A novel membrane-anchored cytochrome *c*-550 of alkaliphilic *Bacillus clarkii* K24-1U: Expression, molecular features and properties of redox potential

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Abstract A membrane-anchored cytochrome *c*-550, which is highly expressed in obligately alkaliphilic *Bacillus clarkii* K24-1U, was purified and characterized. The protein contained a conspicuous sequence of Gly^{22}–Asn^{34}, in comparison with the other *Bacillus* small cytochromes *c*. Analytical data indicated that the original and lipase-treated intermediate forms of cytochrome *c*-550 bind to fatty acids of C_{15}, C_{16} and C_{17} chain lengths and C_{15} chain length, respectively, and it was considered that these fatty acids are bound to glycerol-Cys^{18}. Since there was a possibility that the presence of a diacylglycerol anchor contributed to the formation of dimeric states of this protein (20 and 17 kDa in SDS-PAGE), a C18M (Cys^{18}→ Met) -cytochrome *c*-550 was constructed. The molecular mass of the C18M-cytochrome *c*-550 was determined as 15 and 10 kDa in SDS-PAGE and 23 kDa in blue native PAGE. The C18M-cytochrome *c*-550 bound with or without Triton X-100 formed a tetramer as the original cytochrome *c*-550 bound with Triton X-100, as determined by gel filtration. The midpoint redox potential of cytochrome *c*-550 as determined by redox titration was +83 mV, while that determined by cyclic voltammetric measurement was +7 mV. The above results indicate that cytochrome *c*-550 is a novel cytochrome *c*.

Keywords alkaliphilic, *Bacillus clarkii*, cytochrome *c*, expression, posttranslational modification, membrane-anchored, midpoint redox potential

Introduction

It is considered that the presence of a large amount of cytochrome *c* in alkaliphilic *Bacillus* spp. is important for adaptation to alkaline condition (Goto et al. 2005). Alkaliphile mutant strains that cannot grow under alkaline conditions, derived from two obligate alkaliphilic *Bacillus* strains, have lower cytochrome content than the original strains (Lewis et al. 1980). Furthermore, the facultative alkaliphiles *Bacillus cohnii* YN-2000 and *Bacillus pseudofirmus* OF4 produce larger amounts of cytochromes *c* and *b* when grown at pH 10 than when grown at neutral pH (Guffanti et al. 1986; Yumoto et al. 1991). Three low-molecular-weight cytochromes *c* have been purified from alkaliphilic *Bacillus* strains (including the genus *Sporosarcina*) and characterized (Davidson et al. 1988; Yumoto et al. 1991; Benini et al. 1998). All these cytochromes exhibit a low midpoint redox potential (lower than +100 mV) and are acidic proteins (p*I* lower than 4). Benini et al. posited that the reason behind the low midpoint redox potential is the decrease in the entropy of reduction with the extrusion of water molecules from the protein hydration shell, which occurs upon heme reduction (Benini et al. 1998).

Although there are many studies on the soluble class I cytochrome *c* from various organisms (Bertini et al. 2006), a limited number of studies on the membrane-anchored class I cytochrome *c* have been published (Sone et al. 1989, 2001; von Wachenfeldt and Hederstedt 1990; Fujiwara et al. 1993; Albert et al. 1998; Bengtsson et al. 1999; Suharti et al. 2004). It has been considered that all cytochromes *c* in Gram-positive bacteria are membrane-anchored proteins on the outer surface of the membrane, because Gram-positive bacteria have no periplasmic space. Three types of membrane-anchored cytochrome *c* have been reported to date as follows: those that bind to the membrane via an anchoring domain consisting of a single α-helical transmembrane segment, those that bind to the membrane via two covalently bound fatty acid moieties, and those that fuse as an integral domain of

subunit II in terminal oxidase (Sone and Toh 1994). For example, *Bacillus subtilis* possesses two types of membrane-anchored class I cytochrome *c*: cytochromes *c*-550 and *c*-551. Cytochrome *c*-550 has 120 amino acids with a membrane-anchor domain consisting of a single α-helical transmembrane segment of a hydrophobic 30-amino-acid polypeptide (von Wachenfeldt and Hederstedt 1990). On the other hand, cytochrome *c*-551 is considered to bind to the cellular membrane via a diacylglyceryl-cysteine moiety (Bengtsson et al. 1999). Moreover, cytochromes *c* from thermophilic *Bacillus* sp. strain PS3 (cytochrome *c*-551) (Sone et al. 1989; Fujiwara et al. 1993), *Heliobacterium gestii* (cytochrome *c*-553) (Albert et al. 1998) and *Bacillus azotoformas* (cytochrome *c*-551) (Suharti et al. 2004) are bound to the cellular membrane via a diacylglyceryl-cysteine moiety. The two types of membrane-anchored cytochrome described above are considered to be single-subunit proteins (Sone and Toh 1994; Bengtsson et al. 1999). The third type of membrane-anchoring cytochrome *c* fuses with subunit II of cytochrome *c* oxidase of the caa_3 (Sone & Yanagita 1982; Quirk et al. 1993), cbb_3 (Myllykallio and Liebl 2000; Pitcher and Watmough 2004) or aco_3 (Qureshi et al. 1990; Sone et al. 1994; Yumoto et al. 1993) type. The striking deletion of basic residues in the external, hydrophilic cytochrome *c* anchoring domains of cytochromes caa_3 (Quirk et al., 1993) and aco_3 (Denda et al., 2001) from alkaliphilic *Bacillus* strains has been reported.

Although cytochrome *c* may play an important role in certain alkaliphilic *Bacillus* strains, there is no report on the isolation and characterization of its intact protein with detailed analyses of its anchoring domain. We achieved these by first isolating the obligately alkaliphilic bacterium, *Bacillus clarkii* K24-1U, which exhibits low proteinase activity by screening our culture stock. As a result, intact membrane-anchored acidic cytochrome *c*-550 in *B. clarkii* K24-1U was successively purified, including its diacylglycerol moiety, and its molecular features were characterized in detail.

Materials and methods

Chemicals

Lysozyme, DNase, Triton X-100 and phenazine ethosulfate were purchased from Sigma, phenylmethylsulfonyl fluoride (PMSF) was purchased from Wako Pure Chemical Industries (Osaka, Japan). As a mediator for redox titration, 2-hydroxyl-1,4-naphthoquinone, phenazine methosulfate and *N*,*N*,*N*',*N*'-tetramethyl phenylenediamine (TMPD) were purchased from Wako Pure Chemical Industries (Osaka, Japan), and 2-hydroxyl-1,4-naphthoquinone and 1,2-naphthoquinone were purchased from Aldrich. A marker protein kit for isoelectric point determination was purchased from Oriental Yeast (Osaka, Japan). All the other chemicals used were of the highest grade commercially available.

Bacterial strain

The screening for alkaliphiles from approximately 50 strains isolated from soil samples collected from several sites in Hokkaido, Japan was performed using PYA (peptone/yeast extract/alkaline) agar medium (Yumoto et al. 2003). The strains were incubated at 27ºC. Among the strains isolated, an obligate alkaliphile with a relatively high respiratory cytochrome content was selected for the present study. In a previous report, the same strain was designated as K241 (Yumoto et al. 1997) and the strain was isolated from a soil sample obtained from Yuubari, Hokkaido, Japan. The phenotypic characteristics indicated that the strain exhibits weak protease activity (Yumoto et al. 1997). The level of DNA-DNA relatedness was determined fluorometrically by the method of Ezaki et al. (1989). The

other taxonomic identification methods were performed as described previously (Yumoto et al. 2003). The determined 16S rRNA gene sequence (accession no. AB358959) has been deposited in GenBank/EMBL/DDBJ. It is considered that the characteristic of weak protease is suitable for the purification of intact membrane-anchored cytochrome *c*. The microorganism was cultured in 15 L of PYA medium in a 20-L stainless-steel fermentor (K.K.Takasugi Seisakusho, Tokyo, Japan) at an agitation speed of 106 rpm. The cells were harvested by centrifugation at 10,000 × *g* for 20 min at 4ºC. The collected cells were stored at –85ºC until use.

Estimation of growth rate and cytochrome *c* content

The growth rate of *B. clarkii* K24-1U (pH 10) was estimated by monitoring OD_{650} periodically using a spectrophotometer (Hitachi U-3210, Tokyo, Japan) and compared with that of *B. subtilis* IAM 1026 (pH 7). Growth at neutral pH (pH 7) was carried out using PYA broth containing 100 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 7) instead of 100 mM $NaHCO_3$-Na_2CO_3 buffer. The production of cytochrome *c* in *B. clarkii* K24-1U was estimated and compared with that of *B. subtilis* IAM 1026. The bacterial strain was cultivated in 15 L of PYA medium in a 20-L stainless-steel fermentor (K.K.Takasugi Seisakusho) at an agitation speed of 106 rpm with an air flow rate of 20 L min^{-1}. The harvested cells were suspended in 50 mM Tris-HCl buffer (pH 8.0) and passed through a French pressure cell (SLM-Aminco Instruments, Inc., Rochester, N. Y.) at 18000 lb in^{-2}, followed by centrifugation at 14,000 × *g* for 30 min to remove unbroken cells. The cytochrome *c* content was determined as previously described (Yumoto et al. 1991).

Purification of cytochrome *c*-550 from *B. clarkii* K24-1U

Frozen cells (approximately 100 g wet weight) were suspended in 300 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 100 μM PMSF (buffer A). Then, 5 μg ml^{-1} DNase I and a final concentration of 7 mM $MgCl_2 \cdot 6H_2O$ were added to the suspension. The bacterial cell suspension was passed through a French pressure cell and unbroken cells were removed as described above. The resulting supernatant was centrifuged at 131,000 × *g* for 2 h to obtain the membrane fraction. The obtained pellet was suspended in buffer A, and then 20% Triton X-100 was added to the suspension to a final concentration of 1.5%. The suspension was stirred gently for 60 min at 4ºC and then centrifuged at 131,000 × *g* for 90 min. The reddish supernatant obtained was subjected to anion-exchange chromatography using Q-Sepharose Fast Flow column (2.6 cm × 20 cm; GE Healthcare Bio-Sciences, Buckinghamshire, UK), which has been equilibrated with buffer A containing 0.1% Triton X-100 (buffer B). The column was washed with one-column volume of buffer B, and the cytochrome *c*-550 fraction was eluted with a linear gradient of increasing NaCl concentrations (from 0.3 to 0.8 M) produced from a five-column volume of buffer B. The eluates containing cytochrome *c* were combined and diluted with 10 mM Tris-HCl (pH 8.0) containing 0.1% Triton X-100 (buffer C) and subjected to a second chromatography on a Q-Sepharose Fast Flow column (2.0 cm × 10 cm) equilibrated with buffer C. The adsorbed enzyme was eluted with a linear gradient of increasing NaCl concentrations (from 0.3 to 0.5 M) produced from a five-column volume of buffer C. The eluate was concentrated using Amicon Ultra-15 and then passed through a gel filtration column (Sephacryl S-300 High Resolution, 2.6 cm × 95 cm; GE Healthcare Bio-Sciences) equilibrated with buffer C containing 0.25 M NaCl. The eluates containing cytochrome *c*

were combined and loaded onto a hydroxyapatite column (2.6 cm × 20 cm; Seikagaku Corp., Tokyo, Japan) equilibrated with 10 mM potassium phosphate buffer (pH 6.8) containing 0.1% Triton X-100. Cytochrome *c*-550 was eluted with a linear gradient of 10-400 mM potassium phosphate buffer (pH 6.8) containing 0.1% Triton X-100. The eluates containing cytochrome *c*-550 were loaded onto a Phenyl Sepharose High Performance column (2.0 cm × 10 cm; GE Healthcare Bio-Sciences) equilibrated with 10 mM potassium phosphate buffer (pH 6.8) containing 0.1% Triton X-100 and 0.9 M ammonium sulfate. Cytochrome *c*-550 was eluted with a linear gradient from 0.9 to 0 M ammonium sulfate in the same buffer.

Analytical methods

Spectrophotometry was performed with a Cary 100 UV-Vis spectrophotometer (Varian, Palo Alto, CA, USA) using a 1-cm-light-path cuvette at room temperature. The heme *c* content was determined on the basis of the millimolar extinction coefficient (ε_{mM}) of pyridine ferrohemochrome *c* of 29.1 mM^{-1} cm^{-1} (Drabkin 1942). The molecular mass of cytochrome *c*-550 treated with sodium dodecyl sulfate (SDS) was determined by SDS-PAGE on a 10-20% gradient gel (PAGEL, ATTO, Tokyo, Japan) according to the method of Laemmli and Favre (1973). Native PAGE was performed according to the method of Davis (1964). The gels were stained using Coomassie Brilliant Blue R-250 (CBB) or heme stain with 3,3',5,5'-tetramethylbenzidine (TMBZ) as described by Thomas et al. (1976). A molecular weight standard, the BenchMark Protein Ladder, purchased from Invitrogen (Carlsbad, CA, USA) was used. The molecular weight of the native enzyme was determined by gel filtration using two 7.8 mm × 300 mm Protein PAK 300 columns (Nihon Waters, Tokyo, Japan) equilibrated with 0.1 M potassium phosphate buffer (pH 7.0). For

molecular mass standards, the following proteins were used: thyroglobulin (669 kDa), apoferritin (443 kDa), α-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66.2 kDa), and carbonic anhydrase (29 kDa). The molecular mass of the native enzyme was also determined by blue native PAGE on 4-16% gradient gel (Invitrogen) as described by Schägger et al. (1994). The protein content was determined using a BCA protein assay reagent kit (Pierce, Rockford, IL, USA) with bovine serum albumin as standard (Smith et al., 1985).

Lipase treatment and reverse-phase chromatography

To remove anchor fatty acids, 1 μg of *Rhizopus delemar* lipase (fine grade; Seikagaku Corp.) was added to 1 ml of 0.5 mg ml^{-1} cytochrome *c*-550 solution followed by incubation for 30 and 180 min at 37ºC. The treated solution was diluted with NH_4HCO_3 up to 5 ml. Original and lipase-treated cytochrome *c*-550 were analyzed and separated by reverse-phase high-performance liquid chromatography (HPLC) using a C_4 column (YMC-PAK Pro C_4; 4.6 mm × 150 mm; YMC, Kyoto, Japan). The solvent used was a mixture of water and acetonitrile, both containing 0.1% trifluoroacetic acid. Cytochrome *c* applied onto the column was eluted using a 0% – 100% linear gradient of acetonitrile. The absorbance was monitored at both 408 and 214 nm. The obtained fraction containing cytochrome *c* was freeze-dried and used for further analysis.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)/MS protein profiling

The molecular masses of original, lipase-treated original and *E. coli*-expressed cytochromes *c*-550 fractionated by reverse-phase HPLC were determined by MALDI-TOF/MS (Voyager DESTR, Applied Biosystems). Each sample concentration was adjusted to less than 30 μM by adding an equal volume of a mixture of milli-Q water and acetonitrile. The sample was mixed with an equal volume of matrix (α-cyano-4-hydroxycinnamic acid saturated solution), 1 μl of which was spotted onto one well on the sample plate and then crystallized under an airflow. Horse heart cytochrome *c* was used to standardize the determined molecular masses.

Midpoint redox potential determination

Midpoint redox potential (E_m) by redox titration was measured according to the method of Dutton (1978) using a potential meter (model 744; Metrohm, Herisau, Switzerland) as described previously (Matsuno et al. 2007). The midpoint redox potential was also determined by cyclic voltammetry using a gold disk (diameter: 1.6 mm) that was used as a working electrode. It was polished using a slurry of alumina (0.05 μm, Buehler), rinsed with distilled water, and then thoroughly cleaned by electrochemical oxidation/reduction (–0.2 – +1.5 V vs Ag/AgCl for 30 min) in 0.05 M H_2SO_4 solution. The cleaned gold disk was modified by dip treatment in ethanol-water mixed solution (ethanol:H_2O_2=1:1) containing 1 mM 2-amino-6-purinethiol for 2 h. After modification, the electrode was thoroughly washed with pure ethanol and water. The auxiliary and reference electrodes were a Pt wire and Ag/AgCl (sat. KCl), respectively. Potentials were converted to the standard hydrogen

electrode (SHE). Cyclic voltammograms (CVs) were recorded at room temperature in a 20 mM potassium phosphate buffer (pH 6.8) containing 100 μM cytochrome *c*-550. The midpoint potential from CV was calculated as the average of the anodic and cathodic peak potentials. All the electrochemical responses were measured after deaerating with argon gas.

Protein sequencing

Original cytochrome *c*-550 (approximately 0.5 mg) was digested with proteinase K (Stratagene, La Jolla, CA) and papain (Roche, Basel, Switzerland) for 18 h at 37ºC in 10 mM potassium phosphate buffer (pH 6.8) containing 0.1% Triton X-100 at an enzyme/substrate ratio (w/w) of 1/100. The peptides cleaved with the enzymes were separated by HPLC using the same method used for the separation of original and lipase-treated cytochromes *c*-550. The eluent containing the polypeptides was collected by monitoring the absorbance at 214 nm. The N-terminal amino acid sequences of the polypeptides obtained were determined by Edman degradation (Edman and Henschen 1975) and using a model 491 protein sequencer (Perkin-Elmer, Winter Street Waltham, MA).

Sequence determination of cytochrome *c*-550 gene

The two oligonucleotide primers 5'-GCAGGATGYCATGGNGGNGA-3' and 5'-TTGATCNGCNACCCANGC-3' (Y and N are mixtures of T and C, and A, T, G and C, respectively) were designed on the basis of the determined sequence of the polypeptide by the digestion of cytochrome *c*-550. PCR was performed using the two primers and

chromosomal DNA isolated from *B. clarkii* K24-1U. The amplified 164-bp fragment was further extended by gene walking with a TaKaRa PCR *in vitro* Cloning kit (Takara, Otsu, Japan) and sequenced using a BigDye Terminator Cycle sequencing kit with an ABI 310 Genetic Analyzer (Perkin-Elmer). The determined sequence (accession no. AB358960) has been deposited in GenBank/EMBL/DDBJ. Similarity values for sequences were calculated using the GENETYX computer program (ver. 9; Software Development, Tokyo, Japan). Multiple alignments of the sequences were performed using the CLUSTAL W program (Thompson et al. 1994).

RNA extraction and primer extension analysis

The RNA of *B. clarkii* K24-1U cells was extracted using the SV total RNA isolation system (Promega, Madison, Wis). The cells were grown in PYA broth (pH 10) inoculated with a preculture from the same medium until OD_{600} reached 0.6-1.0. RNA was extracted from the harvested cells and purified according to the manufacturer's instructions. The primer extension reaction was performed as follows: Reverse transcription was performed in a reaction mixture containing 1 μl of transcriptase from avian myeloblastosis virus (AMV) (Takara), 10 pmol of fluorescein isothiocyanate (FITC)-labeled oligonucleotide primer 5'-ATACTGATTCAGCGTTATCCGCATC -3', 1000 ng of RNA of *B. clarkii* K24-1U, and a mixture of each deoxynucleoside triphoshate at 42ºC for 1 h. The primer sequence was complementary to nucleotides 151 to 175 of the cytochrome *c*-550 gene. The transcription initiation site was determined by electrophoresis of the primer extension mixed as previously described (Kiatpapan et al. 2001) with the products of DNA sequencing reactions generated with the non-FITC-labeled primer (control) having the same sequence using an ABI 310 Genetic Analyzer (Perkin-Elmer).

Construction of cytochrome *c*-550 expression plasmid

To construct the wild-type cytochrome c-550 expression plasmid pET101*c*550, the entire genome sequence of 357 bp including the *B. clarkii* cytochrome *c*-550 signal sequence was amplified by PCR using the primers 5'-CACCATGAAAAAAATGCTAGTAGCAATGTTGGGG-3' and 5'-TTACTGATCAGCAACCCATGCAG -3', and a TOPO cloning site of pET101/D-TOPO was introduced (ampicillin resistance; Invitrogen). To construct pET22b*c*550 for the expression of soluble mutant cytochrome *c*-550 in the periplasmic space of *E. coli*, the gene to be inserted was designed using the primers 5'-GTCGCCATGGGAGGAAACGGCGATAATAAC-3' and 5'-GCGAAGCTTTTACTGATCAGCAACCCATGC -3' to remove the signal sequence of 51 bp as well as amino acids, and to replace the C18M in the N-terminal of the mature protein. The amplified sequence was inserted at the *Nco*I/*Hin*dIII site of the pET22b(+) (ampicillin resistance; using a DNA ligation kit (Mighty Mix; Takara). The vectors are under the control of the T7 promoter.

Cytochrome *c*-550 expression and purification

Each constructed vector described above and pEC86 (chloramphenicol resistance) were cotransformed in *E. coli* BL21Star(DE3). Frozen competent cells prepared using calcium chloride were used for the transformation. pEC86 (Arslan et al. 1998) encodes the complete *ccmA-H* gene cluster from *E. coli* (Thöny-Meyer et al. 1995) under the control of the *tet* promoter from pACY4. The coexpression of the *ccm* genes is necessary for the assembly of *c*-type cytochrome in the *E. coli* periplasm under aerobic condition.

Ampicillin- and chloramphenicol-resistant colonies were selected and used for the expression of soluble and membrane-anchored cytochromes *c*.

The transformed *E. coli* cells for the expression of the membrane-anchored or soluble cytochrome *c*-550 were grown at 27ºC in Luria-Bertani medium supplemented with ampicillin (60 μg ml^{-1}) and chloramphenicol (50 μg ml^{-1}). When the culture reached an optical density (OD_{600}) of 0.8-1.0, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and then the cells were harvested after an appropriate period.

The periplasmic protein fraction of the transformed *E. coli* cells was obtained by cold osmotic shock (Neu and Heppel 1965; De Sutter et al. 1992). The membrane-anchored fraction was obtained by solubilization in 1.5% Triton X-100. Both soluble and membrane-anchored cytochromes *c*-550 were purified using Q-Sepharose Fast Flow, Sephacryl S-300 and hydroxyapatite, as described above.

Analysis of diacylglycerol anchor

To ensure that the diacylglycerol connected to cytochrome *c*-550 is covalently bound, Triton X-114 phase separation was performed after removing noncovalently bound lipids by the method of Folch et al. (1957). Phase separation was performed according to the method of Hooper (1992) with modifications.

Fatty acid methyl esters were prepared by treating 0.5 mg of freeze-dried cytochrome *c*-550 with 4 ml of 5% HCl in methanol (v/v) for 3 h at 100ºC. The fatty acid methyl esters were extracted and analyzed as previously described (Yumoto et al. 2003). The fatty acids were identified by gas chromatography (GC) in comparison with fatty acid methyl ester

standards (Supelco) and by gas chromatography-mass spectrometry (GC/MS) (GC-3800 – Saturn2000; Varian).

RESULTS

Identification of isolate

The phenotypic characterization of strain K24-1U suggested that the strain belongs to the genus *Bacillus* (data not shown). The 1534 bp of the 16S rRNA gene sequence of strain K24-1U was determined and the similarity of its sequence with the sequences in the database was estimated by BLAST search. The highest similarity was found with *Bacillus clarkii* DSM 8720^T (98.1%). On the basis of the result, DNA-DNA hybridization between strain K24-1U and *B. clarkii* DSM 8720^T was performed. Results of the phenotypic characterization, phylogenetic analysis based on the 16S rRNA gene sequence (data not shown) and DNA-DNA hybridization indicated that strain K24-1U should be identified as *B. clarkii* (85-93% DNA-DNA relatedness).

Growth rate and cytochrome *c* content

The specific growth rate (μ_{max}) of *B. clarkii* K24-1U (pH 10) was 0.33, while that of *B. subtilis* IAM 1026 (pH 7) was 0.26. It can be said that the alkaliphile grows faster than the neutralophile even under conditions unfavorable for energy production. The cytochrome *c* content of *B. clarkii* K24-1U (14 h growth) was 1.39 nmol mg $proten^{-1}$, while that of *B. subtilis* IAM 1026 (18 h growth) was 0.15 nmol mg $proten^{-1}$. The result indicates that the

cytochrome *c* content of *B. clarkii* K24-1U is approximately ninefold higher than that of *B. subtilis* IAM 1026.

Purification of cytochrome *c*-550 from *B. clarkii* K24-1U

Purified cytochrome *c*-550 was obtained by two steps of anion-exchange chromatography and one step each of gel filtration, hydroxyapatite chromatography and hydrophobic chromatography. The procedure results in a 31-fold purification with 15.5% yield. When purified cytochrome *c* was subjected to native PAGE, it exhibited only one major band (data not shown). However, when purified cytochrome *c*-550 was subjected to SDS-PAGE, it showed two bands of 20 and 17 kDa upon staining with both CBB and TMBZ (Fig. 1). On the other hand, the purified cytochrome *c*-550 exhibited only one band of 23 kDa by blue native PAGE (data not shown). These results suggest that the molecule is homogeneous. However, it seemed to consist of molecules of different masses when the polypeptide chain of cytochrome *c*-550 extended following the SDS attachment. The difference in the molecular mass between the two bands in SDS-PAGE might be due to the difference in the distribution of the hydrophobic part in the molecule including the diacylglycerol anchor.

Characteristics of cytochrome *c*-550

The absorption spectrum of resting-state cytochrome *c*-550 exhibited a Soret band at 408 nm. Cytochrome *c*-550 reduced by a small amount of $Na_2S_2O_4$ exhibited peaks at 550 (α-band), 521 (β-band) and 415 (Soret band) nm. The millimolar extinction coefficient of oxidized cytochrome *c*-552 at 408 nm was determined to be 102 mM^{-1} cm^{-1}. The heme *c*

content of the purified protein was determined to be 94 nmol mg protein^{-1}, which was calculated to be 10,600 Da M heme c^{-1}. The purified cytochrome *c*-550 was electrophoresed on a polyacrylamide gel with a pH gradient of 3.5 to 10, and the isoelectric point was determined to be 4.1. The midpoint redox potential of cytochrome *c*-550 as determined by redox titration was +83 mV at pHs 7 and 9. On the other hand, the midpoint redox potential of the cytochrome using self-assembled momolayers (SAM) of 2-amino-6-purinethiol as determined by cyclic voltammetric measurement was +7 mV.

Internal amino acid sequence analysis

The N-terminus of cytochrome *c*-550 appears to be blocked, because no amino acids were detected using a protein sequencer during the analysis of the protein blotted onto a polyvinylidene difluoride (PVDF) membrane. This may be because the phenylthiohydanation (PTH)-amino acid did not dissociate owing to the protection of the N-terminal amino acid. We analyzed the amino acid composition of cytochrome *c*-550 using an amino acid analyzer (data not shown). Results showed that cytochrome *c*-550 appears to be digestible by trypsin, chymotrypsin and V8 protease. No internal peptide sequence was obtained after treatment with these proteases. It is likely to be caused by the stability of the intact protein. Because specific digestive enzymes are unable to produce peptides, nonspecific digestive enzymes, i.e., proteinase K and papain, were used to obtain the peptides. As a result, 3 and 2 peptides were obtained by HPLC separation after treatment with proteinase K and papain, respectively. Among the obtained peptides, two peptides obtained by proteinase K digestion exhibited amino acid sequences (EGPGSMPA and YAGXHGGDLT) that show similarities with those of *Geobacter metallireducens* GS-15 cytochrome *c* (CP000148) and *Bacillus halodurans* C-125 cytochrome *c*-551

(AP001507-AP001520), respectively. A peptide obtained by papain digestion exhibited an amino acid sequence (AWVADQ) that shows similarity with that of the *Ralstonia metallidurans* CH34 cytochrome *c* class I precursor (Q3RZK6).

Gene cloning

On the basis of the obtained amino acid sequences, two degenerate primers (described in Materials and Methods) were prepared and PCR was performed on *B. clarkii* K24-1U chromosomal DNA. The genome sequence of approximately 3000 bp containing the entire gene sequence of cytochrome *c*-550, *cycA*, was determined using the obtained product (Fig. 2). The cytochrome *c*-550 gene consisted of 357 bp with a Shine-Dalgano (SD) sequence (AGGAGG) preceding the gene 7 bp upstream of a methionine-coding start codon. The cytochrome *c* appeared to consist of 118 amino acids, including a putative 17-residue signal peptide sequence referred to in previous reports (Noguchi et al. 1994; Albert et al. 1998; Sutcliffe and Harrington 2002). The cytochrome *c*-550 polypeptide contained one heme *c* binding motif (CXXCH). Met^{82} could be identified as the sixth heme *c* axial ligand. A striking paucity of basic amino acids was observed in the mature cytochrome *c*-550 amino acid sequence. Lys^{88} is the only basic amino acid in the sequence except the heme *c* binding motif. Cytochrome *c*-550 possesses a high acidic amino acid frequency part and a low basic amino acid content (Fig. 3). In addition, cytochrome *c*-550 possesses a specific amino acid sequence that includes four successive Asn sequences at the N-terminal. Furthermore, the most pronounced difference of this cytochrome *c* from the other cytochromes *c* in the *Bacillus* small cytochrome *c* group (Sone and Toh 1994) is the presence of Gly^{22} – Asn^{34} (cytochrome *c*-550 numbering) (Fig. 3). The amino acid sequence of cytochrome *c*-550 deduced from its gene sequence exhibited the highest

similarity with those of *Oceanobacillus iheyensis* cytochrome *c* (Q9KD41) and *Bacillus* sp. PS3 cytochrome *c*-551 (Q818G9) (40%). Phylogenetic analysis based on the deduced amino acid sequence of cytochrome *c*-550 revealed a distinctive lineage in the *Bacillus* small cytochrome *c* group (data not shown).

Primer extension analysis

The 5' end of the mRNA produced from cytochrome *c*-550 was identified. In the comparison of the mixture of primer extension product and the result of nucleotide sequence with those of the control, a peak associated with FITC was identified. As a result, guanine (G) (-50) 50 bp upstream of the start codon was identified as the transcriptional start point of the gene coding for cytochrome *c*-550 (Fig. 2). The putative promoter consensus sequences [-35: TTCAAA] and [-10: TAAAAT] are located 30 and 10 bp upstream of the transcriptional start point, respectively (Fig. 2). The sequences were similar to those of *Clostridium pasteurianum* ferredoxin [-35: TTTAAA]-16 bp-[-10: TACAAT] (Graves and Rabinowitz 1986), *Staphylococcus aureus* AAD9 [-35: TTCAA]-17 bp-[-10: TATAAT] (Murphy 1985) and *B. subtilis* RNA polymerase [-35: GTGAAA]-17 bp-[-10: TAAAAT] (Wang and Doi 1984). This suggests that the promoter sequence of *cycA* is very similar to a typical Gram-positive vegetative promoter sequence. As the candidate transcription regulator of this cytochrome *c*, an inverted repeat was found between the transcriptional start point and the promoter sequence (Fig. 2).

Analyses of fatty acids

The N-terminal sequence of the cytochrome *c*-550 suggested that the mature protein is diacylglycerol-anchored, as referred to in previous reports (Fujiwara et al. 1993; Noguchi et al. 1994; Albert et al. 1998; Sutcliffe and Harrington 2002). According to the Triton X-114 phase separation of cytochrome *c*-550, the optical absorbance (408 nm) of the Triton X-114 phase was 0.72, whereas that of the water phase was only 0.02. This indicates that fatty acid residues are covalently bound to cytochrome *c*-550. By reverse-phase HPLC, the intact cytochrome *c*-550 was mainly separated into three fractions (peaks 1-3 in Fig. 4). Their molecular masses were found to be 11,086, 11,101 and 11,115 Da by MALDI-TOF/MS. The molecular mass difference of 14-15 Da of intact cytochrome *c*-550 is probably due to the difference in the chain length of the fatty acid. Lipase-treated (30 min) cytochrome *c*-550 was separated into two major fractions by reverse-phase HPLC (peaks 4 and 5 in Fig. 4). MALDI-TOF/MS revealed that their molecular masses were 10,636 and 10,861 Da. The decrease in the molecular masses suggests that 10,636 Da is from the diacylglycerol-anchor-less state and 10,861 Da is from the intermediate state. The molecular mass difference of 225 Da between 10,861 and 10,636 Da suggested that this value corresponds to the molecular mass of the fatty acid of C_{15} length. The results described above suggested that the three fractions that appeared in the reverse-phase HPLC are attributed to fatty acids of C_{15}, C_{16} and C_{17} chain lengths, which are bound to cytochrome *c*-550. The molecular masses of intermediate and fatty-acid-less states exhibited bands at 19 and 16 kDa, and 10 kDa, respectively, in SDS-PAGE (Fig. 1). The results suggest that the cytochrome *c*-550 molecule was not degraded to a monomer following SDS treatment owing to its molecular characteristics and/or attached fatty acids. GC was performed using methyl esters of fatty acids extracted from intact cytochrome *c*.

Results showed that cytochrome *c*-550 was bound to the following fatty acids: iso-$C_{15:0}$ (18.7%), anteiso-$C_{15:0}$ (52.8.%), iso-$C_{16:0}$ (4.5%), $C_{16:0}$ (5.3%), $C_{16:1}$ + iso-$C_{17:0}$ (5.5%), anteiso-$C_{17:0}$ (11.4%), and an unidentified fatty acid (1.8%). This result supported the data of the analyses of the diacetylglycerol moiety by reverse-phase HPLC and MALDI-TOF/MS. This fatty acid composition was similar to the cellular membrane fatty acid composition of *B. clarkii* K24-1U (data not shown).

Purification and characterization of cytochrome *c*-550 expressed in *E. coli*

The expression of membrane-anchored cytochrome *c*-550 in *E. coli* BL21Star(DE3) by introducing the entire genome sequence of 357 bp, including the domestic signal sequence inserted into pET101*c*550, was successful, and the system expressed cytochrome *c*-550 2.3-fold higher (1035 nmol L^{-1}) than that of the original strain (450 nmol L^{-1}). The mutated C18M (Cys^{18}→ Met) -cytochrome *c*-550 was also expressed in *E. coli* BL21Star(DE3) with a cytochrome *c* content 1.3-fold higher than that of the original strain.

Membrane-anchored cytochrome *c*-550 expressed in *E. coli* can be solubilized using Triton X-100 and purified by anion exchange, gel filtration and hydroxyapatite column chromatographies. The purification procedures and yields are almost the same as those in the case of the original strain. It is considered that the diacylglycerol anchor attached through Cys^{18}. The expression of C18M-cytochrome *c*-550 in the periplasmic space of *E. coli* was confirmed with the extraction of cytochrome *c* by cold osmotic shock. This indicates that the N-terminal Cys^{18} of the mature cytochrome *c*-550 is the site of the posttranscriptional modification of fatty acid chains. The purification of the C18M-cytochrome *c*-550 was simplified from 6-7 steps to only 3 steps; the yield became 1.5-fold higher than those in the case of the original strain.

Purified membrane-anchored cytochrome *c*-550 from *E. coli* exhibited two bands of 20 and 18 kDa in SDS-PAGE, which were very similar to those of the original strain (data not shown). On the other hand, purified C18M-cytochrome *c*-550 exhibited two bands of 15 and 10 kDa in SDS-PAGE (data not shown). The possible reason for the difference in the molecular mass between C18M-cytochrome *c*-550 and the lipase-treated (180 min) protein the latter possesses the modified N-terminal residue (acetylation and glycerol). Gas chromatography was performed using methyl esters of fatty acids extracted from the membrane-anchored cytochrome *c*-550 in *E. coli*. Results showed that cytochrome *c*-550 is bound to the following fatty acids: $C_{14:0}$ (5.4%), $C_{16:0}$ (43.6%), $C_{16:1}$ (9.6%), cylo-$C_{17:0}$ (6.3%), $C_{18:1}$ (30.1%), and an unidentified fatty acid (5.0%). Interestingly, the fatty acid composition was almost the same as the membrane fatty acid composition of *E. coli* (Cronan and Rock 1994; Cahoon et al. 1996). Because the fatty acid composition of cytochrome *c*-550 purified from *B. clarkii* K24-1U is also similar to that of its membrane, the fatty acid modification appears to be in accordance with the cellular membrane fatty acids of the host cells. This may involve a particular mechanism of fatty acid modification that occurs in accordance with the fatty acid composition of the host cells. C18M-cytochrome *c*-550 exhibited only one peak in the reverse-phase HPLC profile corresponding to the completely digested original cytochrome *c*-550 by lipase. The molecular mass of C18M-cytochrome *c*-550 was determined to be 10,543 Da, which is almost the same as the sum (10,544 Da) of the predicted molecular masses of C18M without the signal peptide (9,927 Da) and heme *c* (617 Da). These results indicate that C18M is an unmodified protein and has no N-terminal signal peptides. Purified membrane-anchored and soluble C18M-cytochromes *c*-550 expressed in *E. coli* exhibited the same absorption spectra as that of the original cytochrome *c*-550. These results described above suggest that the characteristics of the purified membrane-anchored and C18M-cytochromes

c-550 expressed in *E. coli* are very similar to those of the original cytochrome *c*-550 from *B. clarkii* K24-1U.

Oligomeric states and their size

The original and C18M-cytochromes *c*-550 showed dimeric molecular masses (23 kDa) as determined by blue native PAGE. The molecular masses of the original and C18M-cytochromes *c*-550 in the presence of Triton X-100 as determined by gel filtration (130 kDa) can be explained on the basis of 2 dimeric molecules observed in the blue native PAGE (ca. 23 kDa × 2) of the original and C18M-cytochromes *c*-550 and Triton X-100 (90 kDa) (Metsikkö 1984), respectively. Furthermore, C18M-cytochrome *c*-550 exhibited a tetramer in the absence of Triton X-100. Almost the same result was obtained with the original cytochrome *c*-550 in the presence of 0.1% Triton X-100. The large molecular mass of the original protein without Triton X-100 may be due to the aggregation of the protein by hydrophobic interaction. Thus, it is expected that cytochrome *c*-550 exhibits a tetrameric structure in solution and it may consist of a dimeric molecule bound by hydrophobic interaction.

Discussion

The observed molecular masses of the original mature cytochrome *c*-550 (11,086 Da, 11,101 Da and 11,115 Da) were 47-50 Da larger than the predicted molecular mass, which is the sum of the molecular masses of cytochrome *c*-550 protein (9,899 Da), glycerol residue (73 Da), fatty acids (450-478 Da), and heme *c* (617 Da). These results suggest that

the difference between the determined and predicted molecular masses of the original cytochrome *c* is due to its acetylation (43 Da) at its N-terminal Cys^{18} (Fig. 5A). This prediction is in accordance with the comparison of the molecular mass between the lipase-treated (180 min) original cytochrome *c*-550 (10,543 Da) and the unmodified C18M-cytochrome *c*-550 (10,636 Da).

The molecular masses of cytochrome *c* determined by analytical methods are summarized in Table 1. The original and C18M-cytochromes *c*-550 showed dimeric molecular masses (23 kDa) as determined by blue native PAGE. This result suggested that the two bands of 20 and 17 kDa of the original cytochrome *c* in the SDS-PAGE represent a dimer. The binding strength contributing to the dimer formation may be relatively higher than the ordinary subunit binding strength in other proteins. On the other hand, the C18M-cytochrome c-550 exhibited two bands of 15 and 10 kDa in SDS-PAGE. The results described above probably mean that the protein without the anchor segment formed a dimer (15 kDa) and the formation was enhanced by the presence of the diacylglycerol anchor. This observation is probably due to the diacylglycerol anchor enhancing the hydrophobic interaction between the monomer proteins.

Although several diacylglycerol-anchored cytochromes *c* have been analyzed by reverse-phase HPLC and GC, there is no report on diacylglycerol-anchored cytochrome *c* having three different carbon chain lengths. Furthermore, there has been no report on the appearance of two bands of 17 and 20 kDa upon SDS-PAGE of purified cytochrome *c*, although the minimum molecular mass of the protein is ca. 10 kDa. In addition, although there are a few examples of the molecular mass determination of membrane-anchored cytochrome *c* in a solution, the tetramer in solution in the case of cytochrome *c*-550 is the first to be reported. Comparison of cytochrome *c*-550 and the well-characterized two types of *Bacillus* cytochrome *c* is shown in Table 3. On the basis of the above results, it can be

concluded that cytochrome *c*-550 is a novel cytochrome *c*. Although there is no report concerning the dimeric cytochrome *c* in blue native PAGE, the membrane-binding diheme cytochrome *c* has been reported (Sone et al. 2001). This cytochrome *c* has a molecular mass of 28 kDa and its genomic sequence shows two heme binding sites in one molecule.

Although the amino acid sequence similarities of cytochrome *c*-550 with cytochromes *c* from alkaliphiles were not always higher than those from neutralophiles, cytochrome *c*-550 showed higher similarities with diacylglycerol-anchored cytochromes *c* than with those of the polypepetide-anchored type (Table 2). The amino acid sequences of cytochromes *c* from alkaliphiles exhibited a marked paucity of basic amino acids and a relatively higher amount of acidic amino acids (Fig. 3, Table 3). The lowest number of basic amino acids and the highest number of acidic amino acids of cytochrome *c*-550 were observed among the compared diacylglycerol- anchored cytochromes *c* in Fig. 3. As revealed in its primary structure, *Bacillus* small cytochromes *c* have a region showing no hydrophobic cluster after the signal peptide sequence in the N-terminal side. The presence of the Gly^{22} – Asn^{34} sequence in cytochrome *c*-550 (Fig. 3, Table 3) may indicate the extension of the region, and the sequence contains four proton-dissociable amino acid residues at high pH among the thirteen amino acid residues. Given the oligomeric state of cytochrome *c-550* in solution and that the protein is predicted to react with cytochrome *c* oxidase, it is considered that the tetrameric molecule is attached to the outer surface of the membrane (Fig. 5B). The tetrameric structure of this cytochrome *c* may further enhance the effect of this extended moiety by its accumulation. These properties may cause proton dissociation in acidic amino acids at high pH, and the resulting residues will exhibit a negative electrical charge. The negative charge localized on the outer surface of the membrane may attract protons translocated by the respiratory complexes and prevent proton diffusion to the bulk (Mulkidjanian et al. 2006). Therefore, it is considered that the cytochrome *c*-550 localized

on the outer surface of the membrane contributes to proton reservation in alkaliphilic *Bacillus clarkii* K24-1U.

The properties of the signal peptide amino acid sequences of the membrane-anchored lipoprotein have been reported (Sutcliffe and Harrington 2002; Kamalakkannan et al. 2004; Madan Babu et al. 2006). The signal peptide amino acid sequence of cytochrome *c*-550 had higher similarity with those of a reported Gram-positive bacterial membrane-anchored lipid-modified cytochrome *c* than with those of other types of protein. The signal peptide amino acid sequence of cytochrome *c*-550 showed the highest similarity with that of *O. iheyensis* cytochrome *c* (Q8ENI9) (Table 2). We were able to express cytochrome *c*-550 in *E. coli* cells; we found that lipid modification occurred in accordance with the fatty acid composition of *E. coli*. The signal peptide amino acid sequence of cytochrome *c*-550 might be involved not only in the high expression in *B. clarkii* K24-1U under certain physiological conditions but also in the high expression of cytochrome *c* in *E. coli*. Appropriate fatty acid acylation to cytochrome *c*-550 in accordance with the fatty acid composition of the host cells was observed in both *B. clarkii* K24-1U and *E. coli* on the basis of the original signal peptide sequence of cytochrome *c*-550. This is the first observation in membrane-anchored cytochromes *c*.

The midpoint redox potential determined by redox titration of cytochrome *c*-550 was low compared with those of neutralophiles as in the case of the previously reported cytochromes *c* isolated from alkaliphilic *Bacillus* (Davidson et al. 1988; Yumoto et al. 1991; Benini et al. 1998). However, the midpoint redox potential of cytochrome *c*-550 determined by cyclic voltammetric measurement was even lower than that determined by redox titration. This is the first observation in membrane-anchored *Bacillus* small cytochromes *c*. One possible explanation for this observation is the reorientation of the protein backbone due to the effect of the electric field of the electrode on cytochrome *c*-550.

It has been reported that the electric-field-induced redox potential shifts may occur owing to the Coulombic forces from the electrode (Rivas et al. 2005). Alkaliphilies exhibit a large electrical potential ($\Delta\psi$) compared with that of the neutralophile (Goto et al. 2005). The low midpoint redox potential of cytochrome *c* may be related to allow electron (negatively charged) flow from the outside (cytochrome *c*) to the inner part (cytochrome *a* or binuclear center of cytochrome *c* oxidase) of the membrane to overcome the large electrical potential (negatively charged inner membrane) (Yumoto et al. 1993). The redox potential difference between the redox titration and cyclic voltammetric measurements may be related to changes in the electrical charge in the outer surface of the membrane during the electron transfer step in the respiratory system.

It is considered that the above-described distinctive molecular features and unique redox properties of cytochrome *c*-550 are related to its physiological function for the adaptation to high pH of *B. clarkii* K24-1U.

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Figure legends

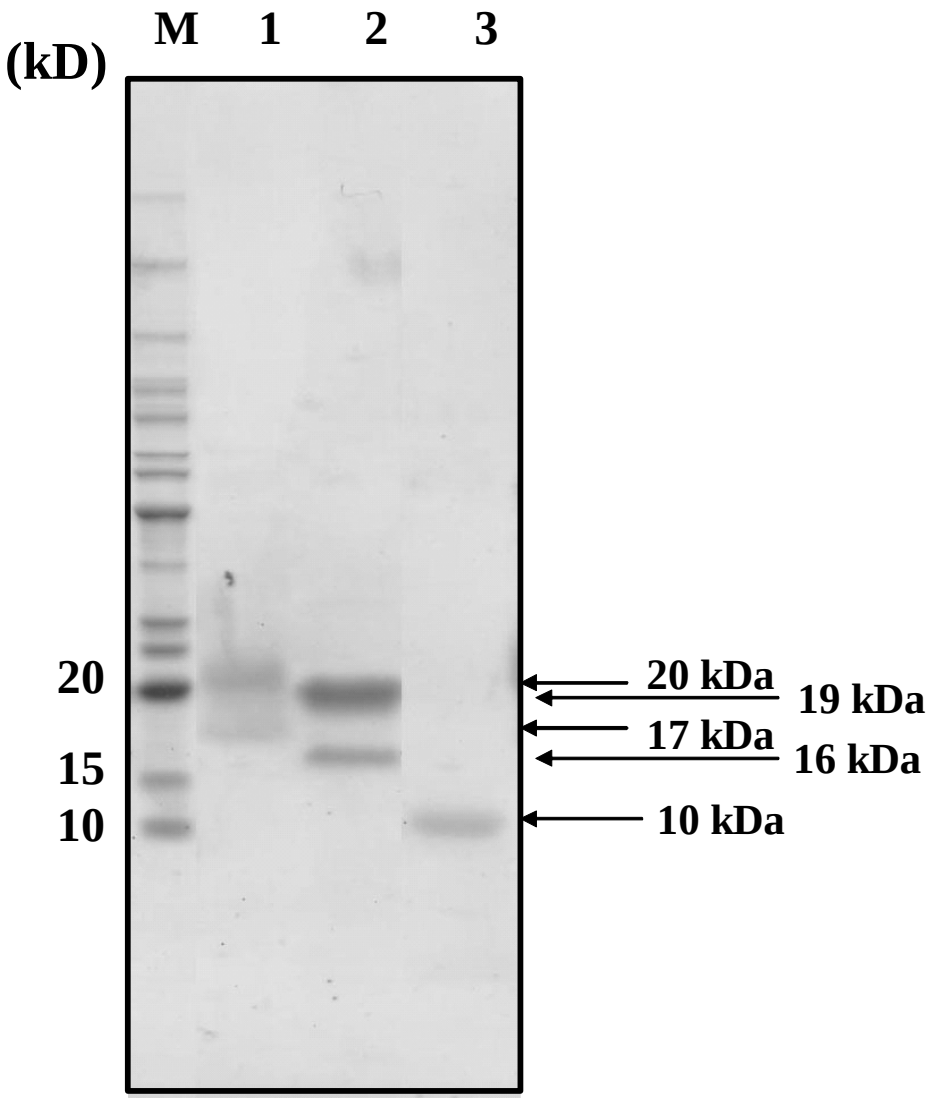
Fig. 1. SDS-PAGE of nontreated and lipase-treated cytochromes *c*-550 purified from *B. clarkii* K24-1U followed by CBB staining. Lane 1, nontreated cytochrome *c*-550; lane 2, intermediate state of lipase-treated (30 min) cytochrome *c*-550; lane 3, lipid-free cytochrome *c*-550 (lipase-treated: 180 min).

Fig. 2. Nucleotide and deduced amino acid sequences of cytochrome *c*-550 of *B. clarkii* K24-1U (*cycA*). A transcriptional start point determined by primer extension is indicated by a thick arrow, and putative -35 and -10 sequences are underlined. The signal sequence is indicated by italic characters. The lipobox (Sutcliffe and Harrington, 2002) is double-underlined. A heme *c* binding motif (CXXCH) and methionine, which is a potential candidate for the sixth iron ligand are enclosed in a box. The diacylglycerol binding site (Cys^{18}) is indicated by an asterisk. Characteristic palindrome structures were found downstream of the transcriptional start point (thick underlined). The putative Sine-Dalgano sequence is indicated by a shaded box. The arrow indicates the site of transcription initiation (+1) in the primer extension.

Fig. 3. Amino acid sequence alignment of cytochrome *c*-550 from *Bacillus clarkii* K24-1U and other diacylglycerol anchored cytochromes *c* from Gram-positive bacteria. Additional sequence of Gly^{22} – Asn^{34} (cytochrome *c*-550 numbering) was observed only in cytochrome *c*-550. The boxed sequences are heme-binding sites. Basic amino acids are highlighted in gray. Asterisks are identical amino acid residues.

Fig. 4. Elution profiles of nontreated (top), 30-min-lipase-treated (middle) and 180-min-lipase-treated (bottom) purified *B. clarkii* K24-1U cytochromes *c*-550 in presence of 0.1% Triton X-100 as determined by reverse-phase HPLC with a column. The upper and lower elution patterns in each elution profile indicate monitoring at 214 and 406 nm, respectively. The molecular masses determined by MALDI-TOF MS were as follows: peak 1, 11,082 Da; peak 2, 11097 Da; peak 3, 11,111 Da; peak 4, 10,857 Da; peak 5, 10,632 Da.

Fig. 5. Model of predicted structure of mature processed cytochrome *c*-550. (A) Model of posttranscriptional modification. The numbers indicate the molecular masses of the respective portions. (B) Model of tetrameric structure binding to outer membrane surface. The upper side is the extracellular membrane and the lower side is the intracellular membrane. The round and membrane-binding moieties are the protein and anchor parts, respectively.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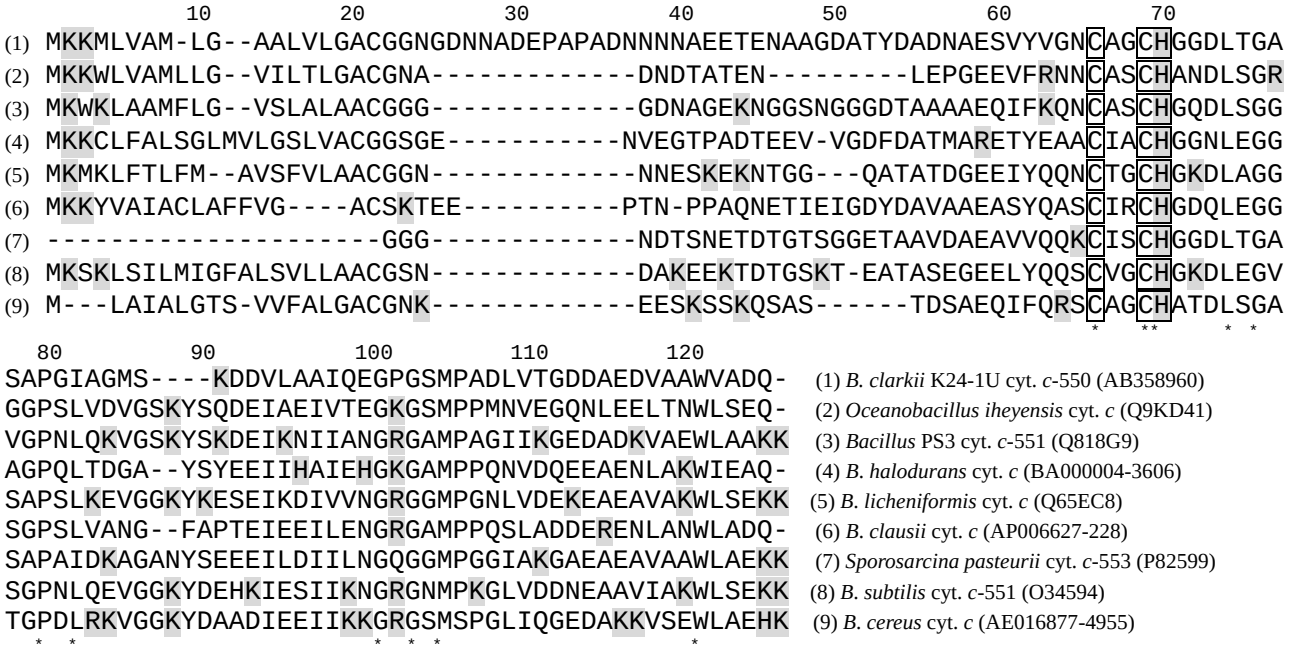
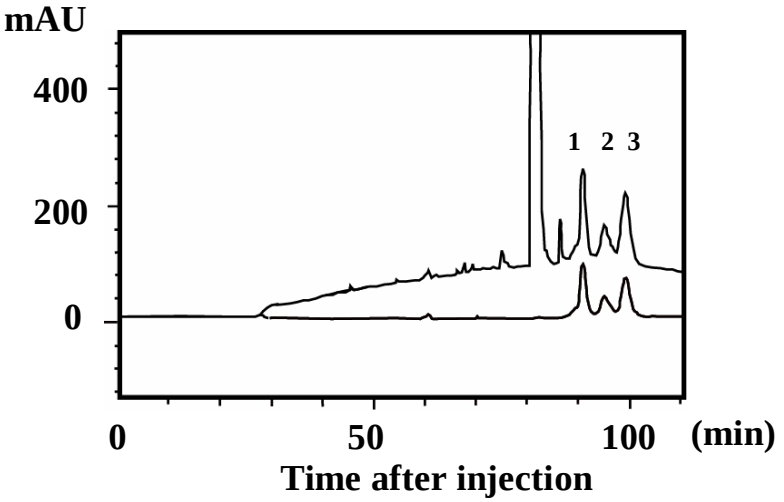
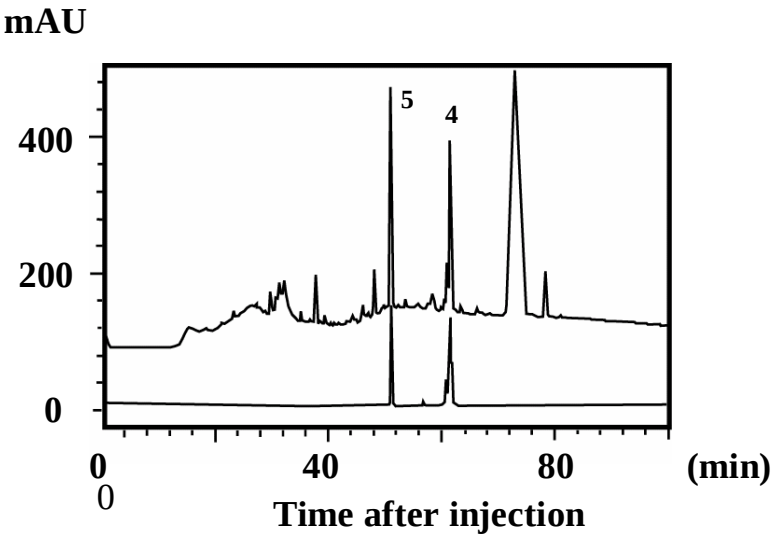
-118 - GCATCCGAGGTACTTGGTGAGGGTTTCAAAAAAAAATAAATAGGTTATAATAAAATGTGG - -57
-35
-10
-56 - ATGGTTGGATAAACCTTCCTGAGTTTATTTTTCTTTTTTTAGGAGGAGGAAATCATATGA - 4
+1
M - 1
5 - AAAAAATGCTAGTAGCAATGTTGGGGGCAGCGCTAGTACTTGGTGCATGCGGAGGAAACG - 64
K K M L V A M L G A A L V L G A C G G N - 21
*
65 - GCGATAATAACGCTGATGAGCCTGCACCGGCTGACAACAACAATAATGCTGAAGAGACAG - 124
G D N N A D E P A P A D N N N N A E E T - 41
125 - AGAACGCTGCAGGGGATGCTACATATGATGCGGATAACGCTGAATCAGTATATGTAGGAA - 184
E N A A G D A T Y D A D N A E S V Y V G - 61
185 - ACTGTGCAGGATGCCACGGAGGCGACCTGACTGGAGCTTCCGCACCAGGAATTGCTGGTA - 244
N C A G C H G G D L T G A S A P G I A G - 81
245 - TGTCTAAAGACGATGTTCTTGCTGCAATCCAGGAAGGACCTGGCTCAATGCCTGCAGATT - 304
M S K D D V L A A I Q E G P G S M P A D - 101
305 - TAGTAACAGGTGACGATGCAGAAGATGTTGCTGCATGGGTTGCTGATCAGTAA - 357
L V T G D D A E D V A A W V A D Q * -



(1) *B. clarkii* K24-1U cyt. *c*-550 (AB358960)
(2) *Oceanobacillus iheyensis* cyt. *c* (Q9KD41)
(3) *Bacillus* PS3 cyt. *c*-551 (Q818G9)
(4) *B. halodurans* cyt. *c* (BA000004-3606)
(5) *B. licheniformis* cyt. *c* (Q65EC8)
(6) *B. clausii* cyt. *c* (AP006627-228)
(7) *Sporosarcina pasteurii* cyt. *c*-553 (P82599)
(8) *B. subtilis* cyt. *c*-551 (O34594)
(9) *B. cereus* cyt. *c* (AE016877-4955)


mAU
400
200
0
1 2 3
0
50
100
(min)
Time after injection


mAU
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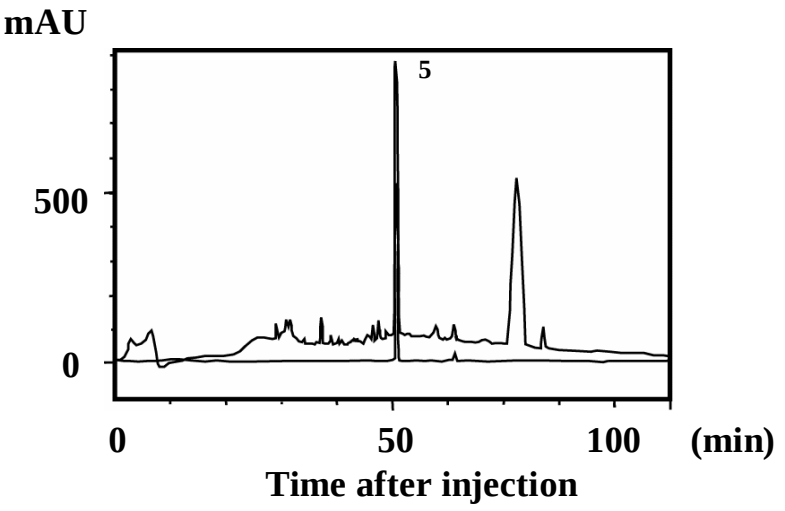
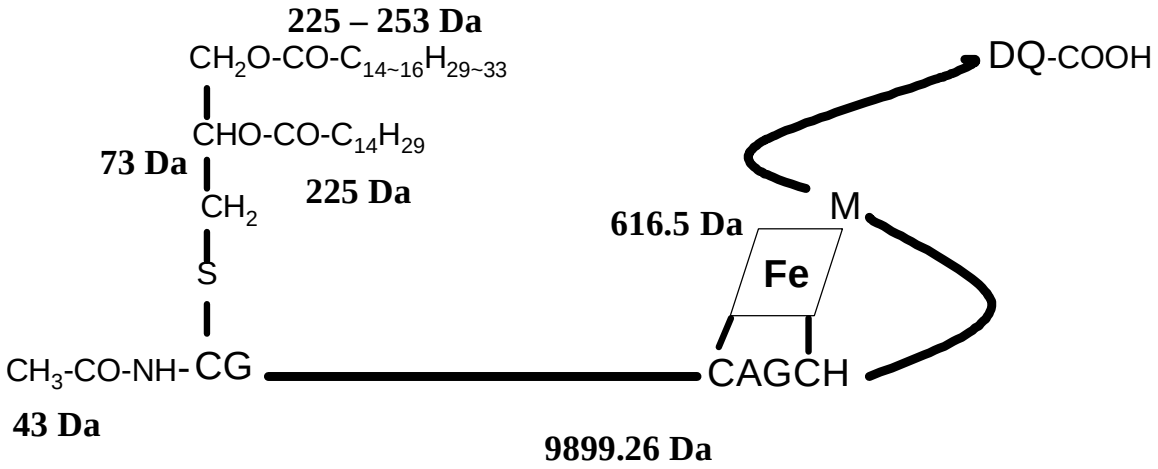
200
0
5
4
0
40
80
(min)
0
Time after injection


mAU
500
0
5
0
50
100
(min)
Time after injection

A


225 – 253 Da
CH2O-CO-C14~16H29~33
CHO-CO-C14H29
73 Da
225 Da
CH2
S
CH3-CO-NH-CG
43 Da
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M
616.5 Da
Fe
CAGCH
9899.26 Da

B

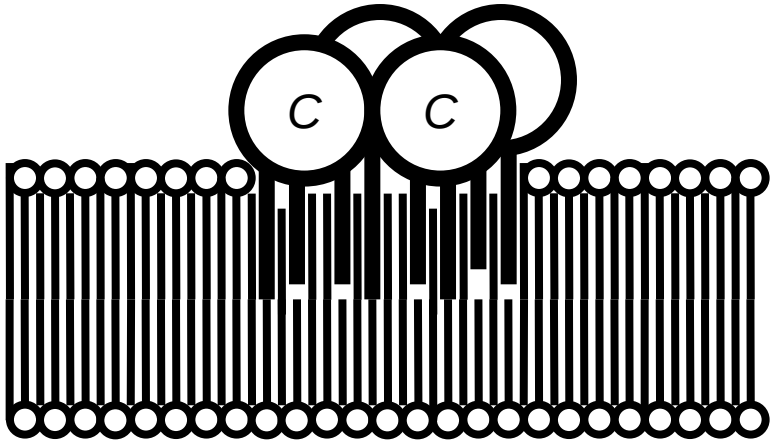
C
C

Table 1. Difference in molecular mass (Da) of original cytochrome *c*-550 and its C18M mutant protein as determined by analytical methods.

Method	Original cytochrome *c*-550	C18M cytochrome *c*-550
MALDI-TOF/MS	11,086, 11,101, 11,115	10,543
Blue native PAGE	23,000	23,000
Gel filtration with Triton X-100*	130,000	130,000
Gel filtration without Triton X-100	800,000, 240,000, 22,000	40,000
SDS-PAGE	20,000, 17,000	15,000, 10,000

* Molecular mass of Triton X-100 is 90,000 Da (Metsikkö, 1984)

Table 2. Comparison of cytochrome *c*-550 of *Bacillus clarkii* K24-1U with those of Gram-positive bacteria.

Organism [a]	N-terminal sequence [b]	Similarity [c] (%)	p*I* [d]	Accession No.
B. clarkii K24-1U*	MKKMLVAMLGAALVLGACGG	100	4.1	AB358960
*Oceanobacillus iheyensis**	MKKWLVANMLLGVILTGACGN	40	4.1	Q8ENI9
Bacillus PS3	MKWKLAAMFLGVSLALAACGG	40	4.0	Q56247
*B. halodurans**	MKKCLFALSGLMVLMLGSLVACGG	39	4.1	BA000004-3606
B. licheniformis	MKMKLFTLFMAVSFVLAACGG	37	5.8	Q65EC8
*B. clausii**	MKKYVAIACLAFFVGACSK	36	4.0	AP006627-228
*Sporosarcina pasteurii** [e]	ND	35	4.0	P82599
B. subtilis (cyt. *c*-551)	MKSKLSILMIGFALSVLLAACGS	34	3.8	O34594
B. cereus	MLAIALGTSVVFALGACGNK	34	6.1	AE016877-4955
*O. iheyensis**	MKKNPVIPYAIIAVIGILAVIIISVV	33	3.8	Q8EPY8
B. cereus	MKRNPLIPFALIAALGIIVMFVFSFQ	31	10	Q818G9
B. subtilis (cyt. *c*-550)	MKWNPLIPFLLIAVLGIGLTFFLSVK	30	5.3	P24469
*B. halodurans**	MKGRPLLPFAIIAVVGILLMISISII	29	3.8	Q9KD41
B. licheniformis	MNRNPLIPFLLIAIMGIGLVFILSIK	29	5.1	CP000002-2593
*B. clausii**	MKGRPLIPFAIIALLGVVLMVALSAV	27	3.9	Q5WHC9

[a]Alkaliphilic bacteria are indicated by an asterisk. [b]Predicted lipoboxes are underlined, and predicted diacylglycerol-cysteines are shaded on the basis of Sutcliffe and Harrington (2002). [c]Similarity with cytochrome *c*-550 of *B. clarkii* K24-1U that includes N-terminal sequence. [d]Although the p*I*s of cyts. *c* from *B. clarkii* K-241U, *Bacillus* PS3 and *B. subtilis* (cyt. *c*-551) were determined by electrophoresis, the other p*I*s were predicted on the basis of amino acid sequences including the N-terminal signal peptide. [e]The sequence lacks an N-terminal sequence.

Table 3. Biochemical properties of cytochrome *c*-550 of *B. clarkii* K24-1U in comparison with those of the membrane-anchored cytochomes *c*

Property	*B. clarkii* K24-1 cytochrome *c*-550	*B. subtilis* cytochrome *c*-551	*Bacillus* PS3 cytochrome *c*-551
Number of amino acid residues[a]	101	92	93
Molecular mass in SDS-PAGE (kDa)	20, 17	10	10.4
Molecular mass in gel-filtration (kDa)[b]	40	ND	33
Number of peaks that appeared in the analysis in reverse-phase chromatography	3	ND	1
Presence of Gly^{22}– Asn^{34} sequence[c]	Yes	No	No
Number of basic residues[d]	2	13	12
Midpoint redox potential (mV)[e]	83	>100	225
Absorption maxima at			
Oxidized form (nm)	408	409	409
Reduced form (nm)	415, 521, 550	416, 522, 551	416, 552, 551

The data of *B. subtilis* and *Bacillus* PS3 are cited from Bengtsson et al. (1999), and Sone et al. (1989) and Fujiwara et al. (1993), respectively. [a]In the processed polypeptide.

[b]The value is the substituted molecular mass of Triton X-100.

[c]Numbering in *B. clarkii* K24-1 cytochrome *c*-550.

[d]In the processed polypeptide. [e]Values are estimated by redox titration.