content was measured with Triton X-100-treated membrane. Other conditions were the same as those shown in the legends for Fig. 3 and Table 1.

Table 3
Summary of purification

Step	Protein (mg)	Cytochrome *c* (%) (nmol)	Specific content (nmol/mg)
Membrane	554	1048 (100)	1.89
1. Cholate extract	140	931 (89)	6.63
2. Polyethylene glycol ppt.	87	578 (55)	6.7
3. 1st DEAE Toyopearl	40	541 (52)	13.5
4. 2nd DEAE Toyopearl	2.8	240 (23)	86.2

3.4. Purification of over-expressed cytochrome c-551

Table 3 summarizes a course of cytochrome *c*-551 purification. The membrane fraction from the transformed cells with the *cccA* gene in the expression plasmid (pSTEc551) were used as the starting material. The main purification step was chromatography in the presence of Triton X-100. This step was repeated again for better purification. The final preparation was sufficiently concentrated (3 mg/ml) and pure. Fig. 4 shows protein-stained SDS-PAGE patterns of membrane fraction of wild K1041 strain, that of K1041(pSTEc551), and the final *c*-551 preparation. We used cells harvested from a 6-l scale culture to prepare 500 mg

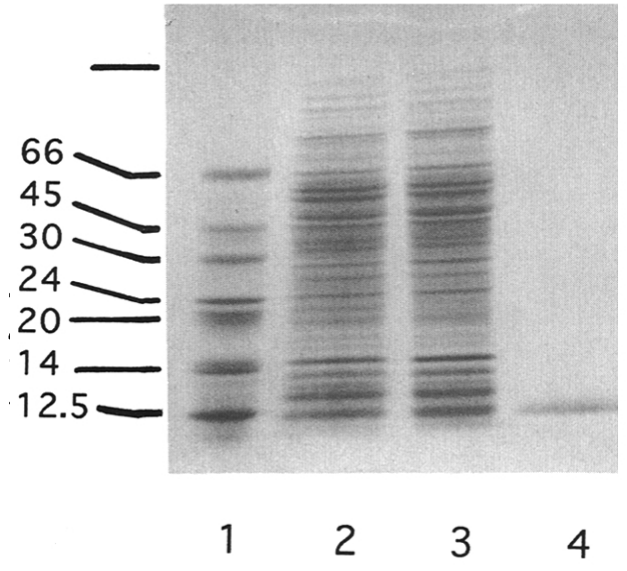

Fig. 4. SDS-PAGE patterns of over-expressed cytochrome *c*-551 during its purification. The 15% gel [17] was stained with Coomassie blue R-250. Lane 1: marker proteins composed of bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), lysozyme (14 kDa) and horse heart cytochrome *c* (12.5 kDa). Lane 2: a membrane fraction of wild K1041. Lane 3: a membrane fraction of K1041(pSTEc551). Lane 4: the purified cytochrome *c*-551.

protein of membrane fraction, and obtained 2.8 mg of cytochrome *c*-551 by treating this membrane fraction.

3.5. Structure of over-expressed cytochrome c-551

The cytochrome *c*-551 over-expressed in *B. stearothermophilus* K1041(pSTEc551) seems to be modified very similarly to the naturally-occurring one which was prepared from PS3 cells grown under air-limited conditions [7,10]. Over-expressed cytochrome *c*-551 and that after *Rhizopus* lipase treatment, showed a very similar chromatographic pattern with a C4 column to the PS3 cytochrome *c*-551 which were obtained from PS3 cells grown under air-limited conditions (not shown, but as shown in Fig. 6 of Ref. [10]). These results thus indicate that release of two fatty acid residues took place, as in the case of naturally occurring cytochrome *c*-551 in PS3 [9]. In order to analyze the molecular species of fatty acids in the over-expressed *c*-551, the *c*-551 fraction of reverse phase HPLC was extracted with $CHCl_3/CH_3OH$ to remove P-lipids and then converted to methyl esters with BF_3.

Fig. 5 shows typical gas chromatograms. The presence of palmitic acid (C16:0) was prominent (2.36 nmol/cytochrome *c*-551) in cytochrome *c*-551 (upper panel), while the content of palmitic acid was low (0.34 nmol/*c*-551) in the deacylated cytochrome *c*-551 (lower panel). Several kinds of fatty acids, mostly branched and saturated, were also found both in the intact *c*-551 and the deacylate *c*-551 as follows (intact *c*/deacylated *c*, nmol): C15:0,iso (0.23/1.21), C16:0,iso (1.26/1.11) and C17:0,iso (0.58/1.08). These fatty acids were very similar to those of total P-lipids of the thermophilic *Bacillus* PS3 [21]. It is thus likely that cytochrome *c*-551 contains two saturated fatty acids (mostly C16:0) in one molecule, even though the specimen also contains small amounts of fatty acids originated from the P-lipids contaminated.

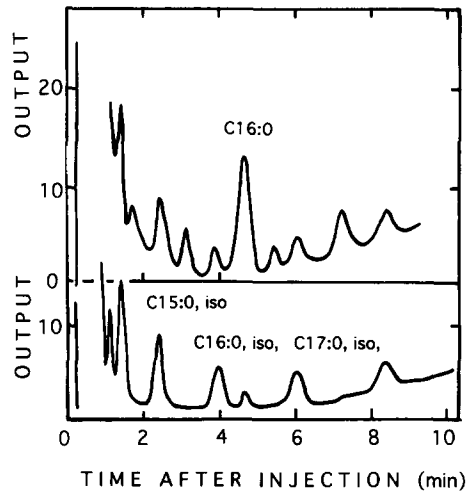

Fig. 5. Gas chromatographic elution profile of methyl esters of fatty acids extracted from intact (upper panel) and deacylated (lower panel) cytochrome *c*-551. The intact and deacylated species of cytochrome *c*-551 were separated in a C4 reverse-phase chromatography as previously described [10]. Shimadzu GC6BM gas chromatograph equipped with 1 m glass column packed with 25% ethylene glycol succinate on Shimalite was used at a carrier gas flow rate of 30 ml/min. The temperature was raised from 160°C at a rate of 5°C /min.

The Edman degradation of the purified over-expressed cytochrome *c*-551 with a gas-phase protein sequencer did not give any peptide sequence, indicating that the expressed cytochrome *c* in *B. stearothermophilus* K1041 also had a blocked N-terminus, as had PS3 cytochrome *c*-551 [10].

Fig. 6 shows the reconstructed mass spectrum of the over-expressed cytochrome *c*-551 and its deacylated form. After the lipase treatment, the cytochrome

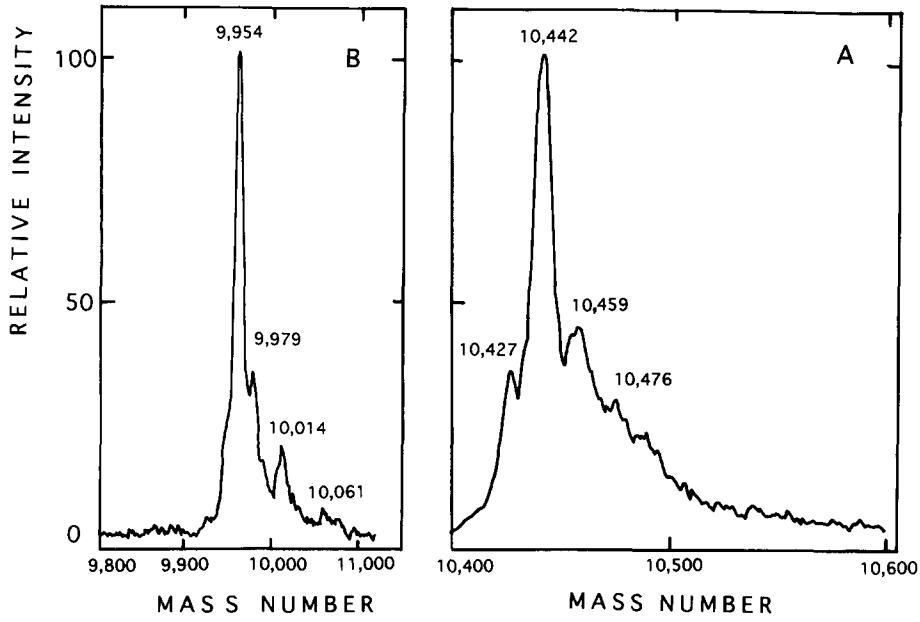

Fig. 6. Ion-spray mass spectra. The original mass spectrum has been deconvoluted. (A) The intact cytochrome *c*-551; (B) deacylated with lipase.

Table 4
Effect of acylation on cytochrome *c*-551 as a substrate of cytochrome *c* oxidase

Substrate	K_m (μM)	Turnover number (e^-/caa_3)
PS3 cytochrome *c*-551	5.2	34.0
Cytochrome *c*-551 over-expressed	5.0	32.7
Cytochrome *c*-551 delipidated	9.1	30.3

Cytochrome *c* oxidase activity was follwed with a pH meter in the presence of various concentrations of three types of cytochromes *c*-551. The delipidated cytochrome *c*-551 was prepared by treating cytochrome *c*-551 with *Rhizopus* lipase and purified on a DEAE-Toyopearl column as in Step 4. PS3 cytochrome *c*-551 was prepared as described previously [10].

showed a mass number of 9964, which is 442 smaller than that before the treatment. The mass numbers of protein (from 19th Cys to C-terminus 111th Lys), heme c and glycerol group are 9234, 616 and 75, respectively, and the sum minus 2 = 9923, is a little smaller than this value. This difference [41] is possibly due to a group which blocks the amino group of N-terminal Cys-residue. From the difference of the mass number acetylation of amino terminus is most likely, since acetylation adds 42 Da to the protein. The mass number of the native cytochrome (10442) is attained if the two fatty acids are two molecules of C16:0.

3.6. Effect of acylation on electron donating activity

Table 4 summarizes the effect of fatty acylation on the cytochrome *c* oxidase reaction. The intact cytochrome *c*-551 showed a lower K_m than that of the deacylated cytochrome, although V_{max} was the same. It is thus likely that modification of fatty acid which transform the cytochrome into a lipoprotein is useful to increase the affinity to the membrane-bound cytochrome *c* oxidase.

3.7. Heat stability of cytochrome c-551

Cytochrome *c*-551 was stable against heat denaturation. The activity of cytochrome *c*-551 as an oxidase substrate was not injured up to 90°C. However, the deacylated cytochrome *c*-551 was less heat-tolerant; the incubation at 85°C for 60 min resulted in 25% decrease, and 60% decrease after 90°C incubation, when electron transfer-mediating activity was measured at 40°C with PS3 cytochrome *c* oxidase and ascorbate (as a final electron donor). Thus the fatty acylation of the cytochrome seems to be useful for stabilization of the protein structure.

4. Discussion

Cytochrome *c*-551 is an interesting cytochrome *c* found in the thermophilic *Bacillus* PS3. This membrane-bound small-sized cytochrome *c* is synthesized under air-limited conditions [2,7] and thus is different from the one found in subunit II of the caa_3-type oxidase [1,22]. A similar C-type cytochrome, named cytochrome *c*-550 was found in *B. subtilis* and reported to be membrane-bound and small-sized (14 kDa) [23,24]. The deduced amino acid sequence of this cytochrome is clearly homologous to PS3 cytochrome *c*-551 [10]. These *Bacillus* sequences are somewhat similar to, but clearly different from the cytochrome c_8 group of denitrifiers and the cytochrome c_6 group of cyanobacteria [25–27]. In spite of sequence similarity, these two *Bacillus* cytochromes *c* choose different ways to be membrane-bound; mature *B. subtilis* *c*-550 being about 14 kDa still has a signal peptide which is a hydrophobic membrane anchor [23], while PS3 *c*-551, being about 10 kDa, has no signal peptide moiety in the mature form and is supposed to be modified into a lipoprotein with two fatty acids, although its precise structure has not been determined [10].

We succeeded in overproducing PS3 cytochrome *c*-551 in *B. stearothermophilus*. The over-produced *c*-551 may be processed as in PS3 cells; the signal peptide part for the secretion is cleaved, and the new N-terminal amino group of the cystein residue is blocked probably with the acetyl group as well as the diacyl glycerol group being introduced to the thiol.

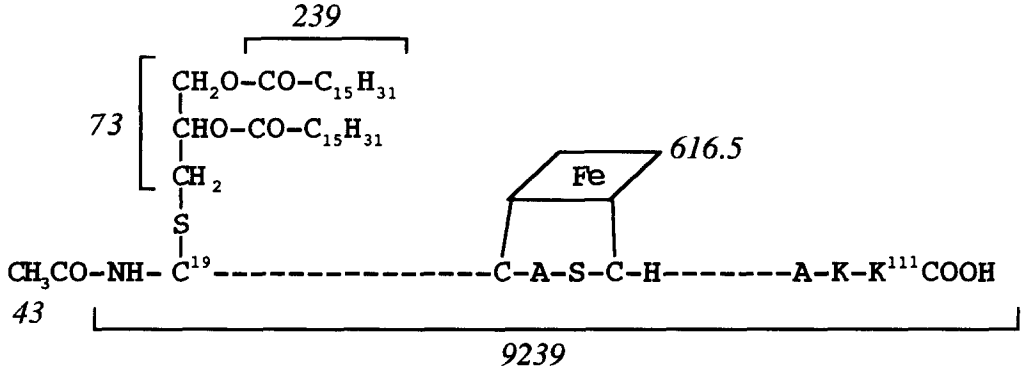

Fig. 7. A tentative structure of cytochrome *c*-551. The numbers are the molecular mass of respective portions.

This derivative of the cytochrome gives a molecular mass of 10442, and the removal of the two C16:0 fatty acids (or one C15:0,iso and one C17:0,iso) from this modified cytochrome by lipase gives a mass of 9964. The most probable structure of cytochrome *c*-551 is shown in Fig. 7. The presence of diacylated N-terminal Cys-residue was reported in cytochrome-subunit having tetra-heme of the reaction center of *Rs. viridis* [11] and the *E. coli* lipoprotein of outer membrane [28], although these lipoproteins have a free N-terminal amino group.

The effects of transformation and expression on the host cells (K1041) are summarized as follows: (1) slight retardation of growth took place in the presence of pSTE12 as well as pSTEc551, suggesting that the retardation may be due to a secondary effect of presence of the plasmids. (2) Over-expression of cytochrome *c*-551 was only observed in the transformant with pSTEc551 and accompanied with an approx. twofold increase of caa_3-type cytochrome oxidase. We do not know the reason why the synthesis of cytochrome oxidase increases upon over-expression of cytochrome *c*-551. But an idea of simple induction of the catalyst upon abundance of its substrate seems not to be the case, since cytochrome *c*-551 is not the physiological substrate for the caa_3-type oxidase. Cytochrome *c*-551, being expressed much under air-limited conditions where the level of caa_3-type cytochrome oxidase is reduced in wild PS3 and *B. stearothermophilus* cell, seems to be a substrate of an alternative terminal oxidase [7,9]. Cytochrome*c*-551 did not really accelerate the electron transport of quinol oxidase activity due to the supercomplex composed of cytochrome $bc_1(b_6f)$ complex and caa_3-type cytochrome oxidase [5]. It is also noteworthy that a deletion mutant of *cccA* gene in *B. subtilis* survives as before [23]. Thus this unexpected increase of cytochrome oxidase may indicate that biosynthesis of the caa_3-type cytochrome oxidase is rather regulated in a sophisticated manner. For example, presence of a reduced form of cytochrome *c*-551 may induce the synthesis of caa_3-type oxidase to get rid of the electron flow from the alternative terminal chain composed of cytochrome *c*-551 and the alternative oxidase, which probably operates with a lower efficiency in forming an electrochemical proton gradient than caa_3-type oxidase does [6,29].

The lipophilic nature of cytochrome *c*-551 with two fatty acid groups seems important for the cytochrome to be an efficient substrate (Table 4). The cytochrome is also heat-stable. These characteristics are unique among various cytochromes *c* which have been used as a conventional electron donor having an E_m of 0.2–0.3 V. In fact the reduced form of this cytochrome *c*-551 can be used in the reduced pulse experiment [3] to show proton pump activity of the terminal oxidase. This cytochrome is now over-produced, and will be used in different ways. We are also studying its behavior in liposomes.

Acknowledgements

We wish to thank Dr. I. Narumi and Prof. Kihara for giving us several strains of *B. stearothermophilus* and thier plasmids for expression. We also wish to thank Dr. Umeda of Takara Shuzo Co. for kindly taking the ion-spray mass spectra, and Dr. M. Nishihara and Prof. Koga of University of Occupational and Environmental Health and Dr. K. Goto of Jichi Medical School for operating gas chromatography and taking GC-mass spectra. This work was supported in part by Grant-in-Aids (04266217) from the ministry of Education, Science and Culture of Japan, and by a Grant from the Ciba-Geigy Foundation (Japan).

References

[1] Sone, N. and Yanagita, Y. (1982) Biochim. Biophys. Acta 682, 216–226.
[2] Sone, N., Kagawa, Y. and Orii, Y. (1983) J. Biochem. 93, 1329–13363.
[3] Sone, N. and Hinkle, P.C. (1984) J. Biol. Chem. 257, 12600–12604.
[4] Kutoh, E. and Sone, N. (1988) J. Biol. Chem. 263, 9020–9026.
[5] Sone, N., Sekimachi, M. and Kutoh, E. (1987) J. Biol. Chem. 262, 15386–15391.
[6] Sone, N., Kutoh, E. and Satoh, K. (1990) J. Biochem. 107, 597–602.
[7] Sone, N., Kutoh, E. and Yanagita, Y.(1989) Biochim. Biophys. Acta 977, 329–3348.
[8] De Vrij, W., Heyne, R.I. and Konings, W.N. (1989) Eur. J. Biochem. 178, 763–770.
[9] Sone, N. and Fujiwara, Y. (1991) J. Biochem. 110, 1016–1021.
[10] Fujiwara, Y., Oka, M., Hamamoto, T. and Sone, N. (1993) Biochim. Biophys. Acta 1144, 213–219.
[11] Weyer, K.A., Schafer, M., Lottspeich, F. and Michel, H. (1987) Biochemistry 26, 2909–2914.
[12] Narumi, I., Sawakami, K., Nakamoto, S., Nakayama, N., Yanagisawa, T., Takahashi, N. and Kihara, H. (1992) Biotechnol. Techn. 6, 83–86.
[13] Nakayama, N., Narumi, I., Nakamoto, S. and Kihara, H. (1992) Biotechnol. Lett. 14, 649–652.
[14] Narumi, I., Sawakami, K., Kimura, T., Nakamoto, N., Nakayama, N., Yanagisawa, T., Takahashi, N. and Kihara, H. (1992) Biotechnol. Lett. 14, 759–764.
[15] Nicholls, P. and Sone, N. (1984) Biochim. Biophys. Acta 767, 240–247.
[16] Deeb, S.S. and Hager, L.P. (1964) J. Biol. Chem. 239, 1024–1031.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Reid, G.A. and Ingledew, W.J. (1980) FEBS Lett. 109, 1–4.
[19] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY.
[20] Yoshida, M., Sone, N., Hirata, H. and Kagawa, Y. (1975) J. Biol. Chem., 250, 7910–7916.
[21] Sone, N., Yoshida, M., Hirata, H. and Kagawa, Y.(1975) J. Biol. Chem. 250, 7917–7923.

[22] Ishizuka, M., Machida, K., Shimada, S., Mogi, A., Tsuchiya, T., Ohmori,T., Souma, Y., Gonda, M. and Sone, N. (1990) J. Biochem. 108, 866–873.
[23] Von Wachenfeldt, C. and Hederstedt, L. (1990) J. Biol. Chem. 265, 13939–13948.
[24] Von Wachenfeldt, C. and Hederstedt, L. (1990) FEBS Lett. 270, 147–151.
[25] Wood, P. (1978) Eur. J. Biochem. 87, 9–19.
[26] Cohn, C.L., Hermodson, M.A. and Krogmann, D.W. (1989)Arch. Biochem. Biophys. 270, 219–226.
[27] Sone, N. and Toh, H. (1994) FEMS Microbiol. Lett. 122, 203–210.
[28] Hantke, K. and Braun, V. (1973) Eur. J. Bioichem. 34, 284–296.
[29] Sone, N. (1990) in The Bacteria vol. XII (Krulwich, T.A., ed.), pp. 1–32, Academic Press, New York.