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Effect of potassium and sodium ions on the cytoplasmic pH of an alkalophilic *Bacillus*

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A bacterium belonging to *Bacillus* and tentatively designated as a facultatively alkalophilic *Bacillus* grows optimally at pH 7.5–10.2 (Koyama, N., Takinishi, H. and Nosoh, Y. (1983) FEMS Microbiol. Lett. 16 213–216). The internal pH of the bacterium was higher than the external pH in the pH range below 8, in the presence of KCl, but in the pH range above 9 the internal pH was lower than the external pH, both in the absence and presence of KCl. The internal pH of the bacterium in the presence of KCl was thus kept at 8.2–9.2 in the external pH range from 7.5 to 10.0. At pH 7.5, an extrusion of H^+ in specific exchange for K^+ was observed. The membrane vesicles, when energized with ascorbate plus tetramethylphenylenediamine, exhibited the formation of a transmembrane pH gradient (acid, interior), the magnitude of which became greater on increasing the extravesicular pH above 8 in the presence of NaCl. The alkalinization and acidification of the cytoplasm by the specific exchange of H^+ for K^+ and Na^+, respectively, were suggested for a pH homeostasis of this bacterium.

Introduction

Many bacteria have been reported to keep the intracellular pH almost constant, and such a pH homeostasis is considered to be essential for the growth of the bacteria [1,2]. In order to regulate the internal pH, the bacteria should transport protons inwardly or outwardly. Some H^+-transport systems such as Na^+/H^+ and K^+/H^+ antiporters and H^+-ATPase have therefore been suggested to regulate the cytoplasmic pH of bacteria [1–11]. *Bacillus alcalophilus*, an obligate alkalophile, grows in the pH range of 9–11, and even in such alkaline pH range, the organism was shown to keep well the internal pH at about 9 through a Na^+/H^+ antiporter [3]. A bacterium, which belongs to *Bacillus* and is considered to be facultatively alkalophilic, was shown to grow over a wide pH range from 7.0 to 10.5 and exhibit almost the same growth rate over a pH range from 7.5 to 10.2 [12]. This bacterium may therefore be expected to regulate the internal pH in response to the external pH over such a wide pH range. It has recently been found that when the culture pH of the bacterium was changed from 10.2 to 7.5, the growth stopped and the bacterium necessitated the addition of KCl to the culture in order to continue the growth. This finding stimulated the authors to examine the roles of K^+, together with Na^+, in pH homeostasis of the bacterium. In this paper, the study on a mechanism for the regulation of the internal pH probably functioning in this organism is reported.

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Abbreviations: ΔpH, transmembrane pH gradient; $\Delta\psi$, membrane potential; $\Delta\bar{\mu}_{H^+}$, proton electrochemical potential.

Materials and Methods

Culture. A bacterium which belongs to *Bacillus* and named tentatively as facultatively alkalophilic *Bacillus* YN-2000, originally designated as YN-2 [12], was used. The bacterium was grown at pH 7.5 or 10.2 at 37°C in peptone medium of the following composition (g/l of deionized water): polypeptone, 10; glucose, 10; yeast extract, 1.5; $Na_2HPO_4 \cdot 12H_2O$, 1.5; NaCl, 1.5; $MgCl_2 \cdot 6H_2O$, 0.1 [12]. The pH of the culture was always kept at 7.5 or 10.2 with 3 M NaOH. The cells grown at pH 10.2 were collected at late logarithmic phase, washed and suspended in 20 mM phosphate buffer (pH 7.5) containing 150 mM NaCl, 0.5 mM $MgCl_2$ and 200 mM sucrose unless otherwise indicated, and used in the following experiments.

Preparation of membrane vesicles. Right-side-out membrane vesicles were prepared, according to the procedure described previously [13]. The membrane vesicles were then washed with 10 mM phosphate buffer (pH 8.2) containing 50 mM KCl and 1 mM $MgCl_2$, and suspended in the same buffer.

Fluorescence measurement. The pH difference ($\Delta pH = pH_{internal} - pH_{external}$) across membranes of whole cells or membrane vesicles was monitored by the change of fluorescence of 9-aminoacridine [14,15].

The assay for whole cells was conducted at 25°C by adding 0.1 ml of the cell suspension to 3 ml 20 mM H_3PO_4-Tris buffer (pH 7.5) containing 0.5 mM $MgCl_2$, 5 mM glucose and 300 mM sucrose (the H_3PO_4-Tris buffer) and 2 μM 9-aminoacridine. Since the cell suspension contained 150 mM NaCl, the reaction mixture contained 5 mM NaCl.

As for the membrane vesicles, the assay was conducted at 25°C in 10 mM phosphate buffer of an appropriate pH value containing 0.5 mM $MgCl_2$ and 50 mM KCl or NaCl. To 3.2 ml of the buffer containing 2 μM 9-aminoacridine and membrane vesicles (2 mg protein) were added 0.1 ml 41 mM potassium ascorbate and then 0.1 ml 1.0 mM tetramethylphenylenediamine. The fluorescence intensity rapidly quenched due to the formation of ΔpH (acid, interior). Upon addition of 20 μl 0.1 mM gramicidin A to the above reaction mixture, the fluorescence intensity rapidly recovered due to the dissipation of ΔpH. The fluorescence intensity thus recovered was somewhat lower than the intensity before the addition of the energy source because of a quenching effect of ascorbate. The intensity thus recovered and the magnitude of quenching on addition of ascorbate plus tetramethylphenylenediamine were expressed as F and ΔF, respectively.

Fluorescence measurements were conducted in a Shimadzu recording spectrofluorimeter RF502, with excitation at 400 nm and emission at 455 nm.

The membrane potential of the organism was monitored with rhodamine 6G [16]. The assay was the same as that for the measurement of the ΔpH with 9-aminoacridine for whole cells, except that the fluorescence was measured with excitation at 520 nm and emission at 550 nm.

Potassium uptake. Potassium uptake was measured at 25°C in the H_3PO_4-Tris buffer (pH 7.5) contaning 1 mM KCl, using a K^+-electrode attached to a Beckman Expandmatic SS-2 pH meter which was connected to a Hitachi recorder 056.

Cytoplasmic pH. The cytoplasmic pH of the bacterium was estimated by determining the distribution across membranes of either [^{14}C]methylamine or 5,5-dimethyl[^{14}C]oxazolidine-2,4-dione [17]. The reaction was started by introducing 50 μl of a cell suspension (10–14 mg dry w/ml) to 6 ml of the H_3PO_4-Tris buffer containing 0.95 μM [^{14}C]methylamine (35 Ci/mol, CEA, France) or 1 μM 5,5-dimethyl[^{14}C]oxazolidine-2,4-dione (32 Ci/mol, CEA, France). Since the pH of the reaction mixture was adjusted with NaOH, the mixture contained Na^+ at a concentration of 40 mmol/l. The reaction mixture was then incubated at 25°C for 10 min with continuous shaking and filtered through a membrane filter (TM-100, Toyo). The cells retained on the filter were transferred into a toluene-Triton scintillation liquid and assayed for radioactivity in Aloka liquid-scintillation system LSC-700.

To calculate the concentration of methylamine or 5,5-dimethyloxazolidine-2,4-dione within the cells, the intracellular space was measured by the method of Winkler and Wilson [18]. The internal water content estimated by use of methoxy[^{14}C]inulin (41.9 Ci/g, New England Nuclear) [7] was 6 μl/mg cell protein.

Membrane potential. Membrane potential ($\Delta\psi$)

of the bacterium was determined at 25°C by measuring the distribution of [^{14}C]tetraphenylphosphonium across membranes. The reaction was carried out in the H_3PO_4-Tris buffer containing Na^+ (about 40 mmol/l) using [^{14}C]tetraphenylphosphonium (3.3 Ci/mol, Amersham), according to the procedure of Schuldiner and Kaback [19]. The proton electrochemical potential ($\Delta\bar{\mu}_{H^+}$) was calculated by the equation, $\Delta\bar{\mu}_{H^+} = \Delta\psi - Z\Delta pH$ ($Z = 2.3\ RT/F$, F is the Faraday constant) using the experimental values of $\Delta\psi$ and ΔpH.

Protein determination. Protein concentration was determined according to the method of Gornal et al. [20].

Results and discussion

Effect of KCl on the growth of the bacterium when the culture pH was changed

The present bacterium is able to grow over a wide pH range from 7.0 to 10.5, and exhibits an optimal growth in the range from pH 7.5 to 10.2 [12].

When the culture pH was changed from 7.5 to 10.2 at logarithmic phase, the bacterium grew with almost the same growth rate as that before the pH shift, although a 15 min of lag period was observed. On changing the culture pH from 10.2 to 7.5, on the other hand, the growth rate gradually decreased, and as shown in Fig. 1, the growth almost completely stopped about 2 h after the pH shift. When the pH shift was immediately followed by the addition of KCl, the growth rate was recovered dependently on the concentration of KCl. The growth rate of the culture on addition of 1–5 mM KCl was almost the same as that without the pH shift. It was suggested that the change of the culture pH from 10.2 to 7.5 may have resulted in lowering the internal pH to decrease the growth rate and that the addition of KCl to the culture may have resulted in an increase of internal pH to recover the growth.

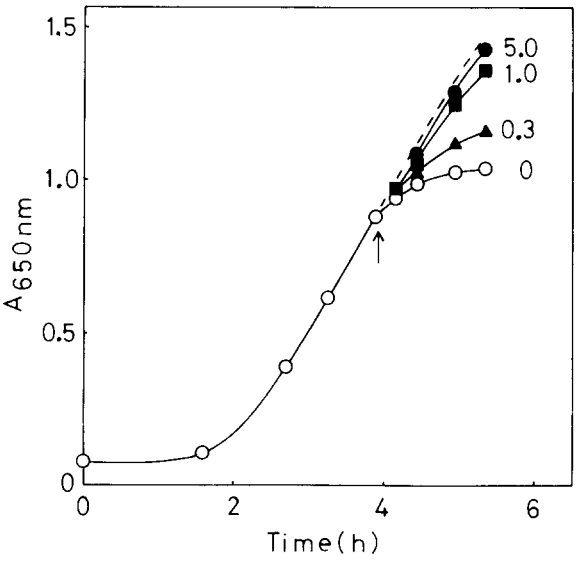

Fig. 1. Effect of KCl on the growth of the bacterium when the culture pH was changed from 10.2 to 7.5. The shift was made with 3 M HCl within 15 s. The arrow indicates the time of the pH shift, which was immediately followed by the addition of the various concentrations of KCl. The numerals on the curves indicate the KCl concentrations (mM). Dashed line represents the growth curve without the pH shift.

Effect of KCl on the internal pH of the bacterium

To confirm the above suggestion, the effect of KCl on the internal pH of the present bacterium was studied first qualitatively by the fluorescence change of 9-aminoacridine.

When the internal pH of a bacterium is lower than the external pH, the fluorescence of 9-aminoacridine is known to decrease on mixing with the organism [14,15]. When the present bacterium grown at pH 10.2 was added to the H_3PO_4-Tris buffer (pH 7.5) containing 9-aminoacridine, the fluorescence of the dye rapidly quenched (Fig. 2A). This suggests ΔpH (acid, interior) across membranes. If the present bacterium is leaky to K^+, a diffusion potential would be formed across membranes, and H^+ influx resulting in a formation of ΔpH would occur, irrespectively of the presence of Na^+. In order to examine whether this is the case of the bacterium, the following experiment was carried out. The cells collected were washed and suspended in the buffer containing CsCl instead of NaCl and the fluorescence experiment in the absence of Na^+ was carried out with the cell suspension. As shown in Fig. 2A, only slight quenching of the fluorescence was observed upon addition of the cell suspension to 9-aminoacridine. As described in Materials and Methods, the dye/cell suspension contained about 5 mM NaCl when the cells were suspended in the

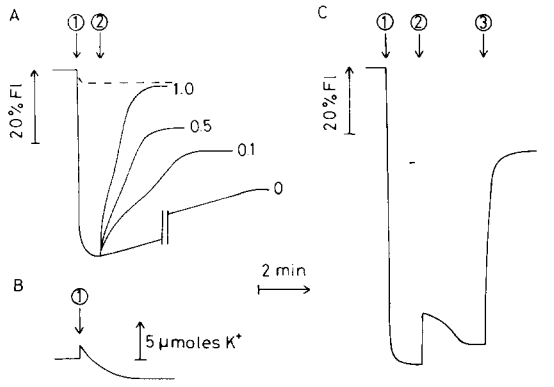

Fig. 2. Effects of KCl on ΔpH (A), potassium uptake (B) and membrane potential of the bacterium grown at pH 10.2. (A) The ΔpH was monitored and expressed by the fluorescence intensity at 455 nm of 9-aminoacridine. At the times indicated by arrows 1 and 2, the bacteria and KCl were added, respectively, to the H_3PO_4-Tris buffer (pH 7.5) containing 9-aminoacridine. The dashes line represents the fluorescence change upon addition of the cells washed and suspended in the buffer containing CsCl instead of NaCl. The final concentration of the bacteria was 0.1 mg protein/ml. Numerals on curves indicate the final concentrations of KCl. In the absence of KCl, the fluorescence intensity attained a steady level within 15 min. Upward deflection shows the increase in fluorescence intensity (decrease of ΔpH). (B) At the time indicated by arrow 1, 0.3 ml cell suspension was added to 10 ml H_3PO_4-Tris buffer (pH 7.5) containing 1 mM KCl. Final concentration of the bacteria was 0.7 mg protein/ml. Downward deflection indicates a decrease in K^+ concentration in the reaction mixture. (C) The membrane potential was monitored by the fluorescence intensity at 550 nm of rhodamine 6G. At the times indicated by the arrows 1, 2 and 3, the bacteria, KCl and dibenzyldimethylammonium were added, respectively. Final concentrations of the bacteria and KCl were 0.1 mg protein/ml and 1 mM, respectively. Upward deflection indicates an increase in the fluorescence intensity (decrease of membrane potential).

buffer containing NaCl. The results may therefore indicate that a rapid quenching of the fluorescence upon additon of the bacterium in the presence of Na^+ is due to the acidification of cytoplasm by Na^+.

When the fluorescence of the dye mixed with the bacteria was at a minimum level in the presence of Na^+, KCl was added to the mixture. As shown in Fig. 2A, the intensity increased rapidly up to a stationary level. The stationary level and the rate of increase in fluorescence intensity were dependent on the concentration of KCl. When 1 mM KCl was used, the stationary level of the intensity was almost the same as that before the addition of KCl. The results indicate that the addition of KCl may have resulted in an increase of the internal pH due to H^+ efflux.

The chloride salts of other monovalent cations such as Na^+, Li^+, Rb^+, Cs^+, NH_4^+ and choline$^+$ did not substitute for KCl. This may indicate the specific effect of K^+ on increasing internal pH.

It can be suggested from the results shown above that K^+ enter the cells as a counter ion in exchange for H^+. K^+ movement was then examined using a K^+-electrode. As shown in Fig. 2B, when the bacteria were incubated in the H_3PO_4-Tris buffer (pH 7.5) containing 1 mM KCl, K^+ entered the cells and the net K^+ influx completed within about 2 min. The results shown in Fig. 2A and 2B may therefore indicate that the bacteria accumulated K^+ in exchange for H^+.

In order to study the electrogenicity of the exchange of K^+ for H^+, the membrane potential was monitored with rhodamine 6G [16]. The addition of the bacteria to the H_3PO_4-Tris buffer (pH 7.5) containing rhodamine 6G produced a rapid and remarkable quenching of the fluorescence of the dye and reached a stationary, minimum level (Fig. 2C). The fluorescence intensity of rhodamine 6G which decreased upon addition of the bacteria was shown to recover by the addition of dibenzyldimethylammonium which is known to dissipate membrane potential [21]. The quenching of fluorescence of the dye on addition of the bacterium was therefore considered to be mainly due to membrane potential (negative, inside) of the bacteria.

When 1 mM KCl was added to the dye/cell suspension, the fluorescence intensity which once instantaneously increased was found to decrease slowly and attain a stationary level about 2 min after the addition of KCl (Fig. 2C). All the results shown in Fig. 2 may indicate that the exchange of K^+ for H^+ was accompanied by the increase of membrane potential and that the organism extruded H^+ with the exchange ratio to K^+ above 1.

Effect of KCl on the internal pH of the bacterium in the external pH range from 7.5 to 10

As described above, the addition of KCl to the bacterium at pH 7.5 increased the internal pH

which had been decreased by Na^+. The effect of KCl on the internal pH of the bacteria was then examined in the external pH range 7.5–10.0, which is optimal for the growth of the bacterium [12].

When the external pH of the bacteria was changed from 7.5 to 10.0 in the absence of KCl, the internal pH changed from 6.8 to 8.9, and the internal pH values were lower than the external pH values at any external pH (Fig. 3). As expected from the result shown in Fig. 2A, the addition of 5 mM KCl to the bacterium at pH 7.5 results in an increase of internal pH from 6.8 to 8.2. As shown in the figure, the internal pH was higher than the external pH in the external pH range below 8. In the pH range above 9, however, the internal pH was lower than the external pH. The internal pH of the bacterium was thus kept in a narrow pH range between 8.2 and 9.2 even when the external pH was changed from 7.5 to 10.0 in the presence of KCl. In the external pH range below 8, the bacteria may extrude H^+ in exchange for K^+ to keep an alkaline internal pH. The involvement of K^+ for the regulation of internal pH through a K^+/H^+ antiporter has been suggested in *Escherichia coli* [6].

Effect of NaCl on the internal pH of the membrane vesicles

As mentioned above (Fig. 3), KCl was shown to increase the internal pH of the bacterium by exchange of K^+ for H^+ in the external pH range below 8. In the range above 9, however, the internal pH was not affected considerably by KCl, and lower than the external pH both in the absence and presence of KCl. It has been suggested that *B. alkalophilus* acidifies the internal pH by a Na^+/H^+ antiporter in the external pH range 9–11 [3]. As described in Materials and Methods, the buffer used for the determination of the internal pH of the present bacterium contained Na^+ at a concentration of 40 mmol/l. The acidification of the cytoplasm of the bacteria as shown in Fig. 3 may be due to the presence of Na^+. Since the bacteria exhibited considerable lysis at an alkaline pH during the experiments as shown in Fig. 3 in the absence of Na^+ (unpublished data), the effect of Na^+ on the internal pH of the bacteria was examined using the membrane vesicles instead of whole cells.

When the membrane vesicles mixed with 9-aminoacridine were energized with ascorbate plus tetramethylphenylenediamine in the presence of 50 mM NaCl at pH 9, the fluorescence of the dye rapidly quenched (Fig. 4A). Since the fluorescence intensity was shown to recover on addition of an

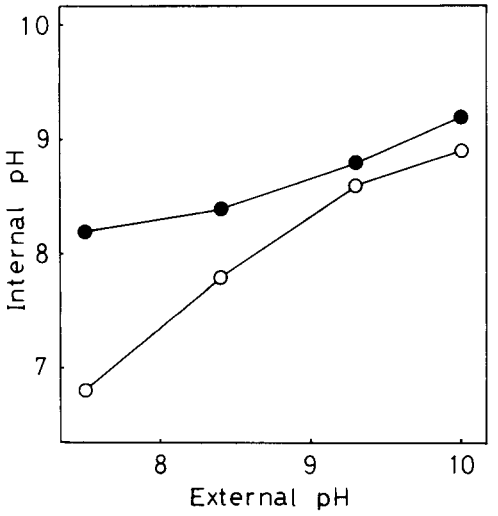

Fig. 3. Effect of external pH on the internal pH of the bacterium in the absence (○) and presence (●) of 5 mM KCl.

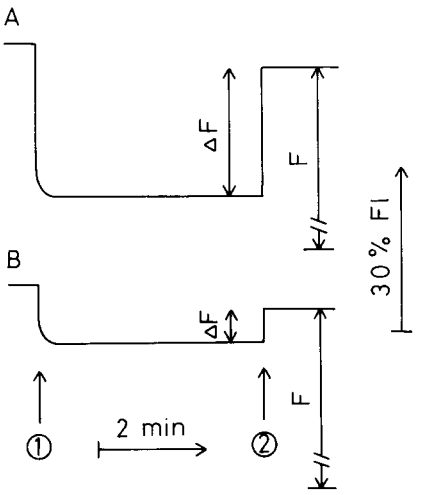

Fig. 4. Formation of ΔpH of the membrane vesicles from the bacterium. Membrane vesicles were added to 10 mM phosphate buffer (pH 9) containing 2 μM 9-aminoacridine, 0.5 mM $MgCl_2$ and 50 mM NaCl (A) or KCl (B). At the times indicated by arrows 1 and 2, ascorbate plus tetramethylphenylenediamine and gramicidine A were added, respectively. Upward deflection shows the increase of the fluorescence intensity (decrease of ΔpH).

ionophore, gramicidin A, the quenching of the dye by ascorbate plus tetramethylphenylenediamine was considered to be due to the formation of ΔpH (acid, interior). In the presence of 50 mM KCl instead of NaCl, the quenching of the fluorescence was also observed, but the value of ΔF (Fig. 4B) in the presence of KCl was much smaller than that in the presence of NaCl. The magnitude of $\Delta F/F$ in the presence of KCl was about one-fourth that in the presence of NaCl at pH 9. The results suggest that the intravesicular pH at pH 9 was more acidic in the presence of NaCl than in the presence of KCl.

Fig. 5 shows the magnitude of $\Delta F/F$ of the membrane vesicles estimated at various pH values in the presence of NaCl. Upon increasing the external pH, the $\Delta F/F$ value increased only slightly up to pH 8, but remarkably above pH 8. The estimation of $\Delta F/F$ was not made above pH 9.5, because of rapid autooxidation of ascorbate. The fact that the cytoplasmic pH of the organism was lower than the external pH in the external pH range above 9 in the presence of Na^+ with or without K^+ (Fig. 3) may therefore be qualitatively explained by an acidification of the internal pH by Na^+. When 5 mM NaSCN was added to the membrane vesicles energized with ascorbate plus tetramethylphenylenediamine at pH 9, the magnitude of $\Delta F/F$ decreased by 50%. This suggests electrogenic exchange of Na^+ for H^+ [22].

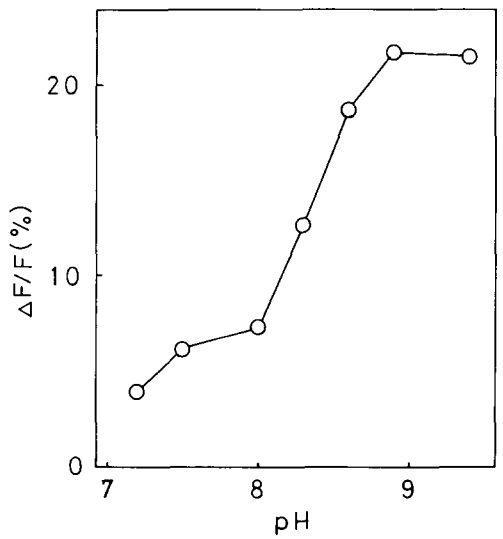

Fig. 5. Effect of external pH on the formation of ΔpH of the membrane vesicles form the bacterium.

From the results described above (Figs. 2–5), the present bacterium was suggested to alkalize the cytoplasm in the external pH range below 8 and acidify in the pH range above 9 by the electrogenic exchange of H^+ for K^+ and Na^+, respectively. Through the regulatory systems, the bacteria may be able to keep the internal pH in a narrow range of 8.2–9.2 in the external pH range of 7.5–10.0. *E. coli* grows optimally in the pH range 6–8, and accordingly, in this pH range the primary proton pumps maintain a proton electrochemical potential which was slightly affected by external pH [1]. The present bacterium also exhibited almost the same magnitudes of proton electrochemical potential at pH 7.5 and 10.0 both in the presence and absence of KCl (Table I). The components of proton electrochemical potential, membrane potential ($\Delta\psi$) and ΔpH, seemed not to be affected considerably by external pH in the absence of KCl. In the presence of 5 mM KCl, however, the magnitudes of membrane potential and ΔpH were drastically affected by external pH. On changing the external pH from 7.5 to 10.0, the membrane potential and ΔpH changed from -71 to -146 mV and from 0.7 to -0.8 unit, respectively. This may indicate that the change of ΔpH was compensated by the membrane potential to keep a proton electrochemical potential constant. The present bacterium is therefore suggested to regulate the internal pH without change in proton electrochemical potential in the external pH range of 7.5–10.0.

TABLE I

EFFECT OF KCl ON $\Delta\bar{\mu}_{H^+}$ AND ITS COMPONENTS IN THE FACULTATIVELY ALKALOPHILIC *BACILLUS*

pH_o and pH_i represent external pH and internal pH, respectively. Δ values are expressed as the $values_{internal} - values_{external}$.

KCl (5 mM)	pH_o	pH_i	$-Z\Delta pH$ (mV)	$\Delta\psi$ (mV)	$\Delta\bar{\mu}_{H^+}$ (mV)
–	7.5	6.8	41	−147	−106
	10.0	8.9	65	−157	−92
+	7.5	8.2	−41	−71	−118
	10.0	9.2	47	−146	−99

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