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Relationship between rates of respiratory proton extrusion and ATP synthesis in obligately alkaliphilic *Bacillus clarkii* DSM 8720^T

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Abstract To elucidate the energy production mechanism of alkaliphiles, the relationship between the rate of proton extrusion via the respiratory chain and the corresponding ATP synthesis rate was examined in obligately alkaliphilic *Bacillus clarkii* DSM 8720^T and neutralophilic *Bacillus subtilis* IAM 1026. The oxygen consumption rate of *B. subtilis* IAM 1026 cells at pH 7 was approximately 2.5 times higher than that of *B. clarkii* DSM 8720^T cells at pH 10. The H^+/O ratio of *B. clarkii* DSM 8720^T cells was approximately 1.8 times higher than that of *B. subtilis* IAM 1026 cells. On the basis of oxygen consumption rate and H^+/O ratio, the rate of proton translocation via the respiratory chain in *B. subtilis* IAM 1026 is expected to be approximately 1.4 times higher than that in *B. clarkii* DSM 8720^T. Conversely, the rate of ATP synthesis in *B. clarkii* DSM 8720^T at pH 10 was approximately 7.5 times higher than that in *B. subtilis* IAM 1026 at pH 7. It can be predicted that the difference in rate of ATP synthesis is due to the effect of transmembrane electrical potential ($\Delta\psi$) on protons translocated via the respiratory chain. The $\Delta\psi$ values of *B. clarkii* DSM 8720^T and *B. subtilis* IAM 1026 were estimated as −192 mV (pH 10) and −122 mV (pH 7), respectively. It is considered that the discrepancy between the rates of proton translocation and ATP synthesis between the strains used in this study is due to the difference in ATP production efficiency per translocated proton between the two strains caused by the difference in $\Delta\psi$.

Keywords Alkaliphilic · Respiration · Membrane electrical potential · *Bacillus clarkii* · *Bacillus subtilis* · Proton translocation · ATP synthesis

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Introduction

On the basis of Mitchell's chemiosmotic theory (1961), the H^+ motive force (Δp) for driving ATPase consists of a transmembrane pH gradient (ΔpH: acidic outside and alkaline inside) and transmembrane electrical potential ($\Delta\psi$: negative inside and positive outside). It has been reported that the cytoplasmic pH of alkaliphilic *Bacillus* species is in the range of 7.9–9.6, when they are grown at pHs 10.2–11.4 (Kitada et al. 1982; Sugiyama et al. 1986; Guffanti et al. 1986; Hoffmann and Dimroth 1991; Sturr et al. 1994). Therefore, it has been considered that a reversed transmembrane proton gradient is generated in alkaliphiles. On the other hand, it has been reported that alkaliphilic *Bacillus* species generate a larger transmembrane electrical potential ($\Delta\psi$) than neutralophilic *Bacillus* species (Kitada et al.

1982; Sugiyama et al. 1986; Guffanti et al. 1986; Hoffmann and Dimroth 1991). However, it has been considered that $\Delta\psi$ is not large enough to compensate for the deficiency of ΔpH as long as the pH in the outer surface of the extracellular membrane is the same as that of the medium. Considering the higher growth rate of alkaliphilic *Bacillus* species than those of neutralophilic *Bacillus* species (Guffanti and Hicks 1991), the larger $\Delta\psi$ in alkaliphiles may have a more effective contribution for ATP production than it has been considered or/and the pH in the outer surface of the extracellular membrane is expected to be much lower than that of the medium.

The effect of medium pH on the growth rate of the facultatively alkaliphilic *Bacillus pseudofirmus* strain OF4 has been studied in pH-controlled cultures at various pHs. The generation time for a culture pH of 10.6 (38 min) is shorter than that for pH 7.5 (54 min) (Sturr et al. 1994). On the other hand, the obligately alkaliphilic *Bacillus clarkii* strain K24-1U exhibits a higher specific growth rate (μ_{max}=0.33 h^{-1}) than neutralophilic *Bacillus subtilis* IAM 1026 (μ_{max}=0.26 h^{-1}) cultured in a fermenter at an agitation speed of 106 rpm and an air flow rate of 20 L min^{-1} (Ogami et al. 2009). The above-mentioned experimental data indicate that even though it has been considered that an alkaline condition is disadvantageous for production of energy theoretically, it seems that the growth of alkaliphilic microorganisms utilizes a specific advantageous feature under alkaline condition for their energy production.

Guffanti and Krulwich (1992, 1994) examined ATP synthesis in ADP + P_i-loaded membrane vesicles prepared using *B. pseudofirmus* strain OF4 grown and assayed at pH 10.5 and pH 7.5, and energized with ascorbate (electron donor) *plus* phenazine methosulfate (electron mediator). Although Δp calculated on the basis of the medium pH as the pH of the outer surface of the extracellular membrane is not high enough to synthesize ATP in the vesicles prepared from cells grown and assayed at pH 10.5, the amount of ATP synthesized per minute by vesicles prepared from cells grown and assayed at pH 10.5 is approximately 1.6 times higher than that of vesicles prepared from cells grown and assayed at pH 7.5. Furthermore, respiratory-driven $\Delta\psi$ energizes ATPase more efficiently than an equal extent of diffusion potential in membrane vesicles. These findings suggested that medium pH cannot be used for the calculation of ΔpH and the respiratory-driven translocation of protons has an important role in ATP synthesis at high pHs in alkaliphiles. Therefore, Guffanti and Krulwich (1994) proposed a hypothetical model in which translocated H^+ by respiratory components is directly transferred to the F_0 moiety in ATPase. Indeed, the interaction between cytochrome *c* oxidase and ATPase of *B. pseudofirmus* strain OF4 is demonstrated by electron paramagnetic resonance and differential scanning calorimetry (Liu et al. 2007).

The steady state pH at the outer surface of the extracellular membrane under an active respiratory chain may be different from that of the surrounding the bulk phase. For example, there are reports that the transfer of respiratory-translocated protons along the membrane surface to the channel gate of ATPase occurs much faster than their equilibration with the bulk aqueous phase (Antonenko and Pohl 1998; Georgievskii et al. 2002a, b; Brändé et al. 2006). On the other hand, Mulkidjanian (2006) advocated that the hypothetical proton well generated by $\Delta\psi$ accumulates protons in the outer surface of the membrane. The concept of the proton well is constructed on the basis of the desolvation penalty for transferring protons into the membrane core.

On the basis of the above-mentioned background, Yoshimune et al. (2010) demonstrated that the protons translocated via the respiratory chain to the extracellular side of the membrane are not directly detected in the bulk water phase at the beginning of respiration. Furthermore, it was also demonstrated that the proton retardation at the outer surface of the membrane is attributed to $\Delta\psi$. From these findings, it can be considered that the pH in the vicinity of the outer surface membrane is different from medium pH and alkaliphilic *Bacillus* species produce ATP under alkaline condition. However, the reasons for the superior growth rate and ATP production rate of alkaliphilic *Bacillus* under alkaline conditions remained to be clarified. To clarify the problem, we estimated the rate of respiratory-driven translocation of protons and ATP production rate and examined the predictable factor for the respiratory-driven extrusion of protons ($\Delta\psi$) for accelerating the driving force for production of ATP.

Materials and methods

Organisms and growth conditions

Bacillus clarkii DSM 8720^T (Nielsen et al. 1994, 1995) isolated from soil was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). *Bacillus subtilis* IAM 1026 (=JCM 20014) was obtained from the Institute of the Applied Microbiology (IAM) culture collection of the University of Tokyo. *B. clarkii* DSM 8720^T and *B. subtilis* IAM 1026 were cultured in peptone/yeast extract/alkaline (PYA) broth containing 8 g/l peptone (Kyokuto, Tokyo, Japan), 3 g/l yeast extract (Merck, Darmstadt, Germany), 1 g/l K_2HPO_4, and 100 mM $NaHCO_3$/Na_2CO_3 buffer (pH 10) and 100 mM NaH_2PO_4/Na_2HPO_4 (pH 7), respectively. The inoculum was prepared by overnight culture at 27 °C. Twenty milliliters of the inoculum was transferred into the broth (500 ml) in a 3-L-volume baffled flask and incubated with rotary shaking (120 rpm)

at 27 °C. Cell growth was monitored on the basis of OD_{660} and cells were harvested by centrifugation (6,400×*g*, 20 min, 4 °C) at the late logarithmic phase of growth (OD_{660}=1.0). The harvested cells were washed twice with a solution containing 0.3 M sucrose and buffer whose component depended on the growth pH (pHs 7 and 8, 20 mM NaH_2PO_4/Na_2HPO_4; pHs 9 and 10, 20 mM glycine) and suspended in the same solution. Cells were prepared at least three times to check the reproducibility of results and statistical analysis. All chemicals were purchased from Wako Pure Chemicals unless otherwise stated. They were of the highest grade available and were used without further purification.

Determination of cytochrome content

The cells grown until the early stationary phase were harvested by centrifugation at 6,000×*g* for 20 min at 4 °C. 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, NY) at 18,000 lb in^{-2}, followed by centrifugation at 6,000×*g* for 20 min at 4 °C to remove unbroken cells. The cytochrome content in the cell extract was determined from the difference spectrum of (dithionite-reduced sample spectrum between *minus* ferricyanide-oxidized sample spectrum) using the following millimollar extinction coefficients of cytochromes: cytochrome *a*, $\Delta\varepsilon_{600-615}$=11.7 mM^{-1} cm^{-1} (Ludwig and Schatz 1980); cytochrome *b*, $\Delta\varepsilon_{558-575}$=17.5 mM^{-1} cm^{-1} (Jones and Pool 1985); and cytochrome *c*, $\Delta\varepsilon_{553-537}$= 22.7 mM^{-1} cm^{-1} (Fee et al. 1980). Spectrophotometry was performed using a Cary 100 UV–vis spectrophotometer (Varian, Palo Alto, CA, USA) using a 1-cm-light-path cuvette at room temperature.

Measurement of respiratory rate

Oxygen consumption rate was measured using a galvanic-type oxygen electrode (Iijima Electronics Corporation, Aichi, Japan) in a closed 2-ml-volume glass vessel with magnetic stirring at 25 °C. A solution containing 0.3 M sucrose and buffer whose component was dependent on the growth pH (pH 7, 20 mM NaH_2PO_4/Na_2HPO_4; pH 10, 20 mM glycine) was used as the reaction solution. A concentrated suspension of cells was added to the reaction solution at a final protein concentration of 0.5 mg ml^{-1}. The reaction was started by adding the cell suspension, and the oxygen consumption rate was recorded until the oxygen contained in the suspension was completely consumed. From the reported data on oxygen solubility in water at various temperatures at 1 atmospheric pressure, the oxygen saturation value of the reaction solution in equilibrium with air was calibrated.

Measurement of H^+/O ratio

The extent of pH change induced by an oxygen pulse with resting cells under anaerobic condition was measured in a closed 4-ml-volume vessel at 25 °C, as previously described (Sone and Fujiwara 1991; Yaginuma et al. 1997). The reaction mixture consisted of 140 mM KCl, 0.4 μg ml^{-1} valinomycin and 83 mM potassium thiocyanate (KSCN). The pH of the reaction solution was adjusted to pH 7 using 0.2 N NaOH. The reaction started after the pH was stabilized by adding oxygen-saturated 140 mM KCl. The amount of extruded protons was calculated from the decrease in pH calibrated by adding 5 μl of 10 mM HCl in the reaction mixture. We present the H^+/O ratio of the two strains only at pH 7 because it hardly changes depending on the pH (Yoshimune at al. 2010) and the most precise value can be obtained under this condition.

Measurement of intracellular ATP concentration

Intracellular ATP concentration was determined using a CellTiter-GloTM Luminescent Cell Viability Assay kit (Promega Corp., Madison, WI) according to the manufacturer's instructions using a 4-ml-volume closed chamber at 25 °C. The same reaction solution was used as in the oxygen consumption experiment. The reaction mixture was kept under anaerobic condition, and then the reaction was initiated by the introduction of air. An aliquot of a sample was periodically obtained from the reaction chamber after oxygen was introduced to the chamber. ATP production was stopped using the aliquot of the sample mixed with the reagent in the assay kit. Luminescence produced from the luciferin-luciferase reaction was measured using a Centro LB 960 luminescence meter (Berthold, Bad Wildbad, Germany), ATP solution of known concentration was used for calibration.

Preparation of right-side-out membrane vesicles

B. clarkii DSM 8720^T and *B. subtilis* IAM 1026 were cultured and harvested as described above. The harvested cells were washed with 20 mM NaH_2PO_4/Na_2HPO_4 (pH 7) buffer and suspended in the same buffer. Right-side-out membrane vesicles were essentially prepared according to the method of Kaback (1971). The cell suspension was incubated with 50 mg ml^{-1} lysozyme (Simga) for 10 min at 30 °C, washed with 20 mM NaH_2PO_4/Na_2HPO_4 (pH 7), and resuspended in the same buffer. The suspension was mixed with fifteen times of the volume of 0.9 % (*w/w*) NaCl containing 100 mM KH_2PO_4/K_2HPO_4 (pH 7) buffer and then treated with 30 μg/ml deoxyribonuclease I for 30 min at 30 °C. The solution was pelleted by centrifugation (30,000×*g*, 40 min, 4 °C). The obtained pellet was resuspended in 50 mM Tris–HCl (pH 8.0).

Measurement of transmembrane electrical potential

A reaction solution was prepared at various concentration of potassium ion (1–50 mM KCl) by adding sodium ion (99–50 mM NaCl) to adjust the total concentration of potassium ion *plus* sodium ion to be 100 mM. The buffer in the reaction solution contained 20 mM glycine buffer (pH 10) and 20 mM HEPES/Tris buffer (pH 7) for *B. clarkii* DSM 8720^T and *B. subtilis* IAM 1026, respectively. The standard reaction mixture (3 ml) consisted of 10 μl of 0.1 mM dis-C_3-(5) (Molecular Probes, Eugene, OR, USA), 20 μl of vesicle solution (10–20 mg ml^{-1}), buffer with components as described above, and KCl and NaCl at various concentrations as described above. The diffusion potential of potassium was generated by addition of 1 μl of valinomycin to the reaction mixture. Fluorescence intensity was measured at ambient temperature at an excitation wavelength of 620 nm and emission wavelength of 670 nm to estimate the difference in fluorescence intensity (ΔF) caused by the generation of diffusion potential. The relationship between ΔF (ordinate axis) and potassium concentration (in logarithmic scale) (abscissa axis) outside of the cells was estimated to determine the intercept of the abscissa axis (Δψ=0). It is considered that the potassium ion concentration inside ($[K^+]_{in}$) of the cells is equal to that outside at the intersection point (Δψ=0 point).

The ΔF due to energization by sodium lactate was estimated for the solution containing 10 μl of 0.1 mM dis-C_3-(5), 20 μl of vesicle solution (10–20 mg ml^{-1}), buffer with components as described above, and sodium lactate (final concentration, 10 mM). The difference in fluorescence intensity (ΔF) was estimated when gramicidin (final concentration, 0.3 μM) was added to the reaction solution after the fluorescence intensity was ultimately decreased. The estimated ΔF was considered as reflection of $[K^+]_{out}$ and Δψ was calculated according to the Nernst equation as follows:

$$\Delta\psi = -2.3RT/F \times \log[K^+]_{in}/\log[K^+]_{out},$$

where R (8.31×10^3 Pa K^{-1} mol^{-1}) is the gas constant, T is the absolute temperature (25 °C, 298 K) and F is the Faraday constant (9.65×10^4 C mol^{-1}).

Protein concentration

The protein concentrations of the cell solution and membrane vesicle were determined using a BCA Protein Assay Reagent kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as the standard.

Results

Growth characteristics and cytochrome content

The growth characteristics and cytochrome contents are listed in Table 1. The growth rate and maximum growth turbidity of *B. clarkii* DSM 8720^T cultivated at pH 10 were higher than those of *B. subtilis* IAM 1026 cultivated at pH 7. The contents of cytochromes *b* and *c* in *B. clarkii* DSM 8720^T grown at pH 10 were higher than those in *B. subtilis* IAM 1026 cultivated at pH 7. Thus, better growth characteristics in spite of the presence of a negative bioenergetic parameter, reversed ΔpH, and a higher amount of respiratory chain components in alkaliphilic *B. clarkii* DSM 8720^T than those in *B. subtilis* IAM 1026 were observed.

Oxygen consumption rate

Sucrose was added as the osmotic agent in the oxygen consumption experiment as well as other experiments using whole cells because sucrose prevents cell lysis during the experiments. Sucrose can be used by both *B. clarkii* DSM 8720^T and *B. subtilis* IAM 1026 as a substrate. However, it is considered that the time scale of sucrose metabolism from incorporation to oxygen consumption is too large to contribute to oxygen consumption. The oxygen consumption rate of *B. clarkii* DSM 8720^T cells grown at pH 10 was the highest at a measurement pH of 10 (0.19 μmol O_2/min/mg cell protein) among measurement pHs of 7–10, and was about 2.4 times higher than that at a measurement pH of 7 (0.08 μmol O_2/min/mg cell protein) (Fig. 1a). The obtained data were similar to those obtained using the endogenous substrate compared with those obtained using malate as the substrate for alkaliphilic

Table 1 Growth characteristics, cytochrome contents, respiratory rates, H^+/O, ΔΨ and ATP synthesis rate of *B. clarkii* DSM 8721^T and *B. subtilis* IAM 1026

Strain	Growth pH	μ_{max} (h^{-1})	OD_{max}	Cytochrome content (nmol • mg protein^{-1})			Respiratory rate[a]	H^+/O	ΔΨ	ATP synthesis rate[b]
				a-type	*b*-type	*c*-type				
B. clarkii	pH 10	0.20	1.06	0.12±0.02	0.37±0.01	0.36±0.01	0.19	6.0	−192	7.21
B. subtilis	pH 7	0.17	0.68	0.12±0.02	0.20±0.02	0.21±0.02	0.50	3.4	−122	0.96

[a] Respiratory rate (μmol O_2/min/mg cell protein)

[b] ATP synthesis rate is ATP synthesis rate (nmol ATP/min/mg cell protein)

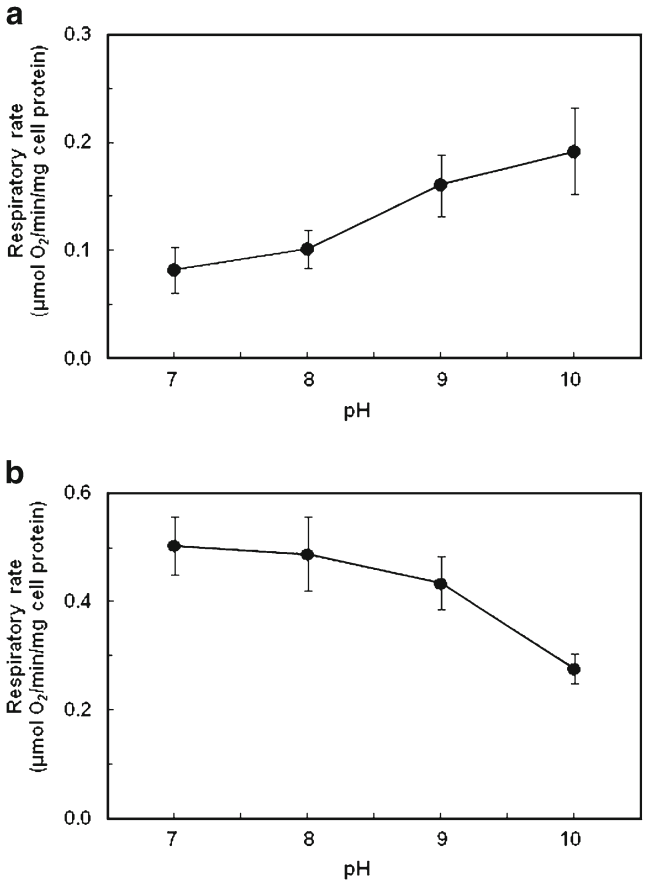

Fig. 1 Respiratory rates of cells of *B. clarkii* DSM 8720^T (**a**) and *B. subtilis* IAM 1026 (**b**). The reaction was started by adding of the cell solution at 25 °C. An air-saturated 20 mM buffer [Na_2HPO_4 (pH7.0 or pH 8.0) and glycine (pH9.0 or 10.0)] and 0.3 M sucrose (1.8 ml) were used for the assay

Bacillus (Lewis et al. 1980; Guffanti et al. 1986; Aono et al. 1996). On the other hand, the oxygen consumption rate of *B. subtilis* IAM 1026 grown at pH 7 was the highest at a measurement pH of 7 (0.5 μmol O_2/min/mg cell protein) among measurement pHs of 7–10 and was about 1.8 times higher than that at a measurement pH of 10 (0.28 μmol O_2/min/mg cell protein; Fig. 1b). The optimum pHs for oxygen consumption rates of *B. clarkii* DSM 8720^T and *B. subtilis* IAM 1026 were in accordance with growth pHs. The oxygen consumption rate of whole cells of *B. subtilis* IAM 1026 at pH 7 grown using the endogenous substrate was approximately 2.5 times higher than that of *B. clarkii* DSM 8720^T grown at pH 10.

H^+/O ratio

To evaluate the efficiency of proton translocation via the respiratory chain (per consumed proton), the endogenous respiration dependent H^+/O ratio was measured by the oxygen pulse method. *B. clarkii* DSM 8720^T exhibited a ratio of 6.0. It has been reported that NADH dehydrogenases in Gram-positive bacteria do not translocate protons (Hisae et al. 1983; Xu et al. 1991; Liu et al. 2008). Therefore, the theoretical H^+/O ratio in *Bacillus* species is 3 (complex III, 2 H^+/O; *plus* complex IV, 1 H^+/O). Hence, it is considered that the proton translocation efficiency of the respiratory chain of alkaliphilic *B. clarkii* DSM 8720^T is higher than that of the theoretical value of *Bacillus* species (e.g., *B. subtilis*). On the other hand, *B. subtilis* IAM 1026 exhibited a H^+/O ratio of 3.4, which is comparable to the theoretically predicted ratio. The proton translocation via the respiratory chain of alkaliphilic *B. clarkii* DSM 8720^T was characterized by its efficiency rather than its turnover frequency. It can be predicted that the efficiency of respiratory proton extrusion related to the difference in the respiratory chain component between *B. clarkii* DSM 8720^T and *B. subtilis* IAM 1026. From the obtained H^+/O ratios and oxygen consumption rates, the rate of translocation of protons via the respiratory chain in *B. subtilis* IAM 1026 was determined to be approximately 1.4 times higher than that of *B. clarkii* DSM 8720^T.

ATP synthesis

From the obtained oxygen consumption rates and H^+/O ratios, the rate of proton extrusion from the intracellular to extracellular membrane can be determined. To examine whether the rate of proton translocation reflects ATP production, ATP production rate was determined. *B. clarkii* DSM 8720^T produced 7.2 nmol ATP/mg protein/min at pH 10 (Fig. 2), which is comparable to that of *Bacillus pseudofirmus* OF4 (6.6±3.9 nmol ATP/mg protein/min [starved cells re-energized with malate]) at pH 10.5 (Guffanti and Krulwich 1992). On the other hand, *B. clarkii* DSM 8720^T produced only 0.3 nmol ATP/mg protein/min at pH 7. *B. subtilis* IAM 1026 produced 0.96 and 0.22 nmol ATP/mg

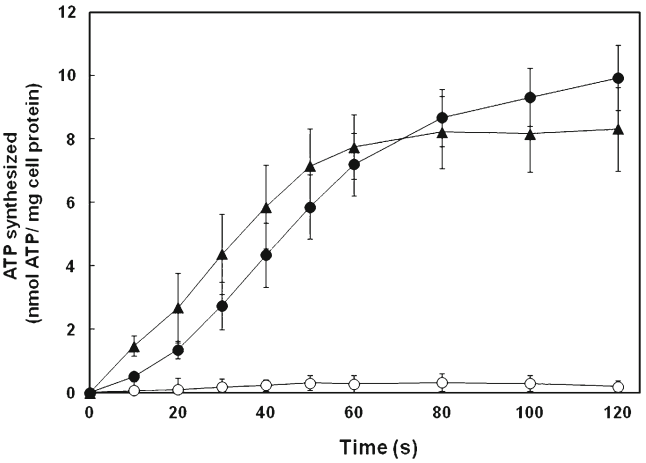

Fig. 2 ATP synthesis of *B. clarkii* DSM 8720^T grown at pH 10. The reaction was performed at 25 °C. pH 7 (*open circle*), 20 mM NaH_2PO_4/Na_2HPO_4; pH 9 (*closed triangle*) and pH 10 (*closed circle*), 20 mM glycine

protein/min at pH 7 and pH 10, respectively (Fig. 3). These results demonstrated that although the proton translocation rate based on the oxygen consumption rate and H^+/O ratio was higher in *B. subtilis* IAM 1026 (pH 7) than in *B. clarkii* DSM 8720^T (pH 10), the rate of ATP produced by *B. clarkii* DSM 8720^T (pH 10) was much higher than that produced by *B. subtilis* IAM 1026 (pH 7). This suggests that protons translocation via the respiratory chain in *B. clarkii* DSM 8720^T exhibited a much higher efficiency than that in *B. subtilis* IAM 1026 to drive the ATPase.

Transmembrane electrical potential (Δψ)

The above-mentioned results suggest that respiratory translocated protons in *B. clarkii* DSM 8720^T exhibit much higher ATPase-driving efficiency than those in *B. subtilis* IAM 1026. It can be predicted that this difference is due to the effect of transmembrane electrical potential (Δψ) on the respiratory translocated protons. Therefore, we attempted to estimate Δψ of *B. clarkii* DSM 8720^T and *B. subtilis* IAM 1026. The determined Δψ of *B. clarkii* DSM 8720^T was determined as −192 mV (pH 10), which is comparable to those of other alkaliphilic *Bacillus* species (−181 mV–−213 mV at pHs 10.2–10.5) (Kitada et al. 1982; Sugiyama et al. 1986; Guffanti et al. 1986; Hoffmann and Dimroth 1991). On the other hand, Δψ of *B. subtilis* IAM 1026 was determined as −122 mV (pH 7). Although the procedure in the present study is different from that in a previous study, Δψ values of *B. subtilis* obtained in both studies are comparable [Shioi et al. 1980 (approximately −130 mV at pH 7)]. The results indicate that Δψ of alkaliphilic *B. clarkii* DSM 8720^T is higher than that of neutralophilic *B. subtilis* IAM 1026. It can be assumed that the difference in Δψ between *B. clarkii* DSM 8720^T and *B. subtilis* IAM 1026 is the main factor for the difference in the efficiency of translocated protons as the driving force of the ATPase between these two strains.

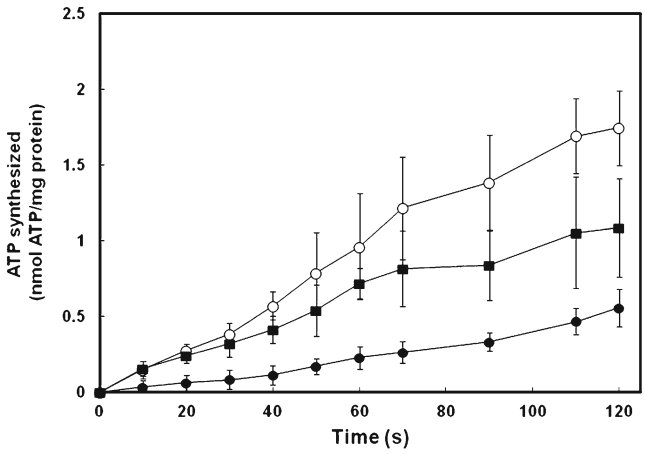

Fig. 3 ATP synthesis of *B. subtilis* IAM 1026 grown at pH 7. The reaction was performed at 25 °C. pH 7 (*open circle*) and pH 8 (*closed square*), 20 mM NaH_2PO_4/Na_2HPO_4; pH 10 (*closed circle*), 20 mM glycine

Discussion

It has been reported that although alkaliphiles can grow under bioenergetically adverse condition, the growth yield of alkaliphiles is higher than that of the neutralophilic *B. subtilis* (Guffanti and Hicks 1991). From our results, the growth characteristics of *B. clarkii* DSM 8720^T and *B. subtilis* IAM 1026 (Table 1) are comparable to those previously reported results in alkaliphiles and neutralophiles (Guffanti and Hicks 1991; Ogami et al. 2009). These findings suggest that alkaliphilic *B. clarkii* DSM 8720^T possesses certain features advantageous for energy production rather than the presence of a disadvantage in terms of ΔpH in their physiological mechanism. To estimate the relationship between respiratory factors and ATP production rate, cytochrome content, respiratory rate, H^+/O and ATP production rate were determined using *B. clarkii* DSM 8720^T and *B. subtilis* IAM 1026. The growth characteristics,

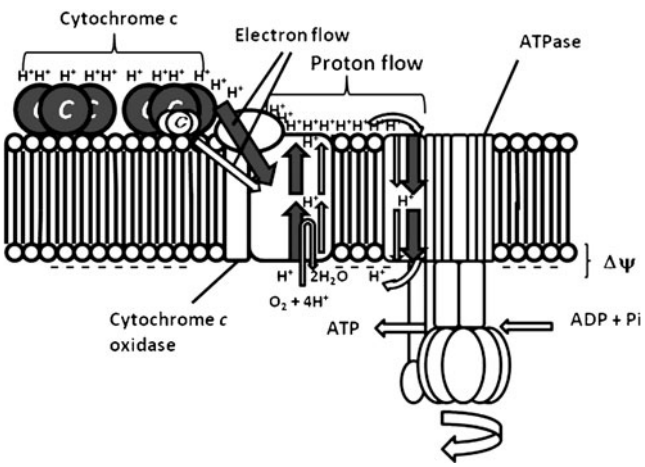

Fig. 4 Hypothetical model of terminal oxidation and ATP production of alkaliphilic *B. clarkii* DSM 8720^T (pH 10) compared with that of neutralophilic *B. subtilis* IAM 1026 (pH 7) in steady state. Proton translocation in cytochrome *c* oxidase occurs under high Δψ. Thus, translocated protons at the outer surface membrane generate a higher potential to drive ATPase than those produced under ordinary Δψ. It is speculated that this energy production is supported by the following factors: (i) A large difference in midpoint redox potential exists between cytochrome *c* and the electron acceptor in cytochrome *c* oxidase, cytochrome *a*. (ii) In addition, cytochrome *c* has a role as an electron reserve (i.e., condenser) in the facilitation of proton transfer across the membrane or on the outer membrane surface rather than to facilitate electron transfer via the respiratory chain. (iii) Proton trapping at a high Δψ in the vicinity of the outer surface of the membrane. Filled and open arrows represent *B. clarkii* DSM 8720^T and *B. subtilis* IAM 1026, respectively. (iv) The negatively charged membrane-anchored cytochrome *c* attracts protons translocated by respiratory complexes and prevents proton diffusion to the bulk

cytochrome contents, bioenergetic parameters, rate of ATP synthesis and transmembrane electrical potential determined in this study are summarized in Table 1. Higher contents of cytochromes *b* and *c* in *B. clarkii* DSM 8720^T than in *B. subtilis* IAM 1026 do not facilitate a higher respiratory rate. In addition, *B. clarkii* DSM 8720^T exhibited an efficient proton extrusion via the respiratory chain rather than by the high turnover of respiratory rate. This may mean that these cytochromes function as an electron reserve (i.e., condenser) in the facilitation of proton transfer across the membrane or on the outer membrane surface rather than in the facilitation of electron transfer via the respiratory chain. This function of cytochrome *c* has also been reported in the study of alkaliphilic *Pseudomonas alcaliphila* (Matsuno et al. 2012). On the other hand, acidic membrane-anchored cytochromes *c* have been isolated from alkaliphilic *Bacillus* species (Hicks and Krulwich 1995; Quirk et al. 1993; Ogami et al. 2009). It is considered that the presence of acidic cytochromes *c* may attract protons translocated by the respiratory complexes.

Although the rate of proton translocation via the respiratory chain in *B. subtilis* IAM 1026 was higher than that in *B. clarkii* DSM 8720^T, the ATP production rate in *B. clarkii* DSM 8720^T was much higher than that in *B. subtilis* IAM 1026. It is considered that the high $\Delta\psi$ observed in *B. clarkii* DSM 8720^T may account for the higher production rate of ATP per proton extruded to the outer surface of the membrane in this strain than in *B. subtilis* IAM 1026. Indeed, it has been reported that alkaliphiles have a higher $\Delta\psi$ than neutralophilic bacteria. However, it has been considered that a large $\Delta\psi$ cannot compensate for the deficiency of bulk-based reversed ΔpH on the basis of the chemiosmotic theory (Krulwich and Guffanti 1989; Guffanti and Krulwich 1994). In our previous study, we observed that at the beginning of respiration, the respiratory-extruded protons are retained in the vicinity of the outer surface of the membrane (Yoshimune et al. 2010). This proton-retaining force is due to $\Delta\psi$ that formed across the membrane. In addition, the present study demonstrated the high $\Delta\psi$ of the alkaliphile used in this study, as previously reported for other alkaliphilic *Bacillus* species. The existence of protons at the outer surface of the membrane indicates that the deficiency of bulk-based reversed ΔpH (inside, acidic; outside, basic) is no longer a disadvantage for ATP production. In the chemiosmotic theory, proton motive force (Δp) is the sum of ΔpH and $\Delta\psi$. Indeed, our previous finding suggests that $\Delta\psi$ contributes to the retention of protons at the outer surface of the membrane. This means that $\Delta\psi$ contributes to the formation of ΔpH in the vicinity of the outer surface of the membrane. Hence, a larger $\Delta\psi$ probably has a greater contribution to Δp than previously considered.

It is known that the addition of $\Delta\psi$-disrupting agents such as valinomicin *plus* K^+ increases the respiration rate (Papa et al. 1983). It has also been reported that ΔpH and $\Delta\psi$ inhibit interelectron transfer in cytochrome *c* oxidase (Gregory and Ferguson-Miller 1989). It can be predicted that the reversed ΔpH in alkaliphiles accelerates proton transfer at the initiation of the proton translocation. However, it is considered that this advantage will not continue owing to the presence of protons in the vicinity of the extracellular membrane (Yoshimune et al. 2010). On the other hand, a high $\Delta\psi$ in alkaliphiles inhibits both electron transfer from the extracellular side toward the intracellular side of the membrane and proton translocation from the intracellular side to the extracellular side of the membrane (owing to the cationic charge) in the respiratory chain. The comparison between *B. clarkii* DSM 8720^T and *B. subtilis* IAM 1026 in terms of rates of proton translocation and ATP production suggests the difference in the efficiency of proton translocation between these two strains. This difference could be explained by the observation that although proton translocation under high $\Delta\psi$ requires a high energy, translocated protons on the extracellular side of the membrane generate a higher potential to drive the ATPase than those produced under ordinary $\Delta\psi$ (Fig. 4). It is considered that for proton translocation by overcoming a high $\Delta\psi$, a larger redox potential difference between electron transfer components on the outer membrane side and those on the inner membrane side is necessary (Yumoto 2002; Goto et al. 2005).

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