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Physiological function of soluble cytochrome *c*-552 from alkaliphilic *Pseudomonas alcaliphila* AL15-21^T

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Abstract It has been found that the alkaliphilic Gram-negative bacterium *Pseudomonas alcaliphila* AL15-21^T produces a larger amount of soluble *c*-type cytochromes at pH 10.0 under air-limited condition than at pH 7.0 under high aeration. Cytochrome *c*-552 was confirmed as the major *c*-type cytochrome among three soluble *c*-type cytochromes in the strain. To understand the physiological function of cytochrome *c*-552, a *P. alcaliphila* AL15-21^T cytochrome *c*-552 gene deletion mutant without a marker gene was constructed by electrotransformation adjusted in this study for the strain. The maximum specific growth rate and maximum cell turbidity of cells grown at pHs 7.0 and 10.0 under the high-aeration condition did not differ significantly between the wild-type and cytochrome *c*-552 deletion mutant strains. In the mutant grown at pH 10.0 under low-aeration condition, marked decreases in the maximum specific growth rate (40%) and maximum cell turbidity (25%) compared with the wild type were observed. On the other hand, the oxygen consumption rates of cell suspensions of the mutant obtained by the growth at pH 10 under low-aeration condition were slightly higher than that of the wild type. Considering the high electron-retaining ability of cytochrome *c*-552, the above observations could be accounted for by cytochrome *c*-552 acting as an electron sink in the periplasmic space. This may facilitate terminal oxidation in the respiratory system at high pH under air-limited conditions.

Keywords Alkaliphilic · *Pseudomonas alcaliphila* · Cytochrome c_5

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Introduction

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). Therefore, it has been considered that cytochrome *c* may play an important role in the adaptation of alkaliphiles to alkaline environments. Membrane-bound cytochromes *c* have been purified from alkaliphilic *B. cohnii* YN-2000 and *Bacillus clarkii* K24-1U and characterized (Yumoto et al. 1991; Ogami et al. 2009). These cytochromes *c* exhibit particular characteristics such as detergent requirement of its soluble state, oligomeric molecular feature, low $E^{\circ\prime}$ values and low p*I*. Although the respiratory system of

Gram-positive alkaliphilic *Bacillus* spp. has been studied using several strains (Goto et al. 2005), there have been few reports on the respiratory system of Gram-negative alkaliphiles for the clarification of alkaline adaptation. In addition, the absence of soluble cytochromes *c* is a specific characteristic of Gram-positive bacteria. To study diversified alkaline adaptation mechanisms based on the respiratory system and clarify the possible contribution of soluble cytochrome *c* to alkaline adaptation, we have studied the function of soluble cytochrome *c* from a Gram-negative alkaliphilic bacterium. We have isolated Gram-negative alkaliphilic *Pseudomonas alcaliphila* AL15-21^T (Yumoto et al. 2001) and have characterized the soluble cytochrome *c*-552 (Matsuno et al. 2007).

We have found that *P. alcaliphila* AL15-21^T produces a larger amount of soluble cytochromes *c* (approximately 3.6-fold) at pH 10.0 under air-limited condition than at pH 7 under high-aeration condition (Matsuno et al. 2007). Cytochrome *c*-552 from *P. alcaliphila* AL15-21^T, one soluble *c*-type cytochrome among the three *c*-type cytochromes in the periplasm of this bacterium, was purified and biochemically characterized. The cytochrome *c*-552 gene consists of a 291-bp ORF corresponding to a protein, a 96-amino-acid pre-apocytochrome that includes an N-terminal signal sequence of 19 amino residues (Matsuno et al. 2007). In the phylogenetic tree constructed on the basis of amino acid sequence, cytochrome *c*-552 and other small cytochrome c_5 in *Pseudomonas* spp. exhibit distinct position among cytochrome c_5.

It is difficult to purify a large amount of cytochrome *c*-552 from *P. alcaliphila* AL15-21^T because the amount of cytochrome *c*-552 is relatively small compared with the large amount of background protein. To understand its physiological role and redox properties, the expression system of cytochrome *c*-552 in *Escherichia coli* was constructed. The recombinant cytochrome *c*-552 was oxidized by *cb*-type cytochrome *c* oxidase from *P. alcaliphila* AL15-21^T. The $E^{\circ\prime}$ values between pHs 5.0 and 10.0 were nearly unchanged ($E^{\circ\prime} = +217 - +225$mV). Cytochrome *c*-552 autooxidizes much slower than horse heart cytochrome *c* between pHs 8.0 and 10.0. Therefore, it is suggested that the high electron-retaining ability of cytochrome *c*-552 at high pH is important for the physiological alkaline adaptation of *P. alcaliphila* AL15-21^T for superior growth at high pH under air-limited condition (Matsuno et al. 2009).

To understand the physiological function of cytochromes *c*, cytochrome *c* deletion mutant strains were constructed using various bacterial strains. Among them, it has been reported that cytochromes *c* are not indispensable for their growth (von Wachenfeldt and Hederstedt 1990; Bengtsson et al. 1999; Braun and Thöny-Meyer 2005: Deeudom et al. 2008). However, there have been few reports on the growth characteristics from the results of deletion of cytochromes *c* under various growth conditions including various pHs. In the present study, a cytochrome *c*-552 deletion mutant was constructed to understand the physiological function of the cytochrome *c* in *P. alcaliphila* AL15-21^T for the adaptation under alkaline condition. We optimized the transformation condition for *P. alcaliphila* AL15-21^T, then constructed a marker less cytochrome *c*-552 gene deletion mutant of *P. alcaliphila* AL15-21^T using the method of Choi and Schweizer (2005). By using the wild type and the obtained cytochrome *c*-552 gene deletion mutant, the growth characteristics, maximum specific growth rate, maximum cell turbidity and oxygen consumption rate were determined under different pHs and aeration conditions.

Materials and methods

Bacterial strains, plasmids, media and culture conditions

Unless otherwise indicated, *P. alcaliphila* AL15-21^T was grown aerobically at 27 °C in PYA broth consisting of 8 g/l peptone (Kyokuto, Tokyo, Japan), 3 g/l yeast extract (Merck, Darmstadt, Germany), 1 g/l K_2HPO_4, 9.4 μM EDTA, 10 μM $ZnSO_4{\cdot}7H_2O$, 36 μM $FeSO_4{\cdot}7H_2O$, 12 μM $MnSO_4{\cdot}H_2O$, 4 μM $CuSO_4{\cdot}5H_2O$, 7 μM $Co(NO_3)_2{\cdot}6H_2O$, 16 μM H_3BO_3, and 100 mM $NaHCO_3/Na_2CO_3$ buffer (pH 10.0) (Yumoto et al. 2001). To prepare competent cells for electrotransformation, *P. alcaliphila* AL15-21^T was grown aerobically in Luria-Bertani (LB) broth at 27 °C. The plasmid pHA10 (carbenicillin-resistant) (Arai et al. 1991), which is a multi-copy broad-host-range plasmid that can be stably maintained in a wide range of gram-negative bacteria, was used to determine the transformation efficiency. *P. alcaliphila* AL15-21^T carrying pHA10 was normally grown at 27 °C in LB medium containing carbenicillin (Cb) (200 μg/ml). The plasmids pDONR221 (kanamycin-resistant) (Invitrogen, CA, USA) and pEX18ApGW (carbenicillin-resistant) (Choi and Schweizer 2005) were used for BP and LR clonase reactions, respectively, as described in the Gateway cloning manual (Invitrogen). For plasmid maintenance in *E. coli*, the medium was supplemented with kanamycin (Km) (35 μg/ml) or carbenicillin (Cb) (200 μg/ml). Cb (200 μg/ml) and gentamicin (Gm) (30 μg/ml) were used as selection markers for *P. alcaliphila* AL15-21^T transformants. Antibiotics were purchased from Wako Pure Chemicals (Osaka, Japan).

Ratio of cytochrome *c*-552 content in soluble cytochromes *c* in *P. alcaliphila*

P. alcaliphila AL15-21^T was cultivated in 15 l of PYA broth using a 20-l stainless-steel fermentor with an agitation speed of 100 rpm and an airflow rate of 5 l/min. The soluble fraction containing cytochromes *c* of this microor-

ganism was prepared using approximately 50 g of wet cells as previously described (Matsuno et al. 2007). The soluble fraction was subjected to anion-exchange chromatography on a QAE-Toyopearl column (2.5×30 cm; Tosoh, Tokyo, Japan) that had been equilibrated with buffer A consisting of 10 mM Tris–HCl buffer at pH 8.0 containing 1 mM EDTA and 100 μM PMSF. After washing with buffer A, the cytochromes *c* were eluted with a linear gradient from 0 to 0.2 M NaCl in buffer A at a flow rate of 1 ml/min. The cytochrome *c*-552 fraction and other cytochromes *c* fractions were separately combined and used for the determination of cytochrome *c* content.

Preparation of cells and electrotransformation conditions

To obtain competent cells, 1 ml of overnight culture of *P. alcaliphila* AL15-21^T in LB medium (pH 7.2) was inoculated into 100 ml of LB medium and incubated at 27 °C with shaking until the mid-log growth phase (OD_{600}=0.4). Glycine was added to the culture at a final concentration of 10 g/l for the glycine treatment and the cultivation was continued for more than 1 h. The cells were cooled in ice-cold water for 10 min and harvested by centrifugation at 6,400 × *g* for 20 min at 4 °C. The cells were then washed three times with the following electroporation media: (a) 0.5 M sucrose, (b) 0.5 M sucrose containing 1 mM HEPES and 1 mM $MgCl_2$, (c) 15% (v/v) glycerol, and (d) 15% (v/v) glycerol containing 1 mM MOPS. The cells were resuspended with the electroporation medium in 1/100 culture volume. Preparation of competent cells was performed on ice. One hundred microliters of competent cells was mixed with 1 μl of plasmid DNA dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) for electroporation. The suspension was transferred to an ice-cooled electroporation cuvette (0.1 cm cuvette gap) on ice. Electroporation was carried out on a Gene Pulser Xcell with a pulse controller (Bio-Rad, CA, USA) at 25 μF and 200 Ω at 12.5 kV/cm with a time constant of approximately 3 ms. Immediately after the discharge, 900 μl of ice-cold LB-broth containing 0.5 M sucrose and 1 mM $MgCl_2$ was directly added to the cuvette and mixed with the cells. After incubation with gentle shaking at 27 °C for 3 h, the cells were selected by plating on LB agar containing Cb (200 μg/ml) and incubated at 27 °C for 48 h.

Construction of a *P. alcaliphila* AL15-21^T cytochrome *c*-552 gene deletion mutant, C552M-2 (AL15-21Δc-552::FRT)

Oligonucleotide primers were synthesized by Hokkaido System Science (Hokkaido, Japan). KOD plus DNA polymerase was purchased from Toyobo (Osaka, Japan). PCR products were purified using a QIAquick PCR purification kit (Qiagen, Tokyo, Japan). A deletion mutant of *P. alcaliphila,* AL15-21Δc-552::Gm^r, was constructed by a previously described procedure (Choi and Schweizer 2005). Briefly, in the first round of PCR, the Gm resistance gene cassette was amplified using Gm-F and Gm-R primers (Table 1). The 5′ and 3′ fragments of the cytochrome *c*-552 gene were amplified by two sets of PCR. The first reaction was carried out with c552-UpF-GWL and c552-UpR-Gm primers and the second reaction was carried out with c552-DnF-Gm and c552-DnRGWR primers for the deletion of the cytochrome *c*-552 gene (Table 1). The PCR mixture for the second round of PCR contained 50 ng each of the 5′ and 3′ fragments of the cytochrome *c*-552 gene and 50 ng of *FRT*-Gm-*FRT* template DNA prepared during the first-round PCR. Three fragments in the PCR mixture were spliced together by overlap extension PCR.

The bacterial strains and plasmids used in this study are listed in Table 2. The resulting DNA fragment was cloned in vitro into the Gateway vector pDONR221 (Invitrogen), by BP clonase reaction (pPAC522M01) and then recombined into the Gateway-compatible gene replacement vector pEX18ApGW (Choi and Schweizer 2005) by LR clonase reaction as described in the Gateway cloning manual (Invitrogen). The resulting vector pPAC552M02 contains functional sequences for recombination-based cloning (*attR1* and *attR2*) and a *ccdB* counterselection

Table 1 Primers used for construction of *P. alcaliphila* AL15-21^T cytochrome *c*-552 deletion mutant

Primer	Sequence (5′→3′)
Gm-F	CGAATTAGCTTCAAAAGCGCTCTGA
Gm-R	CGAATTGGGGATCTTGAAGTTCCT
GW-*attB*1	GGGGACAAGTTTGTACAAAAAAGCAGGCT
GW-*attB*2	GGGGACCACTTTGTACAAGAAAGCTGGGT
c552-UpF-GWL	TACAAAAAAGCAGGCTaccttggcggcactgatgag
c552-UpR-Gm	TTTTGAAGCTAATTCGccgttgaaagacagcgtcataacg
c552-DnF-Gm	CAAGATCCCCAATTCGactgcacggccgaggatta
c552-DnR-GWR	TACAAGAAAGCTGGGTtgcaggccggcataccttc

Sequences in upper case letters are overlapped with the Gm or *attB* primer sequences. Lower case letters indicate *P. alcaliphila* AL15-21^T chromosome-specific sequences

Table 2 Strains and plasmids used in this work

Strain and plasmids	Description	Reference and source
P. alcaliphila		
AL15-21^T	Wild type	Yumoto et al. (1991)
C552M-1	Gm^r; AL15-21^T with φ*c552*::*FRT*-Gm^r-*FRT*	This study
C552M-2	AL15-21^T with φ*c552*::*FRT*	This study
Plasmids		
pHA10	Ap^r; broad-host-range expression vector	Arai et al. (1991)
pDONR221	Km^r; Gateway donor vector	Invitrogen
pEX18ApGW	Ap^r; Gateway destination vector	Choi and Schweizer (2005)
pFLP2	Ap^r; source for Flp recombinase	Hoang et al. (1998)
pPAC522M01	Gm^r, Km^r; pDONR221 with attB1-*c552′*-FRT-Gm^r-FRT-*c552*-attB2	This study
pPAC552M02	Gm^r, Km^r; pEX18ApGW with attB1-*c552′*-FRT-Gm^r-FRT-*c552*-attB2	This study

marker while maintaining a *sacB* counterselection marker for downstream resolution of merodiploids. pPAC552M02 was transferred to the *P. alcaliphila* AL15-21^T chromosome by the optimized electroporation procedure described above. A few colonies were patched on LB plates containing Gm (LB + Gm) and on LB plates containing Cb (LB + Cb) to select double- from single-crossover events. To ascertain the resolution of merodiploids, Gm^r single colonies were isolated from the LB + Gm plates containing 100 g/l sucrose. Gm^r colonies from the LB + Gm + sucrose plates were patched onto LB + Gm plus 100 g/l sucrose, as well as LB + Cb. Colonies growing on the LB + Gm + sucrose plates but not on the LB + Cb plates were considered putative deletion mutants. The resulting strain was designated as C552M-1. The presence of correct mutations was verified by colony PCR with c552-UpF-GWL and c552-DnR-GWR primers (Table 1). Finally, a deletion mutant, C552M-2 (AL15-21Δc-552::FRT), was obtained by Flp-mediated excision of the Gm resistance marker using pFLP2 (Hoang et al. 1998). The presence of AL15-21Δc-552::FRT was verified using colony PCR with c552-UpF-GWL and c552-DnR-GWR primers.

Growth characteristics of wild-type *P. alcaliphila* AL15-21^T and cytochrome *c*-552 deletion mutant strains

Wild-type *P. alcaliphila* AL15-21^T and the soluble cytochrome *c*-552 deletion mutant, C552M-2, were grown at 27 °C in PYA medium prepared at pH 7.0 (containing 100 mM sodium phosphate buffer) and at pH 10.0 (containing 100 mM sodium carbonate-bicarbonate buffer). Precultures of 50 μl (OD_{600}=0.3) were used for inoculation into an L-shaped test tube containing 7 ml of PYA medium. Bacterial growth in the tube was automatically monitored every 15 min by measuring the optical density at 660 nm (OD_{660}) with a model TN-2612 Bio-photorecorder (Advantec, Tokyo, Japan). The test tubes were incubated with shaking at 60 rpm (high-aeration condition) or without shaking (low-aeration condition) at 27 °C for 50 h. Each culture was grown in triplicate and each experiment was repeated three times.

Oxygen consumption of *P. alcaliphila* AL15-21^T wild type and cytochrome *c*-552 deletion mutant strains

The oxygen consumption rate was measured with a galvanic-type oxygen electrode (Iijima Electronics Corporation, Aichi, Japan) in a closed glass vessel with magnetic stirring at 27 °C. In the reaction solution, 50 mM sodium phosphate buffer and 50 mM sodium carbonate-bicarbonate were used for pHs 7 and 10, respectively, in 0.3 M sucrose and 10 mM $MgCl_2$. Sucrose is not utilized by *P. alcaliphila* AL15-21^T as a growth substrate. Therefore, sucrose was used as an osmotic agent. *P. alcaliphila* AL15-21^T wild type and cytochrome *c*-552 deletion mutant strains were grown under different pHs and aeration conditions as described above. After the precultivation of each strain, 1/100 volume of preculture (OD_{600}=0.3) was inoculated into PYA medium. Shaker flasks of 500 ml volume containing 400 ml of PYA medium were shaken at 60 rpm as a low-aeration condition. The same shaker flasks containing 200 ml of PYA medium were shaken at 120 rpm as a high-aeration condition. The cells at mid-log growth phase were harvested by centrifugation at 6,400 × *g* for 20 min at 4 °C and washed twice with the reaction solution. The reaction solution was added to the reaction vessel of the system and incubated at 27 °C. Concentrated cells suspended in the reaction solution were then added to the reaction vessel and the oxygen consumption rate was recorded. From the reported data on oxygen solubility in water at various temperatures, the oxygen saturation value of the liquid in equilibrium with air was calibrated.

Results

Proportion of cytochrome *c*-552 content in the soluble cytochromes *c* in *P. alcaliphila*. AL15-21^T

The soluble cytochromes *c* in *P. alcaliphila* AL15-21^T were fractionated by anion-exchange chromatography with a QAE-Toyopearl column to estimate the ratio of cytochrome *c*-552 content in the soluble cytochromes *c* in this microorganism. Cytochrome *c*-552 and the other cytochromes *c* were eluted with 0.05 M and 0.15 M NaCl in buffer A, respectively. The separation of cytochrome *c*-552 and the other soluble cytochromes *c* was thus achieved. The concentrations of cytochrome *c*-552 and the other cytochromes *c* were 14.4 (64%) and 8.2 (36%) nmol per g of cells, respectively. There are three *c*-type cytochromes in the soluble fraction in this microorganism (data not shown). The results indicated that cytochrome *c*-552 is the major *c*-type cytochrome among the *c*-type cytochromes in the periplasm of *P. alcaliphila* AL15-21^T.

Transformation of *P. alcaliphila* AL15-21^T by electroporation

Glycine is commonly used as a cell-wall-weakening reagent before electroporation (Buckley et al. 1999; Holo and Nes 1989; Ito and Nagane 2001; Stepanov et al. 1990). The effect of glycine added to the growth medium on transformation efficiency was examined (Table 3). Ten grams per liter of glycine was added to the culture at mid-log growth phase (OD_{600}=0.4), and then the cultivation was continued for more than 1 h. It was not possible to prepare glycine-treated electroporation-competent cells in the absence of sucrose because the cells underwent autolysis. In two electroporation media containing sucrose, the transformation efficiency of glycine-treated electroporation-competent cells was lower than that of cells without glycine treatment. The highest transformation efficiency, 1.5×10^4 transformants/μg DNA, was obtained for cells without glycine treatment using 0.5 M sucrose as the electroporation medium. Therefore, electroporation-competent cells were prepared without glycine treatment. To investigate the effect of sucrose concentration in the electroporation medium on transformation efficiency, the concentration was varied from 0.05 to 0.8 M, and 0.3 M sucrose was found to be the optimum concentration of sucrose (Fig. 1). Although 0.5 M sucrose as an osmotic stabilizer was initially used, a drop in transformation efficiency was found above the sucrose concentration of 0.3 M. The effect of electric field strength on transformation efficiency was also examined. The electric field strength was varied from

Table 3 Relationship between glycine treatment of electroporation-competent cells and transformation efficiency

Electroporation medium	Glycine treatment[a]	Transformation efficiency (Transformants/μg DNA)
0.5 M Sucrose	+	3.95×10^3
	−	1.45×10^4
0.5 M Sucrose, 1 mM HEPES, 1 mM $MgCl_2$	+	5.30×10^3
	−	1.10×10^3
15% Glycerol	+	Not prepared[b]
	−	2.00×10^2
15% Glycerol, 1 mM MOPS	+	Not prepared[b]
	−	Not detected

[a] +: 10 g/l glycine treatment, −: No glycine treatment

[b] Electroporation-competent cells were not prepared because the cells underwent autolysis

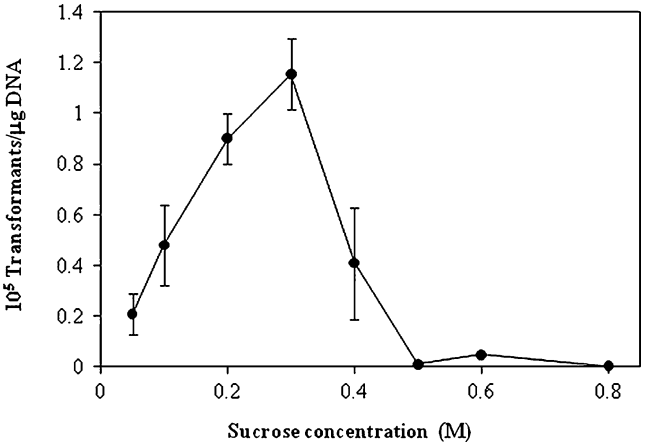

Fig. 1 Effect of sucrose concentration in the electroporation medium on the transformation efficiency of *P. alcaliphila* AL15-21^T with pHA10. The electroporation was varied from 0.05 to 0.8 M sucrose using a Gene Pulser Xcell at the following settings: capacitance of 25 μF, resistance of 200 Ω, and electric field strength of 12.5 kV/cm

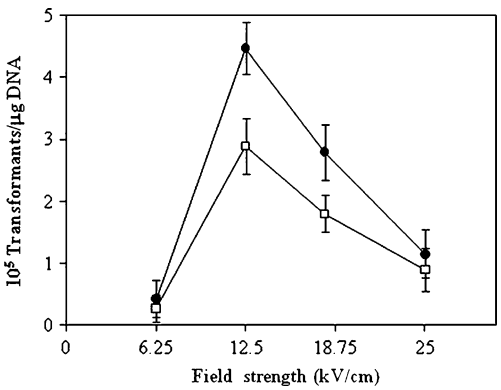

Fig. 2 Effect of electric field strength on the transformation efficiency of *P. alcaliphila* AL15-21^T with pHA10 in 0.2 M sucrose (*open squares*) and 0.3 M sucrose (*closed circles*). The electroporation was varied from 6.25 to 25 kV/cm using a Gene Pulser Xcell at the following settings: capacitance of 25 μF and resistance of 200 Ω

Table 4 Growth characteristics of *P. alcaliphila* AL15-21^T wild type and cytochrome *c*-552 deletion mutant, C552M-2

Strain	Growth condition							
	Low aeration				High aeration			
	pH 10.0		pH 7.0		pH 10.0		pH 7.0	
	$\mu_{max}(h^{-1})$	OD_{max}	$\mu_{max}(h^{-1})$	OD_{max}	$\mu_{max}(h^{-1})$	OD_{max}	$\mu_{max}(h^{-1})$	OD_{max}
Wild type	0.024	0.27	0.024	0.53	0.333	2.06	0.446	2.18
Δ*c*-552	0.014	0.21	0.023	0.50	0.336	2.06	0.455	2.17

Wild type, *P. alcaliphila* AL15-21^T. Δ*c*-552, an unmarked cytochrome *c*-552 deletion mutant, C552M-2. The mutant strain was derived from *P. alcaliphila* AL15-21^T. μ_{max}, maximum specific growth rate. OD_{max}, maximum cell turbidity. The μ_{max} and OD_{max} were estimated from their growth measured at 660 nm with a model TN-2612 Bio-photometer

6.25 to 25 kV/cm, and 12.5 kV/cm was found to be the optimum electric field strength (Fig. 2). Under optimal conditions (electric field strength of 12.5 kV/cm, capacitance of 25 μF with a single pulse, and resistance of 200 Ω by using a Gene Pulser Xcell), 4.5×10^5 transformants/μg of pHA10 plasmid DNA were reproducibly obtained. In addition, the effect of freezing cells at −80 °C in the solution containing 0.3 M sucrose prior to electroporation was examined. *P. alcaliphila* AL15-21^T cells were washed with the solution containing 0.3 M sucrose and then suspended in the same solution. The transformation efficiency with the pHA10 plasmid was determined for a portion of unfrozen cells. The remainder of the concentrated cells was frozen at −80 °C overnight. The transformation efficiency significantly decreased from 4.5×10^5 to 1.5×10^2 transformants/μg of plasmid DNA. This result indicated that the cells are not suitable for storage at −80 °C.

Growth characteristics and oxygen consumption of *P. alcaliphila* AL15-21^T wild type and cytochrome *c*-552 deletion mutant strains

The above-described results indicated that cytochrome *c*-552 is the major cytochrome *c* among the soluble cytochromes *c* in this microorganism. Therefore, to investigate the physiological role of soluble cytochrome *c*-552 in *P. alcaliphila* AL15-21^T, a cytochrome *c*-552 deletion mutant, AL15-21Δc-552::FRT, was constructed using Gateway cloning technology. The growth characteristics of *P. alcaliphila* AL15-21^T wild type and cytochrome *c*-552 deletion mutant strains were examined. The wild type and deletion mutant strains were grown in PYA medium under different growth conditions. Cell density was monitored at 660 nm. The maximum specific growth rate and maximum cell turbidity of the strains were estimated (Table 4). The maximum specific growth rate and maximum cell turbidity of cells grown at pHs 7.0 and 10.0 under the high-aeration condition did not differ significantly between the wild-type and cytochrome *c*-552 deletion mutant strains. Marked decreases in the maximum specific growth rate and maximum cell turbidity were observed in the deletion mutant grown at pH 10.0 under the low-aeration condition (Fig. 3, Table 4). The maximum specific growth rate and maximum cell turbidity of the deletion mutant decreased by 40% and 25%, respectively, compared with those of the wild type.

The oxygen consumption rate of the *P. alcaliphila* AL15-21^T cell suspension grown in the mid-log growth phase was also determined and is shown in Table 5. The oxygen consumption rate of the wild-type strain did not differ significantly from that of the cytochrome *c*-552 deletion mutant under each experimental condition except in the cells grown at pH 10 under air-limited condition. This suggests that the expression level of cytochrome *c*-551 was highest only in the cells grown at pH 10 under air-limited condition among the tested growth conditions. Therefore, it is considered that the cytochrome *c*-552

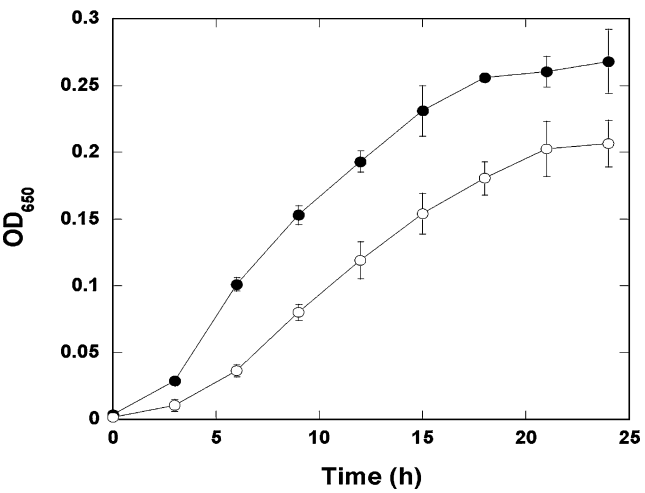

Fig. 3 Time course of growth of *P. alcaliphila* AL15-21^T wild type and cytochrome *c*-552 deletion mutant, C552M-2, strains under the low-aeration condition at pH 10.0. The wild type and the cytochrome *c*-552 deletion mutant are represented as *open circles* and *closed circles*, respectively. The results represent three independent experiments

Table 5 Oxygen consumption rates of cell suspensions of *P. alcaliphila* AL15-21^T wild type and cytochrome *c*-552 deletion mutant, C552M-2. Oxygen consumption rates were exhibited by natom O/min/mg protein

Strain	Growth condition			
	Low aeration		High aeration	
	pH 10.0	pH 7.0	pH 10.0	pH 7.0
Wild type	790±16	751±33	948±53	1030±52
Δ*c*-552	884±27	766±66	1106±69	977±45

Wild type: *P. alcaliphila* AL15-21^T. Δ*c*-552: cytochrome *c*-552 deletion mutant, C552M-2. The mutant strain was derived from *P. alcaliphila* AL15-21^T.Oxygen consumption rate was measured using cells at mid-log phase of growth at 27 °C

deletion mutant retained respiratory activity comparable to that of the wild type because of the presence of the remaining soluble and membrane bound cytochromes *c* in this mutant strain. Consequently, the results indicate that cytochrome *c*-552 is not indispensable in electron transfer in the respiratory chain of *P. alcaliphila* AL15-21^T. The presence of an electron sink, cytochrome *c*-552, may cause a lower oxygen consumption rate of wild type than that of the mutant.

Discussion

To understand the physiological contribution of soluble cytochrome *c*-552 to adaptation under alkaline condition, construction of an unmarked cytochrome *c*-552 deletion mutant strain was attempted using *P. alcaliphila* AL15-21^T. Although during the construction process, the electroporation methods for *Pseudomonas aeruginosa* (Choi et al. 2006; Diver et al. 1990) were adjusted for *P. alcaliphila* AL15-21^T, all the trials to transform strain AL15-21^T were unsuccessful. The adjusted condition for *P. alcaliphila* AL15-21^T was different from that of *P. aeruginosa* in terms of the optimum electric field strength. This may be attributed to the structural difference in around cellular membrane between them.

Although cytochromes *c* may have an important physiological role in bacteria, it has been difficult to observe a pronounced effect of a lack of cytochromes *c* in conventional culture conditions (von Wachenfeldt and Hederstedt 1990; Bengtsson et al. 1999; Braun and Thöny-Meyer 2005). The reasons may be attributed to there being an insufficient expression level difference between the cytochrome *c* deficient mutant and the wild type. The other reason may be attributed to most bacteria having certain adaptation mechanisms that substitute the function of the target cytochrome *c* in the mutant. Noticeable effects due to the lack of cytochrome *c*-552 were observed only in the growth at pH 10 under air-limited condition in terms of growth aspect and oxygen consumption rate. It can be expected that these observations are attributed to the large difference in expression level of cytochrome *c*-552 between the cytochrome *c*-552 deletion mutant and the wild type under the growth condition. It has been found that the alkaliphilic Gram-negative bacterium *P. alcaliphila* AL15-21^T produces a larger amount of soluble *c*-type cytochromes at pH 10.0 under air-limited condition than at the other growth conditions (Matsuno et al. 2007).

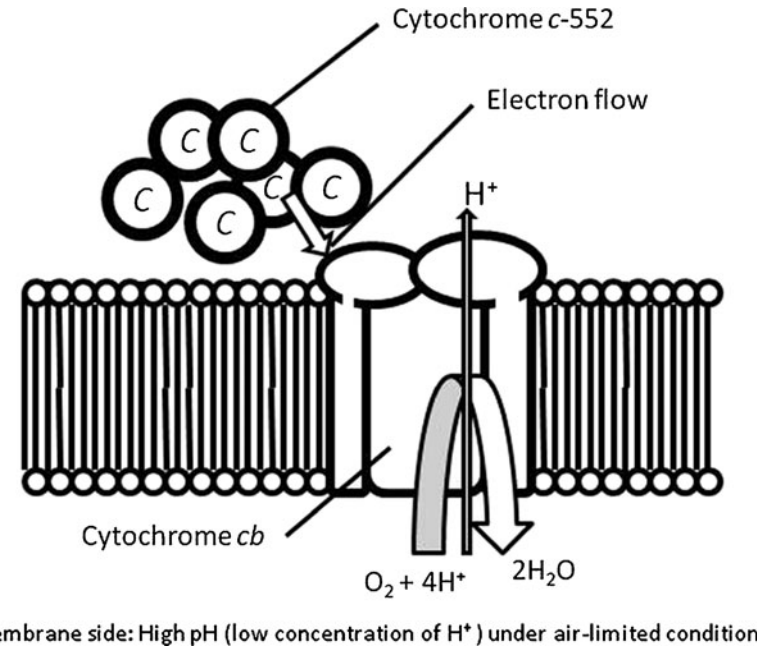

Fig. 4 Hypothetical model of cytochrome *c*-552 function in the terminal oxidation of *P. alcaliphila* AL15-21^T membrane. The function of cytochrome *c*-552 as an electron sink enhances the function of the terminal oxidase, cytochrome *cb*, under air-limited and intramembrane proton-deficient conditions (alkaline condition). Thus, owing to the large electron capacity in the presence of cytochrome *c*-552, cytochrome *cb* is able to translocate protons and reduce oxygen under the growth condition. The hypothetical model shows that cytochrome *c*-552 in large amount in the periplasmic space plays the role of 'condenser' in the terminal respiratory system

On the basis of Mitchell's chemiosmotic theory (Mitchell 1961), it is considered that ATP synthesis under alkaline condition is difficult for microorganisms because the transmembrane pH gradient is reversed (extracellular pH > intracellular pH). It has been reported that the intracellular pH of alkaliphilic *Bacillus* spp. is 2 units lower than the extracellular pH (Krulwich et al. 2007). The intracellular pH increased with extracellular pH (Yumoto 2002). This may mean the avoidance of a big pH difference across the membrane. It is considered that proton translocation from the intramembrane side to the extramembrane side is more difficult in alkaliphiles than in neutralophiles owing to the lower proton concentration in the intramembrane side. A similar aspect in extracellular and intracellular pHs in *P. alcaliphila* AL15-21^T can probably be observed. Therefore, the intracellular pH is probably higher in strain AL15-21^T than in neutralophiles. In general, proton pumping is coupled with electron transfer to the terminal electron acceptor (O_2) in the respiratory chain. Therefore, it means that energy production via the respiratory chain under high-pH and air-limited conditions is difficult if the electron acceptor (O_2) and substrate (proton) are deficient. On the other hand, our previous report indicates the high electron-retaining ability of cytochrome *c*-552 at high pH and that the cytochrome *c*-552 is an electron donor for *cb*-type cytochrome *c* oxidase from *P. alcaliphila* AL15-21^T (Matsuno et al. 2009). Although the oxygen consumption ability of the cells was slightly accelerated by the deletion of the cytochrome *c*-552 gene, the growth of the cytochrome *c*-552 deletion mutant at pH 10.0 under the low-aeration condition was obviously inhibited compared with that of the wild-type strain. The above-mentioned facts suggest that cytochrome *c*-552 plays a role in the maintenance of more appropriate growth at pH 10.0 under the low-aeration condition by facilitating electron flow using the reserved electrons. Cytochrome *c*-552 may manifest an obvious function only in the presence of dual negative factors, i.e., air and intracellular proton limitations, in the respiratory system (Fig. 4). Thus, we hypothesize that cytochrome *c*-552 in a large amount in the periplasmic space plays the function of 'condenser' in the terminal respiratory system. This is the first demonstration of the contribution of cytochrome *c* in the physiology of the alkaliphile under alkaline air-limited conditions.

Although the maximum specific growth rate and maximum cell turbidity of the cells grown at pH 7.0 under either the high- or low-aeration condition did not differ significantly between the wild type and deletion mutant, those of the deletion mutant grown at pH 10.0 were slightly retarded at the initial growth compared with those of the wild type under high-aeration condition (data not shown). This result suggests that the contribution of this cytochrome *c* to adaptation is slightly higher under a high-pH condition than under an air-limited condition. Under a high-pH condition, it is considered that the protons pumped to the extracellular side of the membrane by the respiratory system are important to the ATP synthase. The anionic polymer layer in the cell wall in alkaliphilic *Bacillus halodurans* C-125 is predicted to be equilibrated by cations (Tsujii 2002). The presence of anionic and electron retaining cytochrome *c*-552 (p*I*=4.3) equilibrated by cations (e.g., sodium ion) in the periplasmic space may contribute to the prevention of the diffusion of protons translocated via the respiratory chain in the vicinity of the outer surface membrane.

Moreover, there is a possibility that the cytochrome *c* in the periplasmic space serves as a 'proton condenser' across the membrane to synthesize ATP (Goto et al. 2005). Actually, it has been shown that cytochrome *c* exhibits proton-coupled electron transfer on the basis of the protein 3D structure (Bento et al. 2004; Enguita et al. 2006), results of a Ag electrode experiment using D_2O (Davis and Waldeck 2008; Murgida and Hildebrandt 2001), and thermodynamic and kinetic characterizations (Correia et al. 2004). In addition, it is considered that at above pH 9.5, most of the proton-dissociable side chains in the proton-transferable amino acid residues are deprotonated (becoming low pK_a) and that the protons are transferred from a proton donor to the protonable amino acid residues in the cytochrome *c*. Thus, there is a possibility that cytochrome *c*-552 is able to reserve and supplement protons in the periplasmic space to synthesize ATP. However, the possibility of proton reservation by cytochrome *c*-552 is not always high owing to its localization in the periplasmic space, because it is considered that extramembrane proton transfer occurs in the vicinity of the membrane surface (Antoneko and Pohl 1998; Yoshimune et al. 2010). The clarification of cytochrome *c*-552 function in relation to proton dynamics at the molecular level is necessary to clarify this point.

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