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Contribution of intracellular negative ion capacity to Donnan effect across the membrane in alkaliphilic *Bacillus* spp.

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Abstract To elucidate the energy production mechanism of alkaliphiles, the relationship between the H^+ extrusion rate by the respiratory chain and the corresponding ATP synthesis rate was determined in the facultative alkaliphile *Bacillus cohnii* YN-2000 and compared with those in the obligate alkaliphile *Bacillus clarkii* DSM 8720^T and the neutralophile *Bacillus subtilis* IAM 1026. Under high aeration condition, much higher ATP synthesis rates and larger $\Delta\psi$ in the alkaliphilic *Bacillus* spp. grown at pH 10 than those in the neutralophilic *B. subtilis* grown at pH 7 were observed. This high ATP productivity could be attributed to the larger $\Delta\psi$ in alkaliphiles than in *B. subtilis* because the H^+ extrusion rate in alkaliphiles cannot account for the high ATP productivity. However, the large $\Delta\psi$ in the alkaliphiles could not be explained only by the H^+ translocation rate in the respiratory chain in alkaliphiles. There is a possibility that the Donnan effect across the membrane has the potential to contribute to the large $\Delta\psi$. To estimate the contribution of the Donnan effect to the large $\Delta\psi$ in alkaliphilic *Bacillus* spp. grown at pH 10, intracellular negative ion capacity was examined. The intracellular negative ion capacities in alkaliphiles grown at pH 10 under high aeration condition corresponding to their intracellular pH (pH 8.1) were much higher than those in alkaliphiles grown under low aeration condition. A proportional relationship is revealed between the negative ion capacity and $\Delta\psi$ in alkaliphiles grown under different aeration conditions. This relationship strongly suggests that the intracellular negative ion capacity contributes to the formation of $\Delta\psi$ through the Donnan effect in alkaliphilic *Bacillus* spp. grown at pH 10.

Keywords $\Delta\psi$ · Negative ion capacity · Alkaliphile · Donnan effect

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Introduction

Although there is much difference between alkaliphiles and neutralophiles in terms of their ability to synthesize ATP under alkaline environments, alkaliphiles are not phylogenetically distant from neutralophiles. Alkaliphiles exhibit diverse phylogenetic distributions in phylogeny based on their 16S rRNA gene sequences and they form several alkaliphilic clusters (Yumoto 2007; 2011). Alkaliphiles are distributed in not only specific environments such as alkaline soda lakes but also ordinary environments such as garden soil (Horikoshi 2011; Yumoto 2007; 2011). On the basis of their diverse distributions in both environments and phylogeny, it is predicted that the alkaline adaptation mechanisms of alkaliphiles are diverse between species. Actually, there are many differences between alkaliphilic *Bacillus* spp. in terms of their components related to their environmental adaptation, such as cytochrome contents (Yumoto et al. 1997) and cell wall components (Aono and Horikoshi 1983). It is also predicted that metabolic parameters such as ATP synthesis and oxygen consumption rates

differ depending on the species or strains. Considering these facts and predictions, alkaliphilic bacteria do not adapt to extreme environments by markedly changing their metabolic properties and synthesizing extraordinary special cell components but by accumulating modifications in their metabolic properties and cell components, as shown by studies on the adaptation mechanisms of alkaliphiles. The production of acidic molecules such as the teichurono peptide in the cell wall of alkaliphilic *Bacillus* strains has been studied (Aono et al. 1999). The formation of negatively charged substances contributes to the formation of a negatively charged extracellular cell surface, which attracts H^+ to the cell surface. Tsujii (2002) determined that the cell surface negative charge makes the pH of the periplasmic space (Matias and Beveridge 2005) lower than the extracellular pH (=medium pH). Furthermore, solute transport systems for the Na^+ cycle instead of the H^+ cycle under low H^+ condition have been studied (Kitada et al. 2000; Ito et al. 2004; Padan et al. 2005). It is considered that these transport systems contribute to the adjustment of the pH difference across the membrane and to the creation of a system for the reduction of the frequencies of H^+ usage under H^+-deficient conditions.

Energy production is a basic function of organisms. However, the mechanisms have not yet been completely clarified. It is possible to contribute to a deeper understanding of bioenergetic mechanisms by studying them from the viewpoint of alkaliphiles. Therefore, the bioenergetic problem is one of the most intriguing issues in the study of alkaliphiles. On the basis of Mitchell's chemiosmotic theory (Mitchell 1961), it is considered that the formation of a proton motive force (Δp) to drive F_1F_0-ATP synthase is difficult because of the deficiency in the proper direction of the transmembrane pH gradient (ΔpH) in the bulk base (medium pH). If the outer membrane surface can retain translocated H^+ by the respiratory chain and the pH in the vicinity of the outer membrane surface is not affected by the medium pH, it will not be difficult for neutralophilic bacteria to produce energy by driving the F_1F_0-ATP synthase even under alkaline conditions. Actually, neutralophiles such as *Bacillus subtilis* cannot grow at a medium whose pH is above 10. Therefore, it is considered that the pH at the outer membrane surface is affected by the medium pH, and certain strategies are necessary to retain H^+ in the vicinity of the outer membrane surface. It has been reported that diverse membrane protein segments just outside the membrane have markedly reduced amounts of basic amino acids (Krulwich et al. 2007). If there is no difficulty in accumulating enough H^+ for energy production, the characteristics of the outer surface protein segment should be similar to those of the neutralophiles.

During energy production under bioenergetically adverse conditions, the transmembrane electrical potential ($\Delta\psi$) across the membrane also plays a very important role in retaining H^+ in the vicinity of the outer membrane surface. We observed a lag time in the extrusion of H^+ by the respiratory complex at the beginning of respiration by monitoring the pH change in the bulk space at pH 10 (Yoshimune et al. 2010). This suggests that certain amounts of H^+ translocated by the respiratory complex are retained at the outer membrane surface. However, the lag time in the extrusion of H^+ disappeared following the addition of valinomycin, which eliminates the transmembrane electrical potential ($\Delta\psi$). The experiment indicated that the transmembrane electrical potential ($\Delta\psi$) contributes to the retention of H^+ translocated by the respiratory complex.

The larger $\Delta\psi$ in the alkaliphile than in the neutralophile has been reported (Krulwich 1995; Dimroth and Cook 2004; Yumoto 2003; Goto et al. 2005; Hirabayashi et al. 2012). However, its significance to alkaline adaptation and the basis of the high value have not been determined. The extraordinarily higher ATP synthesis rate in alkaliphiles could be attributed to the high $\Delta\psi$ because the H^+ extrusion rate cannot account for the high ATP synthesis rate in alkaliphiles. It is considered that alkaliphiles have a high rate of ATP synthesis per H^+ rather than frequency for the high ATP synthesis rate (Hirabayashi et al. 2012). However, we still do not know the fundamental mechanisms for achieving a larger $\Delta\psi$ in alkaliphiles. If the larger $\Delta\psi$ in alkaliphiles is spontaneously created, a possible mechanism for the creation is the Donnan effect (Donnan 1924). The Donnan effect is observed when charged particles unable to pass through a semipermeable membrane create an uneven electrical charge. In the present study, we focus on the possibility that the Donnan effect across the membrane has a potential to contribute to the large $\Delta\psi$. To estimate the contribution of the Donnan effect to the large $\Delta\psi$ in alkaliphilic *Bacillus* spp. grown at pH 10, the intracellular negative ion capacity was examined.

In this study, we determined bioenergetic parameters in the facultative alkaliphilic *B. cohnii* YN-2000, the obligate alkaliphilic *B. clarkii* DSM 8721^T and the neutralophilic *B. subtilis* IAM 1024. Much higher ATP synthesis rates and $\Delta\psi$ in the alkaliphilic *Bacillus* spp. than those in the neutralophilic *B. subtilis* grown were observed. It was considered that this high ATP productivity was attributed to the larger $\Delta\psi$ in alkaliphiles than in *B. subtilis*. However, the large $\Delta\psi$ in the alkaliphiles could not be explained only by the H^+ translocation rate in the respiratory chain in alkaliphiles. To understand the contribution of intracellular negative ion capacity to the formation of $\Delta\psi$, we determined negative ion capacity and $\Delta\psi$ in the alkaliphilic strains grown under different aeration conditions. On the basis of the obtained results, we discussed the possibility of the contribution of the intracellular negative ion capacity (which may contribute to the Donnan potential) to the formation of a larger $\Delta\psi$ (diffusion potential *plus* Donnan potential [Helfferich 1962; Lakshminarayanaiah 1969]) in alkaliphiles.

Materials and methods

Organisms and growth conditions

Facultative alkaliphilic *Bacillus cohnii* YN-2000 (Ohta et al. 1975; Yumoto et al. 2000), which can grow at not only alkaline pH but also neutral pH, isolated from an indigo ball was kindly provided by Drs. Y. Nosoh and N. Koyama. Obligate alkaliphilic *Bacillus clarkii* DSM 8720^T (Nielsen et al. 1994, 1995), which cannot grow at neutral pH, isolated from soil was obtained from DSMZ, Germany. Neutralophilic *Bacillus subtilis* IAM 1026 (=JCM 20014) was obtained from IAM, Japan (the IAM culture collection has been transferred to JCM). *B. clarkii* DSM 8720^T and *B. cohnii* YN-2000 were cultured in peptone/yeast extract/alkaline (PYA) broth containing 8 $g \cdot L^{-1}$ peptone (Kyokuto, Tokyo, Japan), 3 $g \cdot L^{-1}$ yeast extract (Merck, Darmstadt, Germany), 1 $g \cdot L^{-1}$ K_2HPO_4 and 100 mM $NaHCO_3/Na_2CO_3$ buffer (pH 10). The alkaline buffer solution was separately sterilized. *B. subtilis* was cultured in peptone/yeast extract/neutral (PYN) broth containing 8 $g \cdot L^{-1}$ peptone (Kyokuto, Tokyo, Japan), 3 $g \cdot L^{-1}$ yeast extract (Merck, Darmstadt, Germany), 1 $g \cdot L^{-1}$ K_2HPO_4 and 100 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7). Culture was performed with rotary shaking (60 or 120 rpm) at 27 °C. Cells were harvested by centrifugation (6400×*g*) for 20 min at 4 °C at the late logarithmic phase. The harvested cells were washed with a solution containing 0.3 M sucrose and 20 mM NaH_2PO_4/Na_2HPO_4 buffer for pHs 7 and 8 or 20 mM glycine buffer for pHs 9 and 10 and suspended at the same pH buffer depending on the measurement condition. All chemicals were purchased from Wako Pure Chemicals unless otherwise stated.

Oxygen consumption rate and determination of cytochrome *c* content

The oxygen consumption of the whole cells using endogenous substrates was measured using a galvanic-type oxygen electrode MD-1000 (Iijima Electronics Corporation Aichi, Japan) in a closed 2-ml-volume glass vessel with magnetic stirring at 25 °C. The reaction was started by adding the prepared cell suspension as described above. The reaction was performed in 20 mM glycine buffer (pH 10) and 20 mM sodium phosphate buffer (pH 7) for alkaliphiles and neutralophile, respectively. The cytochrome *c* content was determined as previously described (Ogami et al. 2009).

Measurement of H^+/O ratio

The ratio of the extent of extruded H^+ to oxygen consumption in the respiratory chain was measured using resting cells under anaerobic condition with an oxygen pulse. The reaction was measured in a 4-ml-volume vessel at 25 °C, as previously described (Sone and Fujiwara 1991; Yaginuma et al. 1997). The reaction mixture consisted of the cell suspension (2–4 mg of dry cells), 0.25 mM MOPS-KOH, 140 mM KCl, 83 mM KSCN and 0.4 μg ml^{-1} valinomycin. The reaction was performed in 20 mM glycine buffer (pH 10) and 20 mM sodium phosphate buffer (pH 7) for alkaliphiles and neutralophile, respectively.

Measurement of transmembrane electrical potential (Δψ)

The fluorescent probe 3′, 3′-dipropyl-thiadicarbocyamine [DiS-C_3-(5)] (Molecular Probes, Eugene, OR, USA) was used for Δψ measurement using right-side-out membrane vesicles (Suzuki et al. 2003). The preparation of the right-side-out membrane vesicles and reaction buffer and the calculation of Δψ were performed as described previously (Hirabayashi et al. 2012). The membrane vesicles were energized by 10 mM sodium lactate for Δψ measurement. The reaction was performed in 20 mM glycine buffer (pH 10) and 20 mM HEPES/Tris buffer (pH 7) for alkaliphiles and neutralophile, respectively. Fluorescence intensity, which is caused by Δψ generated by energized vesicles, and diffusion potential triggered by valinomycin were measured at ambient temperature using a Hitachi F-2000 fluorescence spectrophotometer. From the relationship between the fluorescence intensity change and the extracellular K^+ concentration, the intracellular and extracellular K^+ concentrations were determined. On the basis of these concentrations, Δψ was calculated using the Nernst equation.

ATP synthesis

ATP synthesis using endogenous substrates was determined periodically using a CellTiter-GloTM Luminescent Cell Viability Assay kit (Promega, Madison, WI) according to the manufacturer's instruction. The ATP-producing reaction was performed in a 4-ml-volume cuvette using 20 mM glycine buffer (pH 10) and 20 mM sodium phosphate buffer (pH 7) for alkaliphiles and neutralophile, respectively. The cell suspension was initially kept under anaerobic condition at 25 °C, and then the reaction was initiated by the introduction of air. Luminescence produced by the luciferin-luciferase reaction was measured using a Centro LB 960 luminescence meter (Berthold, Bad Wildbad, Germany).

Measurement of intracellular pH

Intracellular pH was determined by the method of Aono et al. (1997). The bacterial cells were loaded with the pH-sensitive fluorescent probe 2′, 7′-bis-(2-carboxyethyl)-5 (and −6)-carboxyfluorescein (BCECF) as described previously (Aono et al. 1997). The suspension of BCECF-loaded cells (20 μl) was added to 10 ml of 0.1 M TES buffer (pH 7.0–8.5) or

0.1 M glycine buffer (pH 8.5–10.5) containing 20 μM gramicidin. Each cell suspension was measured with the excitation wavelengths of 450 and 510 nm and the emission wavelength of 535 nm using an F-2000 fluorescence spectrophotometer (Hitachi). The correlations between the intracellular pH and the fluorescence intensity at 510 nm (F_{510}) and the fluorescence ratio $F_{510/450}$ were determined. On the basis of the correlations, the intracellular pH was calculated under each extracellular pH condition.

Colloidal titration with clupein

To estimate the intracellular negative ion capacity, a cell extract containing inside-out membrane vesicles was prepared. After the bacterial strains were cultivated as described above, the cells were harvested by centrifugation at 6400×*g* for 20 min at 4 °C. The harvested cells were washed with buffers: pH 6, 250 mM succinate buffer; pH 7, 250 mM NaH_2PO_4/Na_2HPO_4 buffer; pH 8, 250 mM Tris–HCl; pHs 9 and 10, 250 mM $NaHCO_3$/Na_2CO_3 buffer in accordance with the pH measured, and the cells were suspended in the same buffer. The cell suspension was passed through a French pressure cell (SLM-Aminco Instruments, Inc., Rochester, NY) at 18,000 lb/in^2, followed by centrifugation at 6400×*g* for 20 min at 4 °C to remove unbroken cells.

To estimate the negative ion capacity, colloidal titration was carried out using clupein sulfate (Terayama 1962). The cell suspension prepared in each buffer (pHs 6–10) was adjusted to 0.5 mg protein/ml. One milliliter of this cell suspension was mixed with 1 ml of 0.183 % clupein sulfate and the mixture was immediately centrifuged at 3000×*g* for 15 min. The amount of remaining clupein in the supernatant was titrated with 0.663 mM sodium polyvinylsulfate. Experiments on samples for each pH were performed three times and the average was obtained.

Concentrations of vesicle and cell suspensions

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

Results

Oxygen consumption rate and cytochrome *c* content

To understand the contribution of the working rate of the respiratory chain to the formation of Δψ, we determined the oxygen consumption rate (turnover rate) and H^+/O (efficiency) ratio. In the present study, we determined the working rate of the respiratory chain using an endogenous substrate. The oxygen consumption rate of *B. cohnii* YN-2000 cells grown at pH 10 was the highest at a measurement pH of 10 (0.20 ± 0.03 μmol $O_2 \cdot min^{-1} \cdot$ mg cell protein) among measurement pHs of 7–10, and was 3.3 times higher than that at the measurement pH of 7 (0.06 ± 0.02 μmol $O_2 \cdot min^{-1} \cdot$ mg cell protein^{-1}) (Fig. 1). The data were similar to those obtained in *B. clarkii* DSM 8720^T (Table 1; Hirabayashi et al. 2012). The obtained rate is also similar to the data of other alkaliphilic *Bacillus* spp. using the endogenous substrate. These obtained data in facultative and obligate alkaliphilic *Bacillus* spp. measured at pH 10 were lower than that obtained in neutralophilic *B. subtilis* IAM 1026 measured at pH 7 (0.50 ± 0.06 μmol $O_2 \cdot min^{-1} \cdot$ mg cell protein^{-1}). These data suggest that although alkaliphilic *Bacillus* spp. tend to grow more rapidly than neutralophilic *Bacillus* spp., the respiratory rate of alkaliphiles is lower than that of neutralophiles. The cytochrome *c* content of the facultative alkaliphilic *B. cohnii* YN-2000 was determined to be 0.62 ± 0.01 nmol · mg protein^{-1} (Table 1), which was higher than that of the obligate alkaliphile *B. clarkii* DSM 8720^T (0.36 ± 0.01 μmol · mg protein^{-1}).

H^+/O ratio

To evaluate respiration efficiency by means of H^+ translocation per electron flow, H^+/O ratio was measured on the basis of an endogenous substrate depending on respiration. The alkaliphiles *B. cohnii* YN-2000 and *B. clarkii* DSM 8720^T exhibited the H^+/O ratios of 3.5 ± 0.7 and 3.6 ± 0.4, respectively. These ratios are lower than that of the neutralophile *B. subtilis* (5.2 ± 0.2). It has never been reported that *Bacillus* spp. have NADH dehydrogenases that translocate protons. There were several reports on the NADH dehydrogenases that do not translocate protons in the alkaliphilic *Bacillus* spp. (Hisae et al. 1983; Xu et al. 1991; Liu et al. 2008). According to the respiratory complex components, the theoretical H^+/O ratio should be around 6 on the basis of 4 of complex III plus 2 of complex IV (Sone et al. 1999). Therefore, it is considered that the H^+/O ratios of the alkaliphilic *Bacillus* spp. are lower than the theoretical value in the case of neutralophilic *Bacillus* spp. The difference may be related to the functional difference in the respiratory chain or differences in the components or quantity of the respiratory chain.

ATP synthesis

If we can consider that the respiratory extruded H^+ in *B. subtilis* is equivalent to that in the alkaliphilic *Bacillus* spp., the ATP synthesis rate could be predicted on the basis of the oxygen consumption rate and H^+/O ratio. In the case of *B. subtilis* and *B. cohnii* YN-2000 at pHs 7 and 10, respectively, the oxygen consumption rate of *B. subtilis* IAM 1026 is

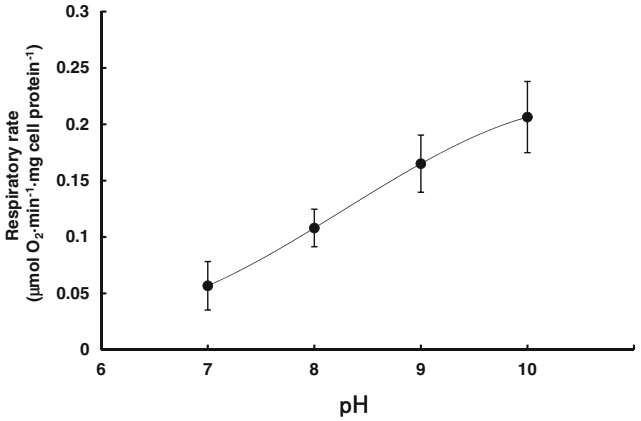

Fig. 1 Changes in respiratory rates of whole cells of facultative alkaliphilic *B. cohnii* YN-2000 depending on pH. The reaction was started by adding the cell solution to the buffer maintained at 25 °C. An air-saturated 20 mM buffer [Na_2HPO_4 (pH 7.0 or 8.0), glycine (pH 9.0 or 10.0)] and 0.3 M sucrose (1.8 ml) were used for the assay. Data represent the means of at least 3 independent replicates and bars are the standard deviations

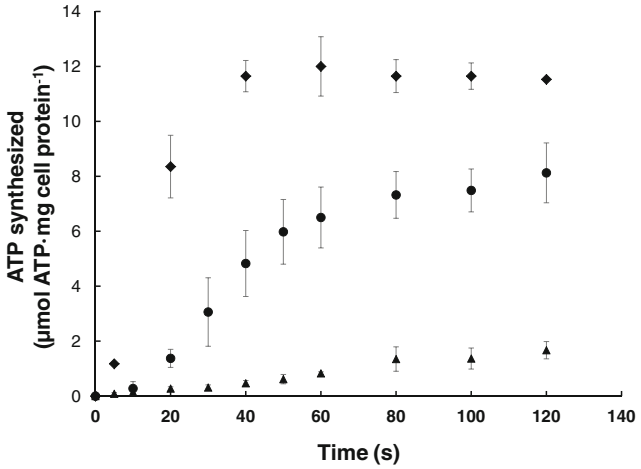

Fig. 2 Changes in ATP synthesis of whole cells of facultative alkaliphilic *B. cohnii* YN-2000 (pH 10; *closed circles*) and obligate alkaliphilic *B. clarkii* DSM 8720^T (pH 10; *closed diamonds*) grown at pH 10, and neutralophilic *B. subtilis* IAM 1029 (pH 7; *closed triangles*). The reaction was performed at 25 °C. Buffers of 20 mM NaH_2PO_4/Na_2HPO_4 and 20 mM glycine were used for pH 7 and pH 10, respectively. Data represent the means of at least 3 independent replicates and bars are the standard deviations

2.5 times higher than that of *B. cohnii* YN-2000 and the H^+/O ratio of the former is 1.5 times higher than that of the latter. Therefore, it can be predicted that the ATP synthesis rate of *B. cohnii* YN-2000 is much lower than that of *B. subtilis* IAM 1026. However, *B. cohnii* YN-2000 produced 10.1 nmol ATP · min^{-1} · mg $protein^{-1}$ as the maximum rates at pH 10 (Fig. 2). The value was much higher than that of *B. subtilis* IAM 1026 at pH 7 (1.56 nmol ATP · min^{-1} · mg $protein^{-1}$ as the maximum rate). The ATP synthesis rate of *B. cohnii* YN-2000 was much lower that of the obligately alkaliphilic *B. clarkii* DSM 8720^T (28.7 nmol ATP · min^{-1} · mg $protein^{-1}$ as the maximum rate at pH 10).

Transmembrane electrical potential (Δψ)

On the basis of the oxygen consumption and H^+/O ratio, it can be predicted that Δψ produced by respiration is lower in the alkakiphiles *B. cohnii* YN-2000 and *B. clarkii* DSM 8720^T than in *B. subtilis* IAM 1026. On the other hand, the much higher ATP synthesis rate of the two alkaliphilic strains than in *B. subtilis* IAM 1026 cannot be explained without the high Δψ in strain YN-2000, which is determined as 173 ± 5 mV (shaking at 120 rpm under pH 10) and was lower than that of the obligately alkaliphilic *B. clarkii* DSM 8720^T (192 ± 3 mV with shaking speed of 120 rpm at pH 10) (Table 1). However, these values became lower under low aeration condition (shaking at 60 rpm under pH 10), which were determined as 100 ± 2 mV and 135 ± 8 mV in *B. cohnii* YN-2000 and *B. clarkii* DSM, respectively. The Δψ in strains YN-2000 and DSM 8720^T with shaking at 120 rpm was also comparable to those in other obligately or facultatively alkaliphilic *Bacillus* spp. (181 – 213 mV) (Kitada et al. 1982;

Table 1 Bioenergetic parameters of *B. clarkii* DSM 8720^T, *B. cohnii* YN-2000 and *B. subtilis* IAM 1026

Strain	Cytochrome *c* content (nmol · mg $protein^{-1}$)	Respiratory rate[a] (μmol O_2 · min^{-1} · mg cell $protein^{-1}$)	H^+/O	Δψ (mV)	Negat ive ion capacity[b] ($\times 10^{-6}$ eq · mg $protein^{-1}$)	Maximum ATP synthesis rate[c] (nmol ATP · min^{-1} · mg cell $protein^{-1}$)
B. clarkii DSM 8720^T	0.36 ± 0.01	0.19 ± 0.04	3.6 ± 0.4	−192 ± 3	3.3 (pH 8.1)	28.7
B. cohnii YN-2000	0.62 ± 0.01	0.20 ± 0.03	3.5 ± 0.7	−173 ± 5	2.9 (pH 8.1)	10.1
B. subtilis IAM 1026	0.23 ± 0.01	0.50 ± 0.06	5.3 ± 0.2	−121 ± 7	0.7 (pH 6.7)	1.56

Cells of alkaliphiles and *B. subtilis* IAM 1026 were prepared using media of pH 10 and pH 7, respect ively. Determinations of respiratory rate, H^+ /O and ATP synthesis rate were performed using whole cells. A right-side-out membrane vesicle was used for the determination for Δψ. Cytochrome *c* content and negative ion capacity were determined using cell extracts prepared by disruoting the cells by French pressure. Data represent the means with standard deviat ions of at least 3 independent experiments replicates.

[a] Respiratory rate was estimated for alkaliphiles and *B. subtilis* IAM 1026 at pH 10 and pH 7, respectively

[b] Intracellular negative ion capacity was estimated in accordance with the intracellular pH in each strain. Intracellular pH is indicated in the parentheses

[c] Maximum ATP synthesis rate was estimated for alkaliphiles and *B. subtilis* IAM 1026 at pH 10 and pH 7, respectively

Sugiyama et al. 1986; Hoffman and Dimroth 1991; Hirabayashi 2012). The obtained $\Delta\psi$ in strains YN-2000 and DSM 8720^T was much higher than that in *B. subtilis* IAM 1026 (121 ± 3 mV) with shaking at 120 rpm. Yoshimune et al. (2010) indicated that $\Delta\psi$ can attract H^+ extruded by respiration in the vicinity of the outer membrane surface at high pH in an alkaliphile. Therefore, it is considered that a high $\Delta\psi$ is important for energy production in alkaliphiles. The obtained result suggested that the larger $\Delta\psi$ in strain YN-2000 provides the larger potential to extrude H^+ to the outer membrane surface and produce more ATP than in the case of the neutralophilic *B. subtilis* IAM 1026.

Intracellular pHs of *B. cohnii* YN-2000, *B. clarkii* DSM 8720^T and *B. subtilis* IAM 1026

We determined the intracellular pHs of *B. cohnii* YN-2000 and *B. clarkii* DSM 8720^T grown at pH 10 and *B. subtilis* IAM 1026 grown at pH 7 (Fig. 3). The intracellular pHs of the two alkaliphilic strains *B. cohnii* YN-2000 and *B. clarkii* DSM 8720^T were the same (8.1) when their external pH was 10 at high aeration (shaking at 120 rpm). The intracellular pH did not change when these alkaliphiles were grown at 60 rpm under pH 10 (data not shown). These results are in accordance with the fact that the Na^+/H^+ antiporters in alkaliphiles play an important role in regulating intracellular pH (Paden et al. 2005; Krulwich at al. 2007). The intracellular and extracellular pH changes in alkaliphiles in the present study are comparable to those reported previously using other alkaliphilic *Bacillus* spp. (Sugiyama et al. 1986; Sturr et al. 1994; Aono et al. 1997; Kitada et al. 2000). The intracellular pH (pH 6.7) of neutralophilic *B. subtilis* IAM 1026 was slightly lower than the extracellular pH (pH 7). The intracellular and extracellular pH changes in *B. subtilis* IAM 1026 were similar to those in *B. subtilis* GSY1026 (Shioi et al. 1980), which is of the same species.

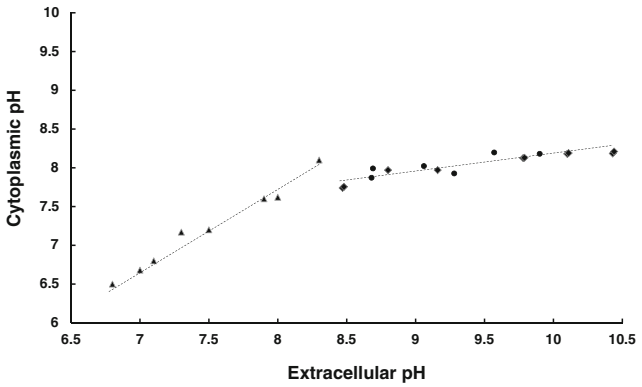

Fig. 3 Cytoplasmic pHs of facultative alkaliphilic *B. cohnii* YN-2000 (*closed circles*) and obligate alkaliphilic *B. clarkii* DSM 8720^T (*closed diamonds*) and grown at pH 10, and neutralophilic *B. subtilis* IAM 1026 (*closed triangles*) grown at pH 7. The suspension of BCECF-loaded cells (20 μl) was added to 10 ml of 0.1 M TES buffer (pHs 7.0–8.5) or 0.1 M glycine buffer (pHs 8.5–10.0)

Estimation of intracellular negative ion capacity

Although it was suggested that a high $\Delta\psi$ plays a key role in the high ATP synthesis in YN-2000, the high $\Delta\psi$ cannot be explained by the respiratory H^+ translocation. It can be predicted that the intracellular negative ion capacity will contribute to the high $\Delta\psi$ through the formation of the Donnan potential. Therefore, we determined the negative ion capacity of the intracellular extract by the titration of positively charged substances using the intracellular extracts of *B. cohnii* YN-2000 and *B. clarkii* DSM 8720^T grown at pH 10 and *B. subtilis* IAM 1026 grown at pH 7. The negative ion capacity of the intracellular materials in the strains of alkaliphiles increased in accordance with the increase in the measured pH between pH 6 and pH 8 under high aeration growth condition (Fig. 4). On the other hand, the negative ion capacity of the neutralophilic *B. subtilis* IAM 1026 barely changed with the change in the measured pH. As described above, the intracellular pH of the two alkaliphilic strains *B. cohnii* YN-2000 and

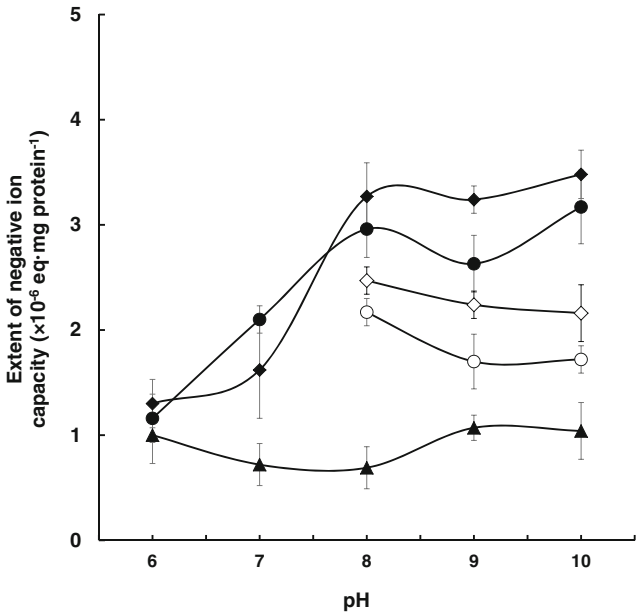

Fig. 4 Colloid titration of the intracellular cell materials from facultative alkaliphilic *B. cohnii* YN-2000 (*closed circles*) and obligate alkaliphilic *B. clarkii* DSM 8720^T (*closed diamonds*) grown at pH 10, and neutralophilic *B. subtilis* IAM 1026 (*closed triangles*) grown at pH 7 under high aeration condition (shaking at 120 rpm). Results of *B. cohnii* YN-2000 (*open circles*) and *B. clarkii* DSM 8720^T (*open diamonds*) under low aeration condition (60 rpm) at pH 10 are also shown. Cell extracts prepared by disrupting cells by French pressure were used for this experiment. Each intracellular material is disolved in buffer: pH 6, 250 mM succinate buffer; pH 7, 250 mM NaH_2PO_4/Na_2HPO_4 buffer; pH 8, 250 mM Tris–HCl; pHs 9 and 10, 250 mM $NaHCO_3/Na_2CO_3$ carbonate buffer. Data represent the means of at least 3 independent replicates and bars are the standard deviations

B. clarkii DSM 8720^T was 8.1. At each pH, the intracellular negative ion capacities of the intracellular materials of *B. cohnii* YN-2000 and *B. clarkii* DSM 8720^T were 2.9 and 3.3 ($\times 10^{-6}$ eq · mg protein^{-1}), respectively. On the other hand, these values became lower, exhibiting 2.1 and 2.4 for *B. cohnii* YN-2000 and *B. clarkii* DSM 8720^T, respectively, grown under low aeration condition (60 rpm). The negative ion capacity of the intracellular materials of *B. subtilis* IAM 1026 at pH 7 was 0.7 ($\times 10^{-6}$ eq · mg protein^{-1}) at intracellular pH 6.7. Therefore, there is a possibility that the difference in the negative ion capacity of intracellular materials contributes to the Donnan effect, which is the potential produced by intracellular impermeable negatively charged materials. In addition, it is considered that the intracellular negative ion capacity may contribute to the large $\Delta\psi$ (diffusion potential *plus* Donnan potential [Helfferich 1962; Lakshminarayanaiah 1969]) of the two alkaliphilic strains. The relationship between the negative ion capacity and the membrane electrical potential ($\Delta\psi$) was estimated using the data of alkaliphiles used in this study grown under different aeration conditions (Fig. 5). There is a proportional relationship between the negative ion capacity and $\Delta\psi$ in the alkaliphiles used in this study, and the result strongly suggests the contribution of the intracellular negative ion capacity to the formation of a large Donnan potential.

Discussion

It has been reported that $\Delta\psi$ is larger in alkaliphilic *Bacillus* spp. than in neutralophilic *Bacillus* spp. (Krulwich 1995; Dimroth and Cook 2004; Yumoto 2003; Goto et al. 2005; Hirabayashi et al. 2012). Even if the medium pH is considered as the actual extracellular pH for the calculation of the proton motive force, $\Delta\psi$ is not sufficiently high to compensate for the low ΔpH to produce a sufficiently high proton motive force (Δp) to produce ATP in alkaliphilic *Bacillus*. Yoshimune et al. (2010) provided evidence that $\Delta\psi$ can attract H^+ that is extruded by respiration in the vicinity of the outer membrane surface at high pH in an alkaliphile. Although it has long been described that the medium pH is the extracellular pH in the calculation of Δp (Sturr et al. 1994; Krulwich et al. 2007), the results of Yoshimune et al. (2010) indicate that the pH at the outer membrane surface is much lower than the pH of the medium. $\Delta\psi$ not only contributes to Δp as the constituent of Peter Mitchell's formula but also attracts respiratory extruded H^+ in the vicinity of the outer membrane surface. Therefore, it is considered that $\Delta\psi$ plays a key role in the production of energy by alkaliphiles under the strict one-thousandth H^+-less condition in comparison with that at neutral pH. On the other hand, in the case of an acidophile, $\Delta\psi$ exhibits negative values in the direction of positive Δp (van de Vossenberg et al. 1998) (Fig. 6). Δp should theoretically be 220 mV to energize ATPase for ATP synthesis (Elston et al. 1998). However, if the medium pH is considered as the extracellular pH, the Δp in the alkaliphile is much lower than the value of 220 mV (Fig. 6). The pH of the outer membrane surface in the alkaliphile should be lower than the medium pH owing to the H^+-attracting force of $\Delta\psi$. Considering the case of alkaliphiles in combination with the results for acidophiles, $\Delta\psi$ may regulate the distance between the respiratory extruded H^+ and the outer membrane surface (Fig. 6).

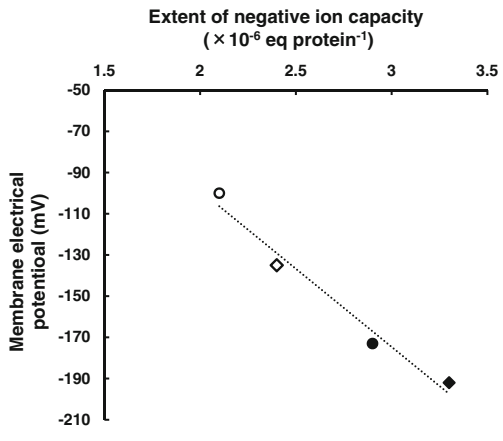

Fig. 5 Relationship between negative ion capacity and membrane electrical potential ($\Delta\Psi$) in facultative alkaliphilic *B. cohnii* YN-2000 grown under different aeration conditions (120 [closed circle] or 60 [open circle] rpm) at pH 10 and obligate alkaliphilic *B. clarkii* DSM 8720^T grown under different aeration conditions (120 [closed diamond] or 60 [open diamond] rpm) at pH 10. Each of the negative ion capacity refracted each of the intracellular pH when the extracellular pH is 10

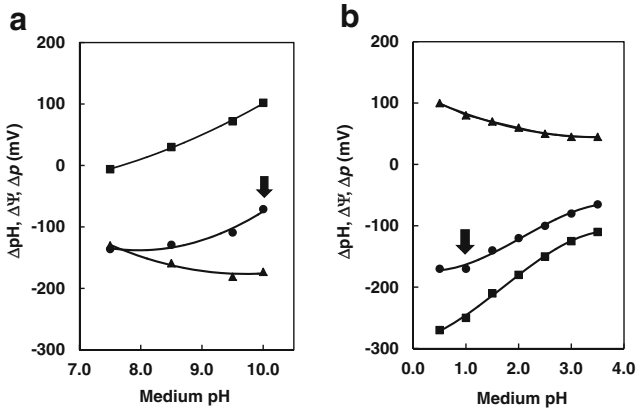

Fig. 6 Bioenergetic parameters of alkaliphilic (**a**) and acidophilic (**b**) bacteria. ΔpH (*closed squares*) was calculated on the basis of Mitchell's formula as the medium pH is considered as the extracellular pH. Membrane electrical potential ($\Delta\Psi$) (closed triangles) is shown as a measured value. Δp (proton motive force; *closed circles*) was calculated as the sum of $\Delta\Psi$ and ΔpH. The arrows indicate the Δp in each bacterial sample under each living condition. The bioenergetic parameters of alkaliphilic bacterium (*B. cohnii* YN-2000) are data obtained in this study except $\Delta\Psi$ at pH 7.5–9.5. Data of $\Delta\Psi$ at pH 7.5–9.5 were cited from Sugiyama et al. (1986). The bioenergetic parameters of acidophilic bacterium (*Picrophilus oshimae* DSM 9789) were cited from van de Vossenberg et al. (1998)

As described above, $\Delta\psi$ plays a key role in the production of energy by alkaliphiles under strict conditions from the bioenergetic viewpoint. From the respiratory rate and H^+ extrusion efficiency (H^+/O ratio), it is difficult to explain the formation of the high $\Delta\psi$ by the respiratory activity depending on the H^+ translocation across the membrane in the facultatively alkaliphilic *B. cohnii* YN-2000. A similar observation was obtained in the case of the obligately alkaliphilic *B. clarkii* DSM 8720^T. Therefore, it is considered that the Donnan effect produced by intracellular negatively charged unpermeable materials contributes to the formation of the high $\Delta\psi$, because the bulk $\Delta\psi$ consists of the diffusion potential of the permeable positive ion and the Donnan potential caused by intracellular unpermeable negatively charged materials (Helfferich 1962; Lakshminarayanaiah 1969). For the estimation of the Donnan potential, we measured the negative ion capacity of the intracellular materials of the two alkaliphilic *Bacillus* spp. *B. cohnii* YN-2000 and *B. clarkii* 8720^T, by the titration of a positively charged substance. We demonstrated that there is a relationship between the intracellular negative ion capacity and $\Delta\psi$ in the alkaliphiles used in this study (Fig. 6). This strongly suggested that the negative ion capacity contributes to the formation of a large Donnan potential. A question may arise on the reason for the formation of the large negative ion capacity. This can be explained by the capacity of intracellularly contained materials such as negatively charged acidic proteins. It was predicted that the obligately alkaliphilic *Bacillus selenitireducens* MLS10 has proteins of low p*I* in the cytoplasm and cytoplasmic membrane in comparison with those in neutralophiles (Janto et al. 2011).

The comparison of bioenergetic parameters among the obligately alkaliphilic *B. clarkii* DSM 8720^T, the facultatively alkaliphilic *B. cohnii* YN-2000 and the neutralophilic *B. subtilis* IAM 1026 is shown in Table 1. It was found that the obligate or facultative alkaliphiles exhibit characteristics different from those of the neutralophilic *B. subtilis* in all the items in Table 1, whereas the obligate alkaliphile is similar to the facultative alkaliphile except for the ATP synthesis rate. The facts mentioned above suggested that both obligate alkaliphilic *B. clarkii* DSM 8720^T and the facultative alkaliphilic *B. cohnii* YN-2000 possess the same mechanisms of producing ATP efficiently, for example, the respiratory translocated H^+ has a larger energy potential than that of *B. subtilis* IAM 1026. The ATP synthesis rate of *B. cohnii* YN-2000 (10.1 ATP $\cdot$ min^{-1} $\cdot$ mg protein^{-1} as the maximum rate at pH 10) was much lower that of the obligately alkaliphilic *B. clarkii* DSM 8720^T (28.7 nmol ATP $\cdot$ min^{-1} $\cdot$ mg protein^{-1} as the maximum rate at pH 10). This fact indicates that the obligate alkaliphile has a higher efficiency of ATP production than the facultative alkaliphile. This may be explained by the larger $\Delta\psi$, which attracts H^+ at the extracellular surface of the membrane in the obligate alkaliphile (Table 1). In addition, it can be predicted that the presence of the H^+-transferable Asn (Doukov et al. 2007)-rich segment at the N-terminal amino acid sequence of the membrane-binding cytochrome *c*, which is specifically observed in obligate alkaliphiles (Ogami et al. 2009), plays a role in the effective H^+ transfer in the vicinity of the extracellular surface of the membrane.

We considered that the bioenergetic basis of the alkali adaptation of alkaliphiles can be explained mainly by the high $\Delta\psi$ and the characteristics of the outer membrane surface structure, which consists of membrane-anchored proteins such as cytochromes *c* and *b*. Although the turnover rate of the extrusion of positively charged H^+ is lower in the alkaliphiles than in the neutralophile, the alkaliphiles exhibit a high $\Delta\psi$ owing to the larger negative ion capacity, which produces the Donnan potential. From the opposite direction, the larger $\Delta\psi$ may be the reason for the low turnover rate of H^+ extrusion by respiratory activity, because it can be predicted that it is difficult to extrude positively charged H^+ under a high $\Delta\psi$ from intracellular (electrically negative) to extracellular (electrically positive) sides across the membrane (Goto et al. 2005). Therefore, alkaliphiles extrude a higher potential of extracellular membrane H^+ than neutralophiles. In other words, alkaliphiles prefer efficiency over quantity of H^+, and this strategy is quite reasonable under the H^+-less condition. Another important factor is the efficient H^+ transfer mechanisms at the extracellular surface of the membrane. In addition to the electrical force of $\Delta\psi$ across the membrane, it can be predicted that cytochrome *c* plays an important role in protecting the diffusion of H^+ extruded by respiratory activity. Cytochrome *c*-550 from *B. clarkii* K24-1U possesses an extra segment between the anchoring part and the main body of the cytochrome *c* molecule (Ogami et al. 2009). This extra segment has a high Asn content. This segment exists only in cytochromes *c* in obligate alkaliphiles. The efficiency of this segment may account for the lower cytochrome *c* content in *B. clarkii* than in *B. cohnii* (Table 1). Between Asn21 and Asn31 (the numbering is based on the amino sequence of cytochrome *c*-550 from strain K24-1U), Asn comprises 41 % of the sequence, and these structural characteristics combined with the tetrameric nature of the protein may enhance the function of this specific structure. On the basis of particular structural characteristics, it can be predicted that the structure contributes to the formation of the H^+ network on the extracellular surface of the membrane. These characteristics may be related to the larger H^+ acceptance capacity of alkaliphiles than that of neutralophiles. In addition to the above-mentioned characteristics, cytochrome *c* has a much lower redox potential in the alkaliphiles than in the neutralophiles (Hicks and Krulwich 1995; Yumoto 2002; Goto et al. 2005). This causes a large redox potential gap between cytochrome *c* and cytochrome *c* oxidase. It is considered that this large redox potential gap is necessary for translocating H^+ under the high $\Delta\psi$ (Yumoto et al. 1993).

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