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Makio Kitada · Shinya Morotomi · Koki Horikoshi
Toshiaki Kudo

K^+/H^+ antiporter in alkaliphilic *Bacillus* sp. no. 66 (JCM 9763)

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Abstract A K^+/H^+ antiport system was detected for the first time in right-side-out membrane vesicles prepared from alkaliphilic *Bacillus* sp. no. 66 (JCM 9763). An outwardly directed K^+ gradient (intravesicular K^+ concentration, K_{in}, 100 mM; extravesicular K^+ concentration, K_{out}, 0.25 mM) stimulated uphill H^+ influx into right-side-out vesicles and created the inside-acidic pH gradient (ΔpH). This H^+ influx was pH-dependent and increased as the pH increased from 6.8 to 8.4. Addition of 100 μM quinine inhibited the H^+ influx by 75%. This exchange process was electroneutral, and the H^+ influx was not stimulated by the imposition of the membrane potential (interior negative). Addition of K^+ at the point of maximum ΔpH caused a rapid K^+-dependent H^+ efflux consistent with the inward exchange of external K^+ for internal H^+ by a K^+/H^+ antiporter. Rb^+ and Cs^+ could replace K^+ but Na^+ and Li^+ could not. The H^+ efflux rate was a hyperbolic function of K^+ and increased with increasing extravesicular pH (pH_{out}) from 7.5 to 8.5. These findings were consistent with the presence of K^+/H^+ antiport activity in these membrane vesicles.

Key words K^+/H^+ antiporter · Na^+/H^+ antiporter · pH homeostasis · Alkaliphilic *Bacillus* sp. · Right-side-out membrane vesicles

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M. Kitada (✉) · T. Kudo
Microbiology Laboratory, The Institute of Physical and Chemical Research (Riken), 2-1 Hirosawa, Wako-shi, Saitama 351-01, Japan
Tel. +81-48-462-1111 ext. 5723; Fax +81-48-462-4672
e-mail: mkitada@postman.ribon.go.jp

S. Morotomi · K. Horikoshi
Bioengineering Laboratory, Department of Applied Chemistry, Toyo University, Kawagoe, Saitama 350, Japan

Introduction

Investigations of how pH homeostasis is achieved in various bacterial species have focused on the role of a Na^+/H^+ antiporter or K^+/H^+ antiporter in the cell membrane (Booth 1985; Krulwich 1986; Padan and Schuldiner 1994). It has been suggested that a Na^+/H^+ antiporter is involved in regulation of intracellular pH in alkaline environments. *Escherichia coli* possesses at least two distinct Na^+/H^+ antiporters (Nha A, Nha B), and the cell becomes sensitive to high salinity and alkaline pH when the *nhaA* gene is deleted from the chromosome (Karpel et al. 1988, 1991; Pinner et al. 1992, 1993; Taglicht et al. 1991).

In our previous reports, we reported that a membrane potential (ΔΨ)-dependent Na^+/H^+ antiporter played a crucial role in pH homeostasis within an alkaline range in alkaliphilic *Bacillus* species (Hamamoto et al. 1994; Kitada et al. 1989, 1994; Kitada and Horikoshi 1992; Kudo et al. 1990). On the other hand, there were indications that a K^+/H^+ antiporter functioned in pH homeostasis when the cytoplasmic pH was alkalinized. The glycolyzing cells of *Streptococcus faecalis* (*Enterococcus hirae*), suspended in an alkaline medium, extruded K^+ against a K^+ concentration gradient by exchange for H^+ via an ATP-driven K^+/H^+ antiport system (Kakinuma and Igarashi 1995). Thus, the pH_{in}-responsive primary K^+/H^+ antiport system worked for pH_{in}-regulation of this strain growing at a high pH. Cells of *Vibrio alginolyticus* expelled K^+ through a K^+/H^+ antiporter that was driven by an outwardly directed K^+ gradient (Nakamura et al. 1984). This K^+/H^+ antiporter functioned as a regulator of cytoplasmic pH to maintain a constant value of 7.8. However, K^+/H^+ antiport systems in membrane vesicles of these strains have not been demonstrated. Brey et al. first discovered a K^+/H^+ antiporter in everted membrane vesicles of *Escherichia coli* (Brey et al. 1980). This K^+/H^+ antiport system was characterized by the effect of K^+ on the pH gradient formed by oxidation of lactate, and functioned to extrude K^+ from inside at the expense of the ΔpH component of the proton motive force (pmf). There was also an indication that a K^+/H^+ antiporter was present in an alkaliphile. Mandel et al. (1980) have suggested that a K^+/H^+ antiporter could be involved in regulation of the cytoplasmic pH in a lower pH range. However, the exact physiological role of the K^+/H^+ antiporter has not yet been elucidated.

During studies on the $\Delta\Psi$-dependent Na^+/H^+ antiporter in *Bacillus* sp. no. 66, we detected uphill H^+ influx into the right-side-out vesicles produced by an outwardly directed K^+ gradient without imposition of $\Delta\Psi$. The present work was conducted to characterize the K^+/H^+ antiporter in membrane vesicles of this strain. We also studied its kinetic properties and the reverse reaction.

Materials and methods

Bacterial strain and growth conditions

An alkaliphilic *Bacillus* sp. no. 66 (JCM 9763) was grown at 37°C in an alkaline medium (pH 10) with shaking as described previously (Kitada and Horikoshi 1987). Taxonomical properties of this strain will be described elsewhere.

Preparation of membrane vesicles

The right-side-out membrane vesicles were prepared using a modification of Kaback's method (Kitada et al. 1982). K^+-loaded vesicles were prepared by lysing protoplasts in 20 mM Tris chloride buffer (pH 9) containing 100 mM KCl and 5 mM $MgSO_4$ (the dilution buffer). The salt composition was suitably changed according to each experiment and the internal cation content was kept at 100 mM. Fluorescein isothiocyanate (FITC)-dextran-containing vesicles were prepared by diluting protoplasts into the dilution buffer containing FITC-dextran (average MW 40 000) at 7.5 μM concentration. External probe was removed by washing with dilution buffer. The everted vesicles were prepared by French press lysis of protoplasts. Protoplasts were suspended with 10 mM Tris chloride buffer (pH 9) containing 100 mM choline chloride, 5 mM $MgSO_4$, 0.05 mM dithiothreitol, and 10% glycerol, and were lysed in a French pressure cell at 500 kg/cm^2. The lysed suspension was treated with 5 μg/ml of DNase and centrifuged at $12\,000 \times g$ for 10 min. Then, the supernatant suspension was centrifugred at $180\,000 \times g$ for 1 h and washed once with the same buffer. The vesicles were suspended at a final protein concentration of 30 mg/ml in the buffer solution.

Measurement of K^+/H^+ antiporter activity

The H^+ movement through the K^+/H^+ antiporter was monitored using quenching of quinacrine dye (Tsuchiya and Takeda 1979) or FITC-dextran fluorescence (Brierly 1988). Right-side-out vesicles prepared as described were concentrated to about 15 mg protein per ml with the dilution buffer. Aliquots (5 μl) of vesicle suspension were diluted into 2 ml of 20 mM Tris chloride buffer (pH 9) containing 100 mM choline chloride, 5 mM $MgSO_4$ (the reaction buffer), and 2 mM quinacrine. The fluorescence quenching of quinacrine was measured in a Hitachi 650-40 fluorometer (Hitachi, Tokyo, Japan) with excitation at 420 nm and emission at 500 nm. The measurement of pH_{in} by FITC-dextran was carried out as follows. Aliquots (5 μl) of FITC-loaded concentrated vesicle suspension (pH 9) were diluted into 2 ml of reaction buffer and the change in fluorescence was monitored with excitation at 480 nm and emission at 520 nm. At the end of each experiment, gramicidin was added into the reaction mixture at 10 μM concentration. Then, $pH_{in}(=pH_{out})$ was varied by successive additions of HCl solution, and changes in $pH_{in}(=pH_{out})$ and fluorescence intensity were measured at the same time. Thus, the relation of fluorescence intensity to pH_{in} was obtained. In this experimental condition, ΔF was linearly related to ΔpH in the pH range from 6.8 to 7.8. A K^+/H^+ antiport activity in everted membrane vesicles was inferred based on its ability to collapse a transmembrane ΔpH generated by the addition of the electron donor. The reaction mixture (2 ml) contained 10 mM Tris chloride (pH 9), 100 mM choline chloride, 5 mM $MgSO_4$, 2 μM quinacrine, and 0.6 mM NADH (Tris salt). After the steady state value of ΔpH was reached, 10 mM KCl or RbCl was added and the extent of the fluorescence enhancement was recorded.

Fluorometric determination of intravesicular K^+ concentration

Decrease in intravesicular K^+ concentration was monitored by measuring fluorescence of potassium fluorescent indicator (PBFI)-loaded right-side-out vesicles (Jezek et al. 1990). The fluorescent K^+ indicator, PBFI, was added into 100 μl of concentrated vesicles at a final concentration of 280 μM. The suspension was then sonicated for about 1 min at 6-min intervals during a 1-h incubation in a bath sonicator. External probe was removed by successive washings with probe-free buffer. The fluorescence of PBFI-loaded vesicles was measured in a fluorometer with excitation at 343 nm and emission at 500 nm.

Determination of membrane potential

The diffusion potential (inside negative) was measured by assaying the quenching of rhodamine 6G as described previously (Kitada and Horikoshi 1992).

Protein determination

Protein amounts were determined by the method of Lowry et al. (1951) with a DC protein assay kit (Bio-rad, Hercules, CA, USA).

Chemicals

Choline chloride, valinomycin, gramicidin, FITC-dextran (average MW 40000), and PBFI were from Sigma (St.

Louis, MO, USA). All other chemicals were obtained commercially at the highest level of purity available.

Results

K^+/H^+ antiport system in right-side-out membrane vesicles of *Bacillus* sp. no. 66

When K^+-loaded vesicles of *Bacillus* sp. no. 66 were suspended in K^+-free Tris chloride buffer (pH 9, 20 mM) containing 100 mM choline chloride, fluorescence quenching of quinacrine was observed, showing that an inside-acidic ΔpH was formed during this process (Fig. 1A). Rb^+ could replace K^+ but Na^+ or Li^+ could not Furthermore, if K^+-loaded vesicles were suspended in 100 mM KCl buffer, fluorescence quenching of quinacrine was not observed. This showed that an outwardly directed K^+ gradient was necessary for the uphill influx of H^+. This reaction strongly depended on the medium pH, and H^+ influx was maximal at a pH around 8.4 (see later). The addition of valinomycin (10 μM) gave a slight inhibitory effect on H^+ influx, showing that the formation of a membrane potential (inside negative) did not stimulate H^+ influx.

The efflux of K_{in} was monitored by the PBFI fluorescence change under the same condition as for H^+ influx. As shown in Fig. 1B, a rapid decrease in PBFI fluorescence was observed when K^+-loaded vesicles were diluted into the K^+-free buffer. Calibration of PBFI fluorescence corresponding to K^+ concentration in vesicles was carried out by adding gramicidin and known amounts of K^+ into the reaction mixture at the end of K^+ efflux. These results indicated that the outwardly directed K^+ gradient was utilized to generate an inside-acidic ΔpH by means of the K^+/H^+ antiporter. This uphill influx of H^+ has not been observed in other alkaliphiles such as *Bacillus alcalophilus* (Mandel et al. 1980), *Bacillus firmus* RAB (Krulwich et al. 1982), *Bacillus* sp. C-125 (Kitada et al. 1994), and *Bacillus* sp. N-6 (Kitada and Horikoshi 1992).

Effect of quinine and Mg^{2+} on K^+/H^+ antiporter

To test whether H^+ might be transported via the K^+/H^+ antiporter, the effect of quinine, a specific inhibitor of the K^+/H^+ antiporter (Garlid et al. 1986), on the K^+/H^+ antiport activity was examined. Figure 2A shows the inhibition of H^+ influx by the addition of quinine into the reaction buffer up to 100 μM. Addition of 25 μM quinine inhibited the H^+ influx by 50%. When quinine was increased to 100 μM, the reaction was inhibited by 70%. A similar result was obtained by the addition of quinidine (not shown).

It has also been shown that the internal Mg^{2+} concentration affects the activity of the K^+/H^+ antiporter (Kakar et al. 1989). The right-side-out vesicles loaded with different levels of Mg^{2+} were diluted into the reaction buffer containing a Mg^{2+} concentration equal to internal Mg^{2+}, and the

Fig. 1. K^+-gradient (ΔK^+)-dependent H^+ influx (**A**) into, and K^+ efflux (**B**) from, right-side-out membrane vesicles of *Bacillus* sp. no. 66. **A** The quenching of quinacrine was initiated by addition of K^+-loaded vesicles (75 μg protein) into choline chloride buffer (pH 9) in the presence (*b*) or absence (*c*) of 10 μM valinomycin. The control experiment (*a*) was performed in KCl buffer (pH 9) instead of choline buffer. **B** The experimental conditions were the same as for Fig. 1A (c). K^+ loss was monitored by the decrease in potassium fluorescent indicator (PBFI) fluorescence. Intravesicular K^+ concentration was determined from calibration of PBFI fluorescence corresponding to K^+

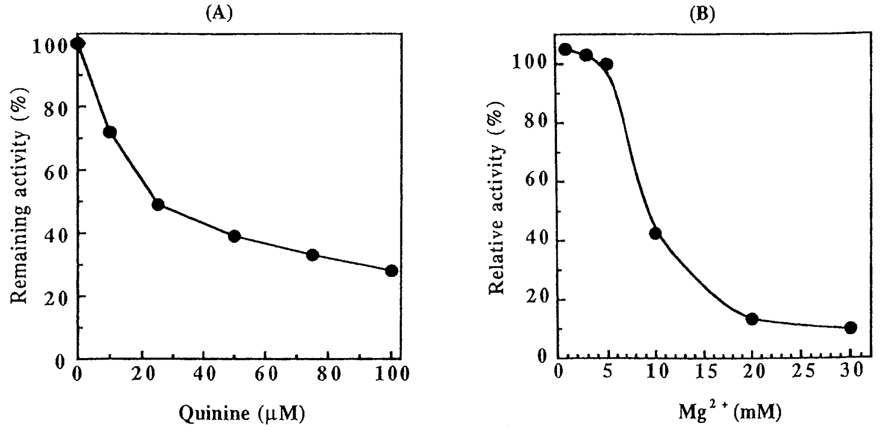

Fig. 2. Inhibition of H^+ influx by quinine (**A**) or Mg^{2+} (**B**). **A** Initial rates of H^+ uptake, obtained from initial slopes of data traces such as those of Fig. 1, were obtained in the presence of various concentrations of quinine. Rates are expressed as percent of the control experiment without inhibitor. **B** Right-side-out vesicles were prepared in KCl buffer (pH 9) containing different amounts of Mg^{2+}. Initial rates of H^+ uptake were obtained as described in **A**, and rates were expressed as percentage of the control experiment with 5 mM Mg^{2+}

quenching of quinacrine fluorescence was observed. As shown in Fig. 2B, the increase of Mg^{2+} concentration from 1 mM to 30 mM inhibited the influx of H^+. These results show that the decrease of internal Mg^{2+} concentration stimulates K^+/H^+ antiport activity, similarly to the K^+/H^+ antiporter in other systems (Bernaldi and Azzone 1983; Brierly and Jung 1990; Wieczorek et al. 1991). When the vesicles loaded with 1 mM Mg^{2+} were diluted into the reaction buffer containing 20 mM Mg^{2+}, the H^+ influx rate was nearly identical to that when diluted into 1 mM Mg^{2+} buffer. In contrast, 20 mM Mg^{2+}-loaded vesicles exhibited no increase in H^+ influx when diluted into 1 mM Mg^{2+} buffer. Therefore, it is clear that the internal Mg^{2+} content had a significant effect on K^+/H^+ antiport activity when it operated in the direction of H^+ influx.

Effect of pH

The effect of pH on K^+/H^+ antiport activity was examined by measuring H^+ influx into right-side-out vesicles at different pH values. Since quinacrine fluorescence was influenced by pH, we used FITC-dextran instead of quinacrine as a fluorescence probe. K^+-loaded vesicles containing FITC-dextran were diluted into K^+-free choline chloride buffer at different pH values and the steady state was attained in 2 min. The magnitude of ΔpH at steady state was calculated by the decrease of FITC-dextran fluorescence. As shown in Fig. 3, ΔpH increased as pH increased from 6.8 to 8.4, indicating this K^+/H^+ antiporter was pH-dependent.

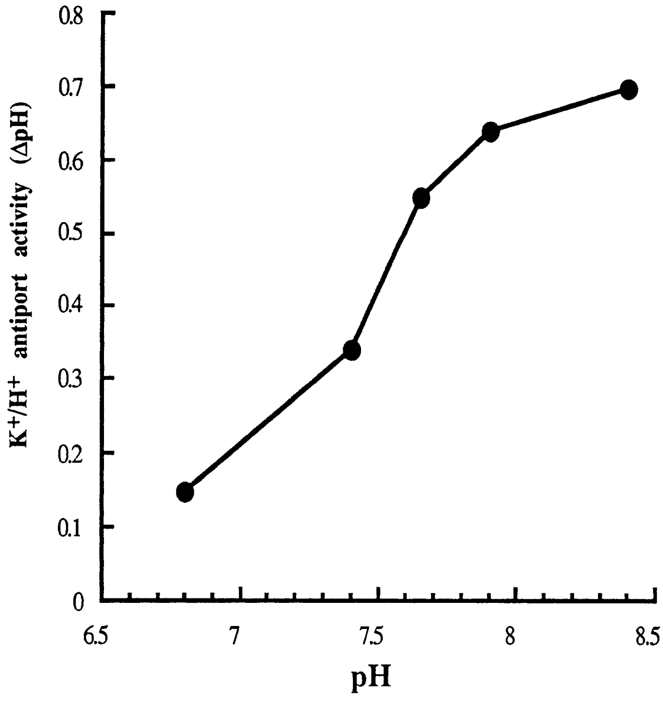

Fig. 3. pH dependence of the K^+/H^+ antiporter. The activity of the K^+/H^+ antiport system was assayed by fluorescein isothiocyanate (FITC)-dextran fluorescence. K^+-loaded vesicles containing FITC-dextran were prepared in Tris-chloride buffer (20 mM) at pHs ranging from 6.8 to 8.4, and 5 μl aliquots (75 μg protein) were diluted in 2 ml of choline chloride buffer at the same pH as the internal pH. The K^+/H^+ antiport activity was expressed as the pH gradient (ΔpH) formed during a 2-min incubation

Effect of extracellular K^+ concentration and pH on the rate of H^+ efflux

As mentioned, when K^+-loaded right-side-out vesicles were diluted into K^+-free buffer, H^+ influx into vesicles was observed and an acid-interior ΔpH was maintained. Addition of K^+ at the point of maximum ΔpH formation caused a rapid H^+ efflux by the inward exchange of K^+ for intravesicular H^+ on the K^+/H^+ antiporter. The response to K^+ was immediate, and Rb^+ and Cs^+ could replace K^+ but Na^+ or Li^+ could not (Fig. 4). This K^+-dependent alkalinization of pH_{in} was determined using FITC-dextran (Brierly et al. 1988). The right-side-out vesicles were prepared in the dilution buffer containing FITC-dextran at pH 9. When the vesicles were diluted into choline chloride buffer at pH 8.5, 8, or 7.5, pH_{in} rapidly decreased and then reached the steady state value of 7.18, 6.97, or 6.86, respectively. Addition of K^+ at this point caused a rapid alkaline shift in pH_{in}. As shown in Fig. 5, the rate of alkalinization showed a hyperbolic relationship to K^+ concentration and increased with increasing pH_{out} from 7.5 to 8.5.

Stoichiometric analysis

In order to test whether this K^+/H^+ antiport reaction is electroneutral or electrogenic, the formation of a membrane potential during the K^+–H^+ exchange reaction was measured by a fluorescence method (Kitada and Horikoshi 1992). As shown in Fig. 6, during H^+ influx via the K^+/H^+ antiporter, there was no change in fluorescence of rhodamine 6G, indicating that an interior-negative membrane potential was not generated. This shows that the stoichiometric ratio between K^+ efflux and H^+ influx is not more than 1. Furthermore, if this ratio is less than 1, i.e., $K^+/H^+ < 1$, the imposition of an interior-negative potential should stimulate H^+ influx. However, this was not the case, be-

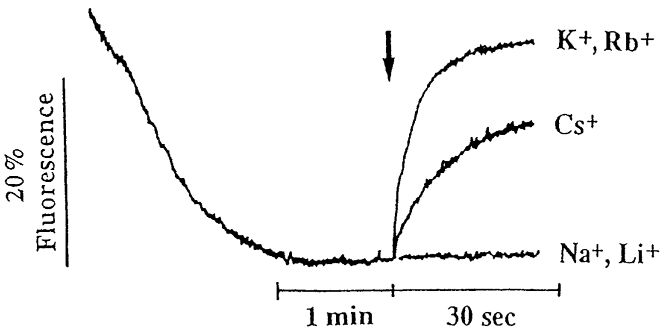

Fig. 4. K^+-dependent H^+ efflux as followed by the reversal of quinacrine quenching. An inside-acidic ΔpH was generated as described in Fig. 1a. At the point of the maximum ΔpH, the various cations indicated were added at the final concentration of 20 mM for Cs^+, Na^+, and Li^+, and 10 mM for K^+ and Rb^+

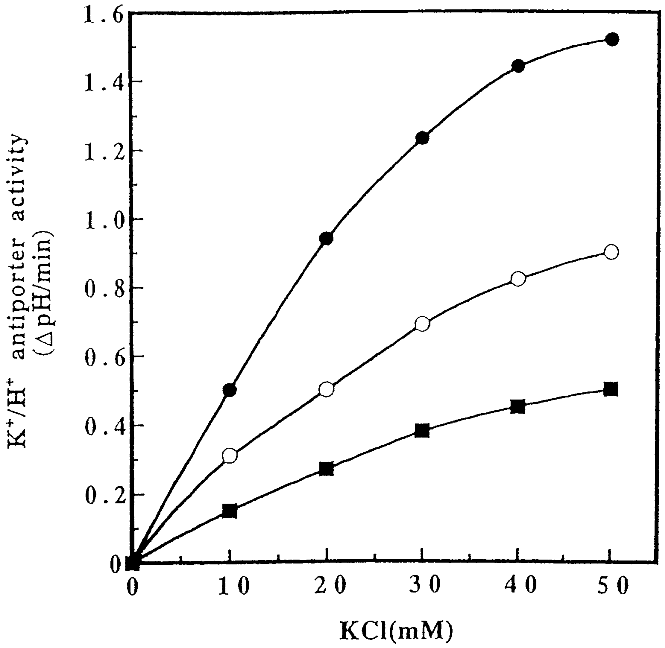

Fig. 5. Effect of extravesicular K^+ concentration on H^+ efflux. An inside-acidic ΔpH was created in the presence of FITC-dextran at pH 7.5 (*squares*), 8.0 (*open circles*), and 8.5 (*closed circles*). Then, the initial rate of H^+ efflux was estimated from the initial rate of increase in FITC-dextran fluorescence following the addition of various amounts of KCl

cause, as shown in Fig. 6, the addition of valinomycin had no stimulatory effect on H^+ influx. In order to clarify the stoichiometric relationship in more detail, the Static Head method was applied to this K^+/H^+ antiport reaction (Ahearn et al. 1990; Turner and Moran 1982). The experiment in Fig. 1a was carried out in the presence of ΔpH (pH_{in} 8.5; pH_{out} 9.5). At 10 mM external K^+, when K^+_{in}/K^+_{out} was equal to H^+_{in}/H^+_{out}, there was no measurable influx of H^+ while at K^+_{out} concentrations less than 10 mM, that is, when K^+_{in}/K^+_{out} exceeded H^+in/H^+_{out}, H^+ influx was observed. These results supported the conclusion that the transport stoichiometry of this K^+/H^+ antiporter was electroneutral.

pH homeostasis

The results obtained raised the question as to whether the K^+/H^+ antiporter plays a role in pH homeostasis in this strain under physiological conditions. Therefore, the K^+/H^+ antiporter was measured in everted membrane vesicles under respiration. The K^+/H^+ antiporter activity was estimated by the partial abolishment of the ΔpH which was formed by oxidation of NADH in everted vesicles. As shown in Fig. 7, addition of KCl (or RbCl) to respiring everted vesicles resulted in transient alkalinization of the interior, indicating that protons were pumped out during respiration and then some of them returned into the vesicles through the K^+/H^+ antiporter. Furthermore, when right-

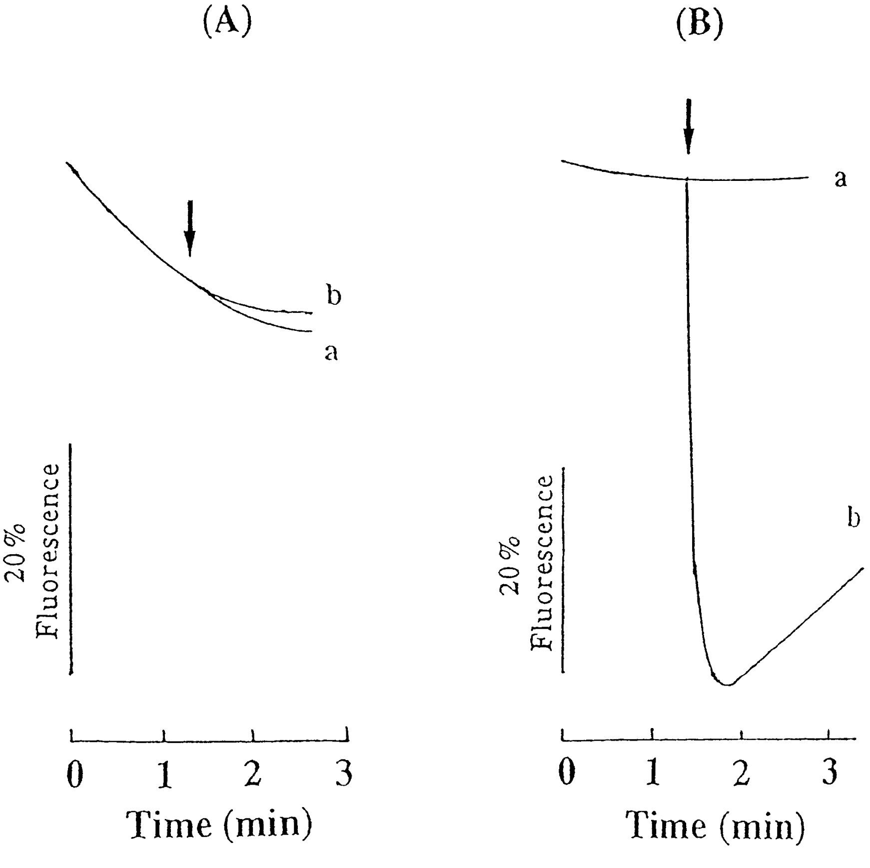

Fig. 6. Changes of a membrane potential during a K^+–H^+ exchange reaction in membrane vesicles of *Bacillus* sp. no. 66. Right-side-out membrane vesicles(50 µg protein) were diluted into the reaction buffer containing 2 µM quinacrine (**A**) or 10 µM rhodamine 6G (**B**), and the H^+ influx (**A**) and the formation of membrane potential (**B**) were monitored. *Arrows* indicate the addition of 0 µM (*a*) or 10 µM (*b*) valinomycin. The quenching of rhodamine 6G (**B** *b*) shows the formation of an inside-negative potential

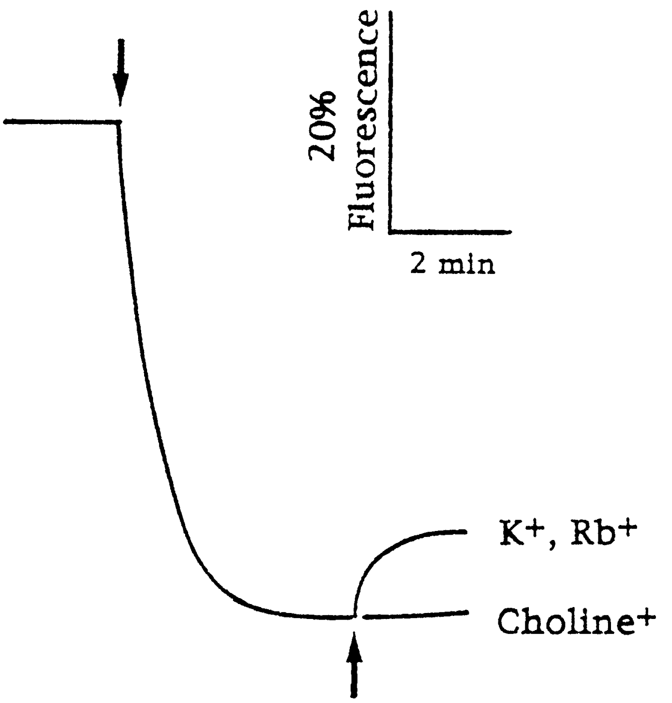

Fig. 7. Antiporter activity of everted membrane vesicles from *Bacillus* sp. no. 66. Antiporter activity was measured by monitoring the fluorescence of quinacrine. As indicated by the *first arrow*, the energy donor Tris-NADH (at the final concentration of 0.6 mM) was added to initiate quenching. After the addition of the appropriate cations at the final concentration of 10 mM (the *second arrow*), antiporter activity was measured as the reversal of quenching

side-out membrane vesicles loaded with K^+ were suspended in Tris-chloride buffer containing K^+ and energized with ascorbate plus tetramethyl-*p*-phenylenediamine (TMPD) at pH 7, these vesicles generated a small, but distinct, inside-acidic ΔpH (data not shown). Therefore, the K^+/H^+ antiporter in this strain contributes to acidification of the vesicle interior at an alkaline region (pH9).

Discussion

Uphill H^+ influx was detected in right-side-out vesicles of *Bacillus* sp. no. 66 strain produced by an outwardly directed K^+ gradient without imposition of ΔΨ (interior negative). This H^+ influx was increased at elevated pH, was sensitive to internal Mg^{2+} concentration, and was inhibited by quinine. The H^+ influx was reduced in right-side-out vesicles prepared in the presence of trypsin (data not shown), indicating that this K^+/H^+ antiporter was trypsin-sensitive, as observed in everted vesicles of *E.coli* (Brey et al. 1980). These properties were the same as those of the K^+/H^+ antiporter found in other systems (Garlid et al. 1986; Kakar et al. 1989; Bernaldi and Azzone 1983; Brierly and Jung 1990; Wieczorek et al. 1991; Brierly et al. 1984), and supported the existence of a K^+/H^+ antiport system in isolated membrane vesicles of *Bacillus* sp. no. 66. Although the physiological properties of this strain have not been described in this paper, this strain was isolated on an alkali minimal medium composed of DL-lactate, $(NH_4)_2SO_4$, K_2HPO_4, and $MgSO_4$ at pH 10. This strain was able to grow in the presence of 200 mM KCl at pH 10 whereas *Bacillus* sp. C-125 was not able to grow at 100 mM KCl. However, the role of the K^+/H^+ antiporter in K^+ tolerance cannot be decided at the present time. For K^+-dependent H^+ efflux, a hyperbolic relation to external K^+ concentration was shown. The efflux of H^+ was much more rapid than the influx of H^+, as seen in Fig. 1A, and decreased with decreasing pH. A similar inhibitory effect of H^+_{out} on K^+-dependent H^+ efflux was observed in beef heart mitochondria, where H^+_{out} acted as a mixed-type inhibitor (Brierly and Jung 1990). On the other hand, in the K^+/H^+ antiporter of the Sf9 insect cell line, the apparent K_m for K^+-dependent H^+ efflux was not very dependent on H^+_{out} (Vachon et al. 1995).

Stoichiometric analysis of the Na^+–H^+ or K^+–H^+ exchange reaction is very important for the study of their cellular functions. The coupling ratio for Na^+–H^+ exchange has usually been determined by measuring the H^+-gradient-driven $^{22}Na^+$ flux. Since the use of radiolabeled K^+ was not available because of its short half life, we examined the stoichiometry of this K^+/H^+ antiport by measuring K^+-gradient-driven H^+ influx with quinacrine fluorescence. Our results supported the conclusion that an electroneutral K^+–H^+ exchange took place in this K^+/H^+ antiporter. It is necessary to determine quantitatively the H^+ uptake by vesicles. In this respect, the use of FITC-dextran for pH_{in} determination seems to be apposite, because the ΔpH thus determined can be converted to H^+ flux by titrating with HCl in the presence of gramicidin. FITC-dextran was effective in a pH range between 6.5 and 8.0 in our experimental conditions. Therefore, it was not proper to apply this fluorescence probe to H^+ influx measurement because the optimum H^+ influx took place at a pH around 9 and the initial rate of H^+ influx could not be determined accurately. On the other hand, once pH_{in} reached a steady state value at pH 6.8–7.2 through the K^+/H^+ antiporter, alkalinization of pH_{in} by the addition of KCl could be measured by the increase of fluorescence intensity, which was linearly related to the increase of pH_{in} at a pH range from 6.5 to 7.8. Further work, including measurements of rates of K^+ transport, will be necessary to determine a precise stoichiometry for this K^+/H^+ antiporter. Since Rb^+ can replace K^+, the coupling ratio for Rb^+–H^+ exchange could be determined by the use of radiolabeled Rb^+ ($^{86}Rb^+$).

The physiological role of this K^+/H^+ antiporter remains to be determined. There are some indications that a K^+/H^+ antiporter plays a role in pH homeostasis in bacteria. Brey et al. (1980) examined properties of the K^+/H^+ antiporter of *E.coli* and postulated that K^+/H^+ exchange via the K^+/H^+ antiport system regulated cytoplasmic pH rather than Na^+/H^+ exchange via the Na^+/H^+ antiport system. In the intact cells of *V. alginolyticus*, it was suggested that the K^+/H^+ antiporter functions as a regulator of cytoplasmic pH to maintain a constant value of 7.8 over a pH range of 6–9, and the Na^+/H^+ antiporter constitutes a part of the pH homeostasis mechanism (Nakamura et al. 1984). Mandel et al. (1980) suggested that the K^+/H^+ antiporter in

Bacillus alcalophilus could be involved in the regulation of cytoplasmic pH in a lower pH range. As already mentioned, the K^+/H^+ antiporter in this strain also seems to be involved in pH homeostasis in a similar way to that of *Bacillus alkalophilus*. However, the right-side-out vesicles loaded with Na^+ exhibited a larger ΔpH (acid in) than K^+-loaded vesicles upon energization with ascorbate and TMPD. This shows that the Na^+/H^+ antiporter in this strain plays a primary role in pH homeostasis at an alkaline pH range, just as seen in other alkaliphiles. The exact role of the K^+/H^+ antiporter remains to be elucidated.

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