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**Cytochrome *c*-552 from Gram-Negative Alkaliphilic *Pseudomonas alcaliphila* AL15-21<sup>T</sup> Alters the Redox Properties at High pH**

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Running title: CYTOCHROME C FROM *P. ALCALIPHILA*

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A soluble class I cytochrome *c* of an alkaliphile was purified and characterized, and its primary structure was determined. This is the first example of a soluble class I cytochrome *c* in alkaliphiles. Cells the alkaliphilic gram-negative bacterium *Pseudomonas alcaliphila* AL15-21<sup>T</sup> grown at pH 10 had a soluble cytochrome *c* content that was more than twofold that of strain AL15-21<sup>T</sup> cells grown at pH 7 under air-limited conditions. Cytochrome *c*-552, a soluble cytochrome *c* with a low molecular weight, was purified from strain AL15-21<sup>T</sup> cells grown at pH 10 under air-limited conditions. Cytochrome *c*-552 had a molecular mass of 7.5 kDa and exhibited an almost fully reduced state in the resting form, which exhibited absorption maxima at wavelengths of 552, 523 and 417 nm. In the oxidized state, it exhibited an absorption maximum at 412 nm when it was oxidized by ferricyanide, its isoelectric point (pI) was 4.3 and it contained one heme *c* as a prosthetic group. Cytochrome *c*-552 was autoreduced at pH 10, and the autoreduction was reproducible. On the other hand, the autoreduction of cytochrome *c*-552 was not observed at pH 7.0. When pH was increased from 7.0 to 8.3, its midpoint redox potentials ( $E_m$  values) increased from +228 mV to +276 mV as determined by redox titrations, and from +217 mV to +275 mV as determined by cyclic voltammetric measurements. The amino acid sequence deduced by cytochrome *c*-552 gene analysis revealed that the sequence consists of 96 residues, including 19 residues as an amino-terminal signal peptide. A phylogenetic tree based on amino acid sequence indicated that the protein belongs to group 4, cytochrome  $c_5$  in class I cytochrome *c*.

Alkaliphilic bacteria adapt to alkaline conditions physicochemically by producing alkali-tolerant extracellular enzymes that are stable at high pHs (1). Moreover, they must adapt to alkaline conditions bioenergetically. Transmembrane pH gradient ( $\Delta\text{pH}$ ) (extracellular pH < intracellular pH) is thought to be an important factor in the  $\text{H}^+$  motive force ( $\Delta\mu$ ) that drives ATPase according to Mitchell's chemiosmotic theory (2). Therefore, it is thought to be very difficult for alkaliphiles to produce ATP under alkaline conditions (reversed transmembrane pH gradient). However, alkaliphilic *Bacillus* spp. exhibit higher growth rates and yields under alkaline conditions than neutralophilic *Bacillus subtilis* under neutral pH conditions (3). That is, energy production in alkaliphilic *Bacillus* spp. may involve special features for overcoming this disadvantageous condition. Alkaliphilic *Bacillus* spp. have been studied for their physiological ability to adapt to alkaline conditions (4-9). Recently, on the basis of the results of studies on certain alkaliphilic *Bacillus* spp., such as *Bacillus cohnii*, we have proposed a hypothetical alkaline adaptation model in which cytochromes *c* in its outer membrane reserve protons for the respiratory chain under alkaline conditions. (4, 8, 9). Several obligate alkaliphilic *Bacillus* spp. have a higher cytochrome content than the neutralophile *B. subtilis*, and mutant strains derived from alkaliphilic *Bacillus* spp. that cannot grow under alkaline conditions have fewer cytochromes than their parent strains (10, 11). Furthermore, facultative alkaliphilic *Bacillus* spp. produced larger amounts of cytochromes *c* and *b* when grown at pH 10 than those grown at neutral pH (12, 13). Several cytochromes *c* have been purified from alkaliphilic *Bacillus* spp. and characterized. All of these membrane-binding cytochromes *c* from alkaliphilic *Bacillus* spp. exhibit a low redox midpoint potential (less than +100 mV) and an acidic protein *i.e.* isoelectric point (*pI*): lower than 4 (4, 13-15).

Although there are several examples of gram-positive alkaliphiles, there is very little information on the physiology of gram-negative alkaliphilic bacterial strains. We have isolated a gram-negative alkaliphilic bacterium, *Pseudomonas alcaliphila* AL15-21<sup>T</sup>, to investigate the physiological functions of cytochromes *c* needed for its adaptation to alkaline environments (16). Gram-positive bacteria do not possess periplasmic space, and all of the components of their respiratory chain are of the membrane-binding type. Considering the differences between the cell structures of gram-positive and gram-negative bacteria, it would be intriguing to study the characteristics of soluble cytochromes *c* of the gram-negative alkaliphile *P. alcaliphila* AL15-21<sup>T</sup>.

Here, we describe an induction condition and the characteristics of cytochrome *c*-552 from *P. alcaliphila* AL15-21<sup>T</sup>. By comparing the characteristics of cytochrome *c*-552 from strain AL15-21<sup>T</sup> with those of cytochromes *c* from gram-positive alkaliphiles, the roles of cytochrome *c* in the adaptation to alkaline conditions are discussed.

## MATERIALS AND METHODS

**Chemicals** DNase was purchased from Sigma (St. Louis, MO, USA), phenylmethylsulfonyl fluoride (PMSF) was purchased from Wako Pure Chemical Industries (Osaka), the *pI* marker used was purchased from Oriental Yeast (Tokyo), and the molecular weight standard used for SDS-PAGE was purchased from APRO Science (Tokushima). All other chemicals used were of the highest grade commercially available.

**Bacterial strain and cultivation** Cells of the gram-negative facultatively alkaliphilic bacterium *P. alcaliphila* AL15-21<sup>T</sup> were used in this study. The

microorganism was grown in PYA broth medium consisting of 0.8% peptone, 0.3% yeast extract, 0.1% K<sub>2</sub>HPO<sub>4</sub>, 9.4 μM EDTA, 10 μM ZnSO<sub>4</sub>·7H<sub>2</sub>O, 36 μM FeSO<sub>4</sub>·7H<sub>2</sub>O, 12 μM MnSO<sub>4</sub>·H<sub>2</sub>O, 4 μM CuSO<sub>4</sub>·5H<sub>2</sub>O, 7 μM Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O, 16 μM H<sub>3</sub>BO<sub>3</sub>, and 100 mM NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> buffer (pH 10) at 27°C. The growth rates at pH 7 and pH 8.5 were determined in PYA broth medium containing 100 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> buffer and 100 mM Tricine-NaOH buffer, respectively. The bacterial cells were harvested in the early stationary growth phase and stored at -30°C until use.

**Culture condition for estimation of cytochrome *c* content.** To determine the effects of aeration condition and pH on the total soluble cytochromes *c* content, the cells were cultivated in a 2-*l* flask containing 300 ml of PYA broth medium with reciprocal shaking at 60 rpm or 120 rpm at pH 7, 8.5 or 10 at 27°C. The obtained cells were suspended in 10 mM Tris-HCl buffer at pH 8.0 containing 1 mM EDTA and 100 μM PMSF (buffer A), and 1 μg · ml<sup>-1</sup> DNase was added to the suspension to reduce viscosity. Then, the suspension was passed through a French pressure cell (SLM-Aminco, Rochester, NY, USA) at 1,8000 lb/in<sup>2</sup>. The obtained suspension was centrifuged at 6400 × *g* for 20 min to remove unbroken cells and cell debris. The supernatant was subjected to ultracentrifugation at 125,000 × *g* for 90 min to remove the membrane fraction. The supernatant as a soluble fraction was used for determining total soluble cytochromes *c* content.

**Purification of cytochrome *c*-552** For the purification of the soluble cytochrome *c*-552 from *P. alcaliphila* AL15-21<sup>T</sup>, the organism was cultured in 15 *l* of the PYA broth medium using a 20-*l* stainless-steel fermentor with an agitation speed of 100 rpm and an airflow rate of 5 *l* · min<sup>-1</sup>. Approximately 50 g of wet cells was obtained from the 15- *l* culture, and 100 g of wet cells was used to prepare a soluble fraction for the following

purification. The reddish soluble fraction was subjected to anion-exchange chromatography on a QAE-Toyopearl column ( $5.0 \times 30$  cm; Tosoh, Tokyo, Japan), which had been equilibrated with buffer A. After washing with buffer A, the absorbed cytochromes were eluted with a linear gradient from 0 to 0.25 M NaCl in buffer A at a flow rate of  $1.25 \text{ ml} \cdot \text{min}^{-1}$ . The cytochrome *c*-552 fractions were combined and subjected to a second anion-exchange chromatography on a QAE-Toyopearl column ( $2.5 \times 20$  cm), which had been equilibrated with buffer A. The column was washed with buffer A, and the cytochrome *c*-552 fraction was eluted with 50 mM NaCl in buffer A at a flow rate of  $1 \text{ ml} \cdot \text{min}^{-1}$ . Cytochrome *c*-552 fractions were collected and subjected to ultrafiltration in an Amicon Ultra Centrifugal Filter Device (Millipore, Bedford, MA, USA). The concentrated cytochrome *c*-552 solution (about 1 ml) was then applied to a Sephacryl S-100 HR column ( $2.5 \times 100$  cm; GE Healthcare Bio-Sciences, Piscataway, NJ, USA) equilibrated with buffer A containing 0.25 M NaCl at a flow rate of  $0.2 \text{ ml} \cdot \text{min}^{-1}$ . The cytochrome *c*-552 fractions were collected and loaded onto a hydroxyapatite column ( $0.9 \times 6.0$  cm; Seikagaku Kogyo., Tokyo) equilibrated with buffer A containing 100 mM NaCl. Cytochrome *c*-552 was eluted with 50 mM NaCl in buffer A.

**Physical and chemical measurements.** Spectrophotometric measurements were performed using a Cary 100 UV-Vis spectrophotometer (Varian, Palo Alto, CA, USA) using a 1-cm-light-path cuvette at room temperature. Sodium dithionite and potassium ferricyanide were used as the reductant and the oxidant, respectively. The cytochrome *c* content in the soluble fraction was determined from the difference spectrum of dithionite-reduced *minus* ferricyanide-oxidized samples using the following millimolar extinction coefficient of cytochrome *c*:  $\Delta\epsilon_{550} = 22.7 \text{ mM}^{-1}\text{cm}^{-1}$  (17). The molecular

mass of the purified protein was determined by Tricine SDS-PAGE on a stacking gel of 4% (w/v) and a separation gel of 15% according to the method of Schagger and von Jagow (18). Isoelectric focusing in the disc gel was carried out in the presence of 2% (w/v) ampholine in the pH range from 3.5 to 10. The heme *c* content of the purified cytochrome *c* was determined from the millimolar extinction coefficient ( $\epsilon_{\text{mM}}$ ) of pyridine ferrohemochrome *c* ( $29.1 \text{ mM}^{-1} \text{ cm}^{-1}$ ) (19). Protein content was determined using a BCA protein assay reagent kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.

**Measurement of midpoint redox potentials by redox titration.** Midpoint redox potentials ( $E_{\text{m}}$  values) were measured by the method of Dutton (20) using a potential meter apparatus (Model 744, Metrohm, Herisau, Switzerland). During the tests, a calomel electrode was used as the reference electrode and a platinum electrode was used as the auxiliary electrode. Titrations were performed by injecting microliter quantities of potassium ferricyanide through side arms in the redox cell under an anaerobic condition in 100 mM sodium phosphate (pH 7.0) and Tris-HCl (pH 8.3). Redox mediators, *e.g.*, 2,3,5,6-tetramethyl phenylenediamine, 2-hydroxyl-1,4-napthoquinone, 1,2-napthoquinone, phenazine methosulfate and phenazine ethosulfate, were added to a final concentration of 50  $\mu\text{M}$  each except for *N,N,N',N'*-tetramethyl phenylenediamine (TMPD), which was added to a final concentration of 10  $\mu\text{M}$ . A redox titration of the solution was performed by stepwise oxidation with 50 mM anoxic potassium ferricyanide after reducing cytochrome *c*-552 by adding a small amount of sodium dithionite. The extent of the reduction of cytochrome *c*-552 was determined by recording the spectrum between 200-800 nm in approximately 5-mV steps in potential. The absorption spectrum was recorded with a spectrophotometer. Midpoint redox



potential was calculated by the method of Dutton (20).

**Midpoint redox potential using gold electrode** The redox potential of cytochrome *c*-552 was also examined by cyclic voltammetry (CV). A gold disk (diameter: 1.6 mm) was used as a working electrode, and it was thoroughly cleaned by electrochemical oxidation/reduction treatment (-0.2 V – +1.5 V vs Ag/AgCl for 30 min) in 0.05 M H<sub>2</sub>SO<sub>4</sub> solution. The cleaned gold disk was modified by dip treatment in ethanol solution containing 2-amino-6-purinethiol (1 mM) for 30 min. After modification, the electrode was thoroughly washed with pure ethanol and water. The auxiliary and reference electrodes were Pt wire and Ag/AgCl (NaCl), respectively. Cyclic voltammograms (CVs) were recorded at room temperature in 0.1 M Tris-HCl buffer solution (pHs 7.0 - 8.3) containing cytochrome *c*-552 (0.18 mM) and 0.1 M NaClO<sub>4</sub>. All electrochemical responses were measured after deaerating using argon gas.

**Autoreduction of cytochrome *c*-552** The autoreduction of cytochrome *c*-552 was tested as described by O’Keeffe and Anthony (21) without adding glycerol at room temperature. Cytochrome *c*-552 (30-40 μM) was kept anoxic by bubbling with argon for 3 min followed by oxidation with anoxic potassium ferricyanide in 100 mM buffers. TMPD, an electron mediator, was added to accelerate e<sup>-</sup> transfer. TMPD was fully oxidized by potassium ferricyanide at the beginning of the experiment. Sodium phosphate (pH 7.0), Tris-HCl (pH 8.5), or glycine-NaOH (pH 10.0) was used for the buffer. Absorption spectra were recorded immediately and at 1 h intervals until the spectra showed no change in the reaction condition.

**Amino acid sequence determination** To determine the amino acid sequence of cytochrome *c*-552, heme was removed from the purified protein by treatment with *o*-nitrophenylsulfonyl chloride (*o*-NPSC) in acetic acid according to the method of

Fontana *et al.* (22). The heme removed was separated with ethyl acetate from the apo-protein. The cysteine residues were pyridylethylated prior to sequence analysis as described by Friedman *et al.* (23). After desalting by ultrafiltration with a Microcon YM-3 (Millipore), a pyridylethylated apo-protein was purified using a reversed-phase HPLC system equipped with an ODS-80 Ts column (2.0 × 150 mm; Tosoh). The apo-protein was eluted with a linear gradient from 0 to 80% acetonitrile under an acidic condition containing 0.1% trifluoroacetic acid (TFA).

Ten nanomoles of the purified apo-protein was first digested with endoproteinase Asp-N (Wako Pure Chemical Industries) for 16 h at 37°C in 50 mM Tris-HCl (pH 8) at an enzyme/substrate ratio (w/w) of 1/100. The peptides cleaved with the enzyme were separated using the same conditions as those used for the purification of the pyridylethylated apo-protein. To make chemical cleavage, 1 mg of protein was treated with 1% (w/v) cyanogen bromide (CNBr) in 70% (v/v) formic acid for 16 h at 37°C. The fluid obtained was evaporated to dryness under an N<sub>2</sub> stream and resolved in 100 µl of 70% (v/v) formic acid. The polypeptides obtained after protein treatment were separated using a reversed-phase HPLC system equipped with an ODS-80 Ts column (2.0 × 150 mm; Tosoh) and eluted at a flow rate of 0.8 ml/min with a linear gradient from 0 to 80% acetonitrile containing 0.1% TFA. The eluent containing polypeptides was collected by monitoring absorbance at 214 nm. The N-terminal amino acid sequences of the polypeptides obtained were determined by Edman degradation (24) using a model 491 protein sequencer (Perkin-Elmer, Wellesley, MA, USA).

**Sequence determination of cytochrome *c*-552 gene** Genomic DNA was extracted from *P. alcaliphila* AL15-21<sup>T</sup> using an ISOPLANT II kit (Nippon Gene, Tokyo). Two oligonucleotide primers, namely, 5'-TGYGGNGTNTGYCAYAAAYGG-3' and

5'-TARTCYTCNGCNCCRCARTC-3' (Y, R, and N are mixtures of T and C, A and G, and A, T, G and C, respectively), were designed on the basis of the determined primary structure of cytochrome *c*-552. The amplified 170-bp fragment was further extended by gene walking with a TaKaRa PCR *in vitro* Cloning kit (Takara Shuzo, Kyoto) and sequenced using a BigDye Terminator Cycle sequencing kit with an ABI 310 Genetic Analyzer (Perkin-Elmer). The determined sequence (accession no. EF178295) has been deposited in GenBank.

**Phylogenetic analysis of amino acid sequence of cytochrome *c*-552** Multiple alignments of the determined sequences were performed, and nucleotide substitution rate ( $K_{\text{nuc}}$ ) was calculated. A phylogenetic tree was constructed by the neighbor-joining method (25, 26) using the CLUSTAL W program (27). Sequence similarity and *pI* based on the amino acid sequence were calculated using the GENETYX computer program (Software Development, Tokyo).

## RESULTS

**Cytochrome *c* content depending on growth conditions** Cytochrome *c* contents in the soluble fraction of *P. alcaliphila* AL15-21<sup>T</sup> under various growth conditions were estimated from the reduced *minus* oxidized difference spectra. Total soluble cytochromes *c* content (shown as nmol · mg protein<sup>-1</sup>) was determined under various growth conditions (Table 1). Results are averages and standard deviations from at least three independent preparations of cell extracts. Statistical analysis was performed by Student's *t* test at  $P = 0.05$ . The production of cytochrome *c* in the soluble fraction was higher at pH 10 than at pH 7.0 under the air-limited condition and was higher when the organism was grown at 60 rpm than when it was grown at 120 rpm at both pH 7 and

pH 10. The largest amount of cytochrome *c* was found in the soluble fraction of *P. alcaliphila* AL15-21<sup>T</sup> when the organism was grown at pH 10 under the air-limited condition (shaking at 60 rpm). The cytochrome *c* content in cells grown at pH 10 with shaking at 60 rpm was 3.6-fold that in cells grown at pH 7.0 with shaking at 120 rpm.

**Purification of soluble cytochrome *c*-552 from *P. alcaliphila* AL15-21<sup>T</sup>** Table 2 shows a summary of the purification of the soluble cytochrome *c*-552. Cytochrome *c*-552 bound to the hydroxyapatite column in the presence of 100 mM NaCl and was eluted by 50 mM NaCl in buffer A. Cytochrome *c*-552 was purified effectively using the hydroxyapatite column. Cytochrome *c*-552 bound to the column under the condition of a high NaCl concentration and was eluted by 50 mM NaCl in buffer A. Since phosphate buffer is usually used for elution from a hydroxyapatite column, most of the contaminating proteins were not eluted by the buffer without phosphate buffer. When the purified cytochrome *c*-552 was subjected to Tricine SDS-PAGE, cytochrome *c*-552 showed one major band in the gel. Soluble cytochrome *c*-552 was purified to an electrophoretically homogeneous state. The molecular mass of the purified cytochrome *c*-552 as determined by Tricine SDS-PAGE was 7.5 kDa. This is very small compared with the sizes of *Pseudomonas aeruginosa* soluble cytochromes *c* (9-15 kDa) (28). The cytochrome *c*-552 content in the cells grown at pH 10 was 1.7-fold that in the cells grown at pH 7 as determined on the basis of the elution profile (Table 1). In addition to cytochrome *c*-552, two other kinds of cytochromes *c* were found in the soluble fraction of the cell extract of *P. alcaliphila*. However, it was difficult to estimate the differences in their contents in the case of cells grown at pH 10 and pH 7 because they did not elute independently in anion-exchange chromatography.

**Properties of cytochrome *c*-552.** Absorption spectra of resting, reduced and

oxidized purified cytochrome *c*-552 are presented in Fig. 1. The  $\alpha$ ,  $\beta$  and Soret bands of the purified cytochrome *c*-552 in the resting form showed absorption peaks at 552, 523 and 417 nm, respectively. The fully reduced cytochrome *c*-552 showed absorption peaks at 552 ( $\alpha$ ), 523 ( $\beta$ ) and 418 (Soret band) nm. The Soret band of the oxidized form showed a peak at 412 nm. Cytochrome *c*-552 exhibited an almost fully reduced spectrum in the resting form. The spectrum of the oxidized form was taken after the oxidation of cytochrome *c*-552 using potassium ferricyanide, the residual of which was removed by dialysis. The millimolar extinction coefficient of the reduced cytochrome *c*-552 at 552 nm was determined to be  $25.8 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ . The *pI* of cytochrome *c*-552 (*pI*=4.3) was slightly higher than those of the cytochromes *c* of alkaliphilic *Bacillus* spp. (*pI*=3.4-4.0). However, the acidic nature of the isolated cytochrome *c*-552 was similar to that of the reported cytochromes *c* from alkaliphilic *Bacillus* spp. (4, 13-15). The heme *c* content of the purified protein was determined to be 104 nmol/mg protein (Table 2), which was calculated to be 0.78 mol heme *c* in 1 mol cytochrome *c*-552. This indicates that cytochrome *c*-552 contains one heme *c* per molecule. The  $E_m$  values of purified cytochrome *c*-552 were +228 mV and +276 mV at pH 7 and pH 8.3, respectively, as determined by redox titration (Table 1). It was very difficult to determine  $E_m$  values above pH 9 owing to the autoreduction of cytochrome *c*-552.

It has been reported that some organosulfur compounds can be used as modifiers of gold electrode surfaces to realize the interfacial electron transfer of proteins (29-35). Quasi-reversible voltammetric responses were obtained in the measurement of the redox potential of cytochrome *c*-552 using a modified gold electrode under solution conditions. The  $E_m$  values of cytochrome *c*-552 as determined by redox titrations and cyclic voltammetric measurements are summarized in Table 1. The  $E_m$  of cytochrome *c*-552

from *P. alcaliphila* determined at pH 7 was lower than those of cytochromes *c*-550 and *c*-551 from *P. aeruginosa*, *i.e.*, +280 mV and +286 mV, respectively (28).

**Autoreduction** The extent of the autoreduction of cytochrome *c*-552 was estimated by evaluating the generation of a reduced form of cytochrome *c*-552 without an external electron donor under various conditions (pHs 7-10 and the presence or absence of 10  $\mu$ M TMPD) and under anaerobic condition. Cytochrome *c*-552 was almost fully reduced after 40 h of incubation at pH 8.5, while it was almost fully reduced after 4 h under the same reaction conditions at pH 10 in the presence of TMPD. The reaction exhibited a first-order reaction curve with reaction constants of 0.07 h<sup>-1</sup> during the initial 24 h of the incubation and 0.56 h<sup>-1</sup> during the initial 2 h of incubation at pH 8.5 and pH 10, respectively, in presence of TMPD (Fig. 2). Autoreduction occurred at pH 8.5 only in the presence of TMPD, while it was observed at pH 10 in the absence of TMPD. Cytochrome *c*-552 was almost fully reduced after 60 h of incubation at pH 10 in the absence of TMPD. The reaction exhibited a first-order reaction curve with a reaction constant of 0.04 h<sup>-1</sup> during the initial 30 h of incubation in the absence of TMPD (Fig. 2). These results indicate that electron transfer is accelerated by the addition of an electron mediator. Autoreduction was not observed even with the addition of TMPD at pH 7. Although the autoreduction of cytochromes *c* in *Methylobacterium extorquens* AM1 (*Pseudomonas* AM1) is not always reproducible in the absence of glycerol (21), the autoreduction of cytochrome *c*-552 was reproducible in the absence of glycerol.

**Sequence analysis of cytochrome *c*-552 gene.** The amino acid sequence of the purified cytochrome *c*-552 was determined and revealed to consist of 73 amino acids. On the basis of the results of amino acid sequencing, the cytochrome *c*-552 gene sequence was deduced. The cytochrome *c*-552 gene sequence was almost identical to

that revealed by amino acid sequence analysis, except that the former had an N-terminal extension of 19, and 4 amino acid residues in the C-terminal region were found in the gene sequence (Fig. 3). Because of its N-terminal signal peptide sequence and the fact that heme *c* incorporation occurs only in the periplasm of gram-negative bacteria (36), cytochrome *c*-552 is predicted to exist in the periplasmic space (*i.e.*, it is not associated with the cytoplasmic membrane).

In the deduced amino acid sequence, only one single-heme binding sequence pattern, namely, Cys-X-X-Cys-His, was found. This is consistent with the fact that 1 mol of cytochrome *c*-552 has 0.78 mol of heme *c*. This result indicates that cytochrome *c*-552 is a mono-heme protein. On the basis of the number of hemes in it and results of its phylogenetic analysis (Fig. 4), cytochrome *c*-552 was shown to belong to cytochrome *c*<sub>5</sub> in class I (37, 38). Database searches using BLAST indicated that the deduced amino acid sequence of cytochrome *c*-552 from *P. alcaliphila* AL15-21<sup>T</sup> exhibited a high similarity with the amino acid sequences encoded by genes assigned as putative cytochromes *c* from *P. aeruginosa* (Q9HT82; 68% similarity), *P. fluorescens* (Q8GCG9; 60% similarity), and *P. putida* (Q88HM2; 62% similarity) (Fig. 3). These genomic sequences were determined on the basis of results of gene analyses. These cytochromes *c* have not been purified or characterized yet. The predicted *pI*s of these probable cytochromes *c* from *P. aeruginosa* and *P. fluorescens* are 8.3 and 7.0, respectively, and the determined *pI* of *P. alcaliphila* AL15-21<sup>T</sup> cytochrome *c*-552 is 4.3. Amino acid residues related to the heme *c* of cytochrome *c*-552 were identified by referring to the 3D-structure of available ScyA (*Spicytc*) of *Shewanella putrefaciens* (39). Structurally, His<sup>35</sup> and Met<sup>73</sup> are the fifth and sixth heme *c* axial ligands, respectively. Furthermore, it is thought that Arg<sup>54</sup> is the residue that binds with the propionic acid of

heme *c* by hydrogen bonding.

The phylogenetic analysis based on the deduced amino acid sequence of cytochrome *c*-552 revealed that this cytochrome *c* is closely related to the probable cytochromes *c* from the genes of *P. aeruginosa* and *P. fluorescens*. The amino acid sequence indicated that the protein belongs to group 4, cytochrome *c*<sub>5</sub> in class I cytochrome *c*. This group of cytochromes *c* has an extra pair of cysteine residues not required for heme binding (40). The physiological function of cytochromes *c*<sub>5</sub> is not yet fully understood. These cytochromes are mainly found in nitrogen-fixing and denitrifying microorganisms.

## DISCUSSION

One of the common features of alkaliphilic *Bacillus* spp. is a cytochrome content that is manyfold higher than that of neutralophilic bacteria. The synthesis of soluble cytochrome *c*-550 from *P. aeruginosa* is induced by ethanol exposure (28), and the synthesis of cytochrome *c*<sub>B</sub> of *Shewanella violacea* is induced by atmospheric pressure (41). These facts suggest that some soluble cytochromes *c* have a role in environmental adaptation. The most marked induction of soluble cytochrome *c*-552 of *P. alcaliphila* AL15-21<sup>T</sup> was observed at a high pH under air-limited condition, which is an unfavorable condition for this bacterium's energy producing processes (Table 1). This suggests that soluble cytochrome *c*-552 has a role in compensating for the deficiency of metabolism under such unfavorable conditions.

The determined  $E_m$  values of the isolated cytochrome *c*-552 were in the range of the  $E_m$  values for neutralophiles (approximately +180 – +250 mV) but were not in the range of those for general alkaliphilic *Bacillus* spp. (+47 – +95 mV) (4, 15). There has been



only one example of a cytochrome *c* from an alkaliphilic *Bacillus* sp. exhibiting a higher  $E_m$  than those of reported membrane-binding alkaliphilic *Bacillus* spp. Alkaliphilic *Bacillus* sp. No. 13 produces a soluble cytochrome *c* that exhibits a higher  $E_m$  than those of reported membrane-binding cytochromes *c* from alkaliphilic *Bacillus* spp. (*i.e.*,  $E_m = +197$  mV) (42).

The values obtained by redox titrations were comparable to those determined by cyclic voltammetric measurements. There is an example of a large difference between  $E_m$  values obtained by redox titration and those obtained by electrode-voltammetric measurements. The  $E_m$  of cytochrome *c*-550 from *Synechocystis* PCC 6803 obtained by electrode voltammetric measurement was -108 mV at pH 7 (43), whereas the  $E_m$  determined by redox titration was -250 mV at pH 7 (44). This difference was thought to be due to the adsorption of cytochrome *c*-550 on the electrode, and such adsorption may induce an interaction of the electrode with a corresponding reorientation of the protein backbone (possibly due to the exclusion of water molecules) near the heme-edge-involving residues (45). The agreement of the results obtained using different procedures may mean that there is no drastic difference between the molecular features of the isolated state and the electrode-attached state of cytochrome *c*-552.

The  $E_m$  of cytochrome *c*-552 increased as ambient pH increased. Although  $E_m$  changes have been studied as biochemical aspects of cytochromes *c*, the physiological meaning of the value changes has not yet been elucidated (45-48). The redox potential of cytochrome *c* is greatly influenced by the fifth and sixth iron ligands and by the nature of the residues surrounding the heme environment (46). For example, the ionization state of polypeptide groups close to the heme influences the redox potential of cytochrome *c* (47). In such a case, the redox potential is influenced by the  $pK_a$

values of the residues surrounding the heme. Indeed, the redox potential of *P. aeruginosa* cytochrome *c*-551 depending on the surrounding pH has been interpreted as being caused by a change in the  $pK_a$  of heme propionate. Cytochrome *c*<sub>6</sub> from *Cladophora glomerata* also exhibits a pH-dependent  $E_m$ . Its molecular basis is due to the deprotonation of heme propionate-7 and the replacement of an axial methionine with lysine (48). Ishikita and Knapp predicted the redox potential of cytochrome *c*-550 from *Thermosynechococcus elongates* on the basis of its crystal structure, and the redox potential they obtained is highly consistent with that determined by redox titration (45, 49). Therefore, they concluded that the pH dependence is predominantly due to the deprotonation of the heme-propionic group D close to Asp<sup>49</sup>. The phenomena described above mean that the protonated state of the residues surrounding the heme changes depending on the redox state and surrounding pH. Similar phenomena might occur in the case of cytochrome *c*-552 from *P. alcaliphila* AL15-21<sup>T</sup>. Consequently, cytochrome *c*-552 exhibits characteristics in  $e^-$  transfer ability ( $E_m$ ) concomitant with surrounding-pH-coupled  $H^+$  transfer.

Cytochrome *c*-552 was isolated in an almost fully reduced state and exhibited autoreduction when oxidized. The autoreduction of the oxidized cytochrome *c*-552 was observed at a high pH under an anaerobic condition. Autoreduction velocity decreased when ambient pH decreased compared with that in the case between pH 10 and pH 8.5 in the presence of an  $e^-$  transfer mediator, *i.e.*, TMPD. This phenomenon cannot only be explained by the deprotonation of the heme *c*-propionic group, because cytochrome *c*-552 is not autoreducible at pH 8.5 without the addition of TMPD, at which its redox potential changes (Table 2). The increase in  $E_m$  at a high pH may partially explain the occurrence of autoreduction in the  $e^-$  acceptability of cytochrome

*c*-552. A certain number of  $e^-$  may come from dissociated  $e^-$  from the region of  $H^+$ -dissociated amino acid residues. We consider a similar scheme of  $e^-$  dissociation from the region of  $H^+$ -dissociated residues for the hypothetical model of autoreducible cytochromes *c* in *M. extorquens* AM1 (21). In the case of yeast iso-1 cytochrome *c*, it has been suggested that the reason for the autoreduction is the presence of a redox-active amino acid, perhaps Tyr, in the molecule (50). The autoreducible nature of cytochrome *c*-552 may be related to the characteristics of cytochrome *c* in its dissociations of  $H^+$  and  $e^-$  at high pHs. A comparison of the results of aligning of the primary structures of cytochromes *c* (Fig. 3) indicates that the candidate of the  $H^+$ -transferable autoreduction-causing amino acid might be Lys<sup>48</sup>. Although it has been thought that cytochrome *c* is just an electron carrier protein, it has been reported that cytochrome *c* exhibits  $H^+$ -coupled  $e^-$  transfer with an Ag electrode (51) and  $H^+$ -assisted  $e^-$  transfer to hydrogenase (52, 53).

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## Legends for Figures

FIG. 1. Absorption spectra of resting (solid line), reduced (dotted line) and oxidized (dashed line) cytochrome *c*-552 from *P. alcaliphila* AL15-21<sup>T</sup> showing  $\alpha$ ,  $\beta$ , and Soret bands. The protein was reduced with sodium dithionite and oxidized with potassium ferricyanide.

FIG. 2. Time course of autoreduction of cytochrome *c*-552. Cytochrome *c*-552 in the oxidized form (30  $\mu$ M) was monitored at 552 nm in the presence or absence of 10  $\mu$ M TMPD in 100 mM glycine-NaOH (pH 10) or Tris-HCl (pH 8.5) buffer. Open circles, closed circles, open triangles, and closed triangles indicate glycine-NaOH (pH 10) with TMPD, glycine-NaOH (pH 10) without TMPD, Tris-HCl (pH 8.5) with TMPD, and Tris-HCl (pH 8.5) without TMPD, respectively.

FIG. 3. Alignment of the amino acid sequences of cytochrome *c*-552 and putative cytochromes *c* of *P. aeruginosa* (Q9HT82), *P. fluorescens* (Q8GCG9), *P. putida* (Q88HM2), *S. putrefaciens* (O52685). The black bar shows the signal peptide predicted by the N-terminal sequence of the mature cytochrome *c*-552. The heme-binding motif (CXXCH) and the methionine that is the potential candidate for the sixth iron ligand are indicated by asterisks.

FIG. 4. Phylogenetic tree for class I cytochrome *c*. The phylogenetic tree was constructed by the neighbor-joining method. The numbers at nodes are bootstrap values based on 1000 replicates. Bootstrap values greater than 500 are shown. Bar:

$0.1 K_{\text{nuc}}$ .

Table 1. Effect of pH on characteristics of soluble cytochrome *c*-552

	pH		
	7.0	8.5	10
Soluble cytochromes <i>c</i> content (nmol/mg protein)			
60 rpm	0.18±0.1	0.21±0.04	0.47±0.05
120 rpm	0.13±0.01	0.16±0.01	0.16±0.02
Soluble cytochrome <i>c</i> -552 content (nmol/g cells)			
	6.7	-	12.1
Autoreducibility (min <sup>-1</sup> )			
With TMPD	None	7.7 × 10 <sup>-2</sup>	2.6 × 10 <sup>-1</sup>
Without TMPD	None	None	1.9 × 10 <sup>-2</sup>
<i>E</i> <sub>m</sub> (mV)			
Redox titration	+228	+276 <sup>a</sup>	-
Cyclic voltammetric measurement	+217	+275 <sup>a</sup>	-

a: Data at pH 8.3

Table 2. Purification of cytochrome *c*-552

Purification step	Total protein (mg)	Total amount of cytochromes <i>c</i> (nmol)	Cytochrome <i>c</i> content (nmol/mg)	Yield (%)	Purification factor (fold)
Cell-free extract	9710	4070	0.4	100	1.0
Soluble fraction	4090	1670	0.4	41	1.0
First QAE-Toyopearl	563	1120	2.0	27.5	4.7
Second QAE-Toyopearl	29	183	6.3	4.5	15
Sephacryl S-100 HR	5.5	167	30.4	4.1	73
Hydroxyapatite	1.1	111	104	2.7	248

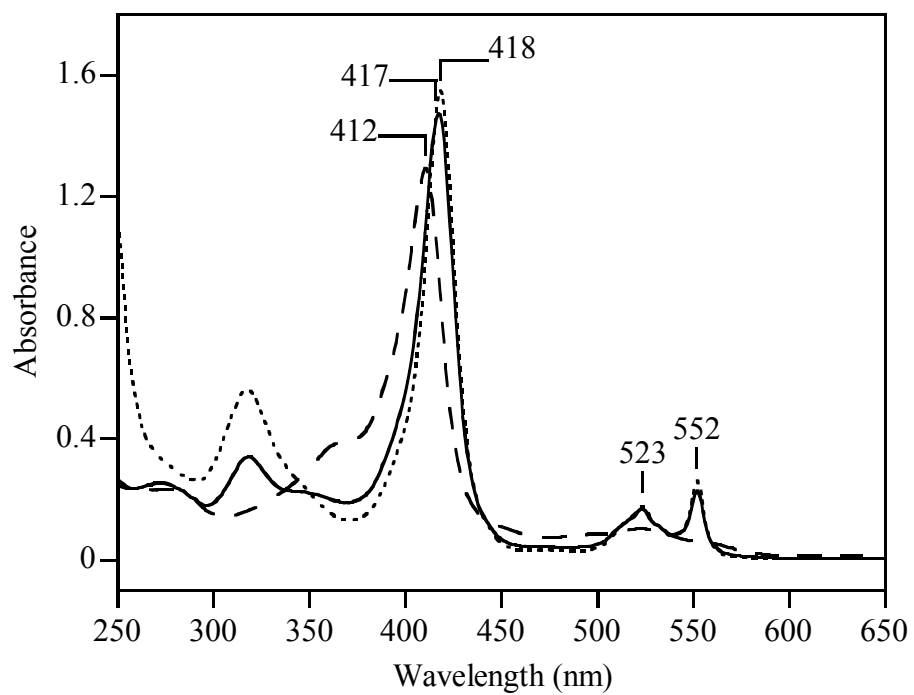


FIG. 1

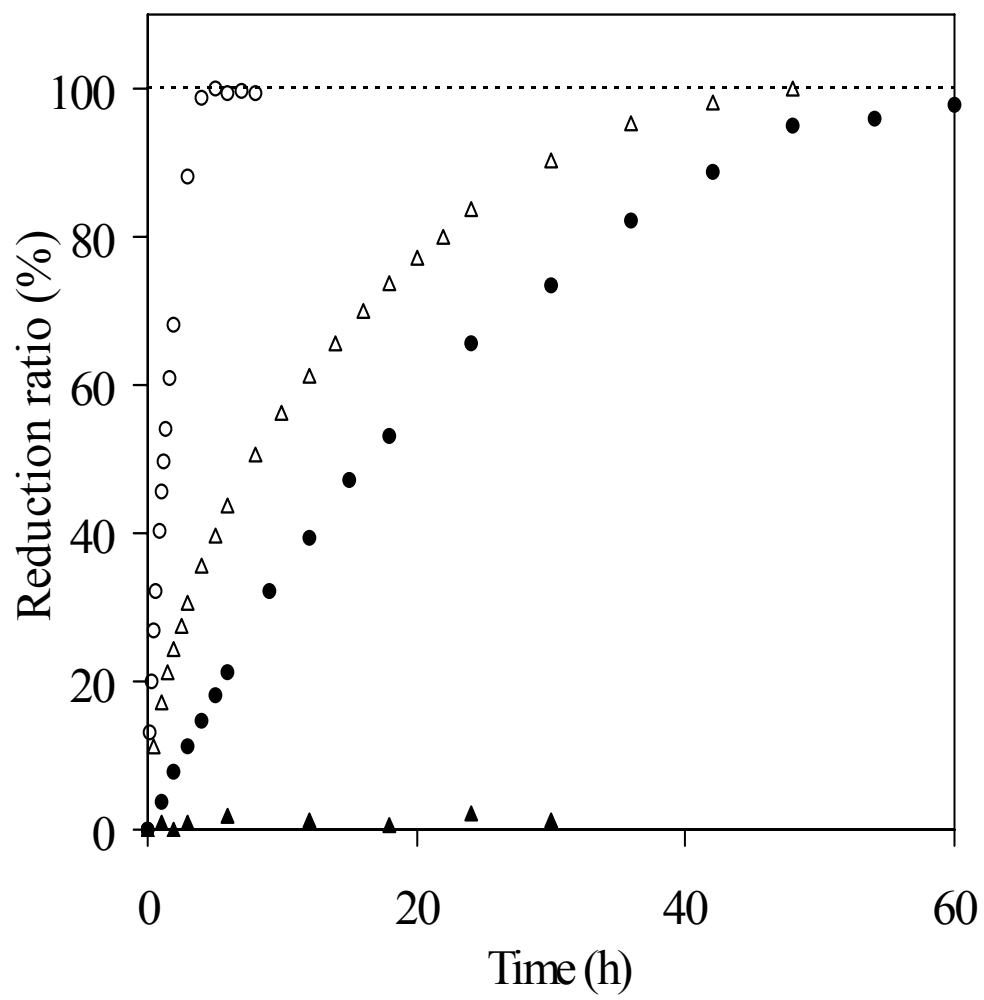


FIG. 2



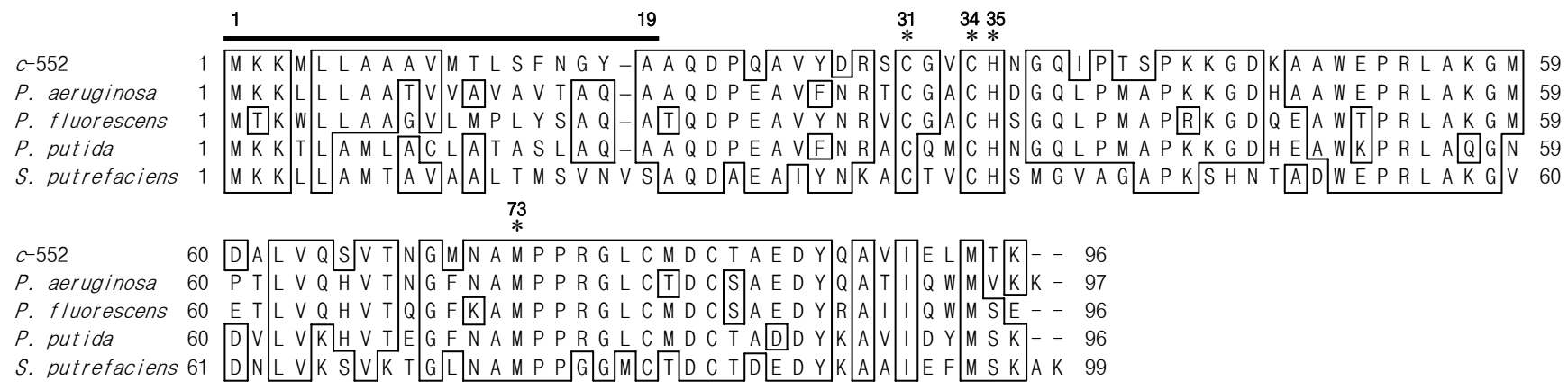


FIG. 3

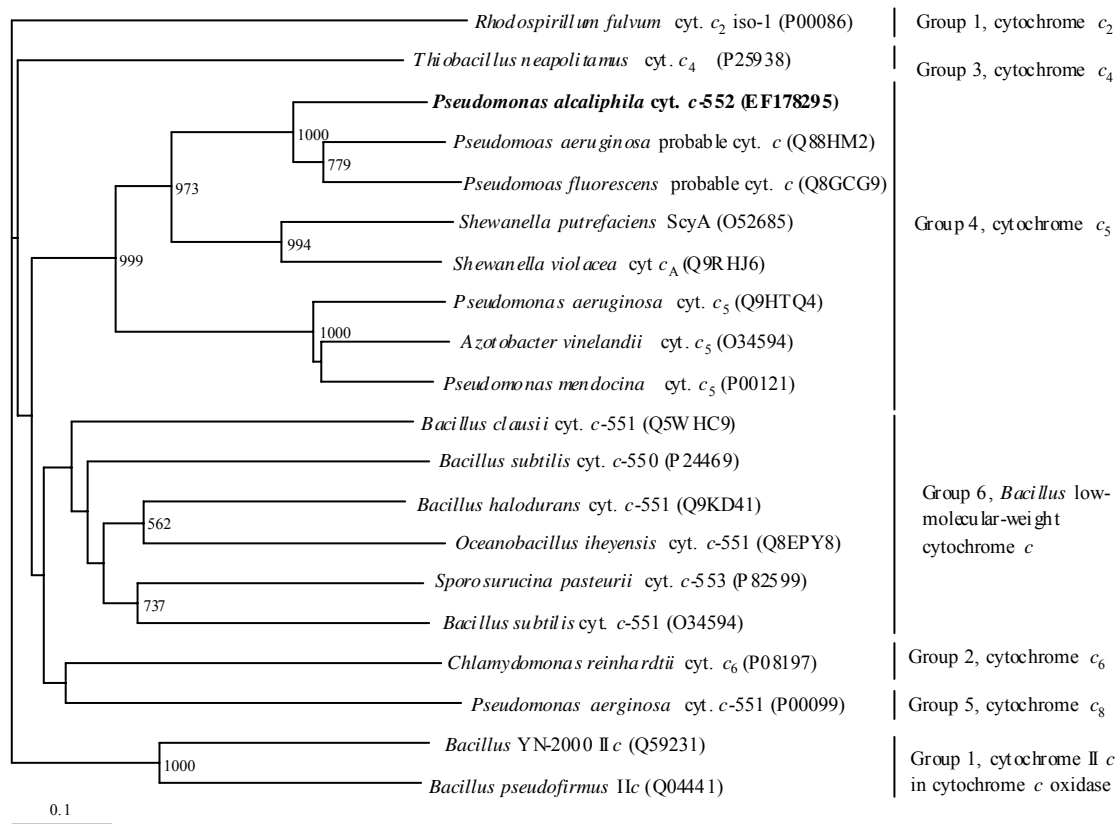


FIG. 4