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K^+/H^+-antiporter in membrane vesicles of an alkalophilic *Bacillus*.

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ABSTRACT

K^+-or Na^+ -gradient dependent movement of H^+ was observed with membrane vesicles of an alkalophilic *Bacillus* (sp. A-007). H^+ -gradient dependent release of K^+ was also observed. The K^+ -release activity was inhibited by NEM. Furthermeore, addition of ATP inside of the vesicles decreased the K^+ -release from the vesicles, and DCCD abolished the ATP effect.

FOOTNOTES

ATP ; adenosine-5' -triphosphate.
DCCD ; N, N' -dicyclohexlcarbodiimide.
FCCP ; carbonylcyanide *p*-trifluoromethylyhenylhydrazone.
NEM ; N-ethymaleimide.
Tris ; tris(hydroxymethyl) aminomethane.

INTRODUCTION

For the growth of an alkalophilic *Bacillus* (sp. A-007), in addition to alkaline pH, both Na^+ and K^+ are essential (ANDO et al, 1981a ; ANDO et al, 1983a). At the optimal growth condition, H^+ -gradient (in>out), which is reverse of that of mesophiles, is formed (ANDO et al, 1981a). As far as we examined, Na^+ -gradient is major drive force of many solutes (ANDO et al, 1981b ; ANDO et al, 1982). K^+ -or H^+ -gradient is responsible for phosphate transport (ANDO et al, 1983c). The information of the regulation mechanism of these cation gradients across the membrane is clearly important for understanding the bioenergetics of alkalophiles.

Most of above studies were done on the view point of solutes which were carried by these ion gradients. We examined the movement of H^+ and K^+ (or Na^+) using membrane vesicles. Some evidence that indicates the existence of K^+/H^+ -antiporter and Na^+/H^+ -antiporter are obtained. And the possibility that K^+ -pumping ATPase does some role in the transport is discussed.

MATERIALS AND METHODS

1. Cultivation of bacterium and preparation of membrane vesicles.

Bacillus sp. A-007 was grown aerobically as before (ANDO et al, 1981b), except glutamate synthetic medium (L-glutamic acid, 0.5 ; D-glucose, 1.0 ; Na_2CO_3, 2.0 ; KCl, 0.15% and minerals[1]) was used for liquid culture instead of GPY menium.

Membrane visicles were prpared according to lysozyme-protoplasts method (ANDO et al, 1981b) and loaded with Tris/HCl pH 7.5 (2.0 mM for the assay of H^+ -movement ; 25 mM for the assay of K^+ -release), 2.5 mM $MgCl_2$ and 0.4 M salt (NaCl, KCl, RbCl, LiCl or choline-Cl). The loaded vesicles were washed once with the same buffer containing 2.5 mM $MgCl_2$ and 0.4 M choline-Cl by centrifugation (30,000 g 10 min), and resuspended in the same buffer to 10 mg protein per ml.

Estimation of protein concentration was done according to that of Lowry et al (LOWRY et al, 1951), using bovine albumine as standard.

1) ; $MgSO_4 7H_2O$, 200 ; $MnSO_4 4H_2O$, 10 ; $FeSO_4 7H_2O$, 6 ; $CaCl_2$, 100 ; Na_2HPO_4 ; 500 ; NaH_2PO_4, 25 (mg/l).

2. Assay method for H^+ -movement.

Membrane vesicles (3 mg protein), which were prepared as above, were suspended in 0.4 M salt solution containing 2.5 mM $MgCl_2$ in total 12 ml (initial pH was about pH 8.45, at 37°C). pH difference of the mixture was monitored using a pH memter

(Hitachi, F-7ss II) to which a recorder (Hitachi, 056) was connected. Initial rate of the pH change was calculated using control without cation gradient.

3. **Assay method for K^+ -release from vesicles.**

0.4 M KCl loaded vesicles (3 mg protein, prepared as above) was suspended in 25 mM Tris/HCl (pH 6.2, pH 6.8, pH 7.4 or pH 8.5) containing 2.5 mM $MgCl_2$ and 0.4 M choline-Cl at 0°C (total 6 ml). After aliquot was sampled out as control, temperature of the mixture was jumped to 37°C. Aliquots were sampled out at time indicated in the figures, and half of each was immediately filtrated through membrance filter (pore size 0.45 μm, Toyo Roshi), which was washed previously with the same buffer without membrane vesicles for three times. K^+ concentration of the vesicles was calculated from K^+ concentration (which was determine using a flamephotometer) difference of the mixture with or without filtration. Used vesicles volume was 3.3 μl per mg protein (unpublished data).

4. **Chemicals.**

ATP (Mg salt) was purchased from Sigma. Tris, KCl, RbCl and NEM were obtained from Merk. FCCP was from Boehringer. Other chemicals were of the best grade commercially obtainable.

RESULTS

1. **Effect of K^+ -or Na^+ -gradient on H^+ -movement across the membranes.**

When K^+ -or Na^+ -gradient was imposed from outside of the vesicles, H^+ was released from vesicles (*e. g.* medium pH decreased) according to the magnitude

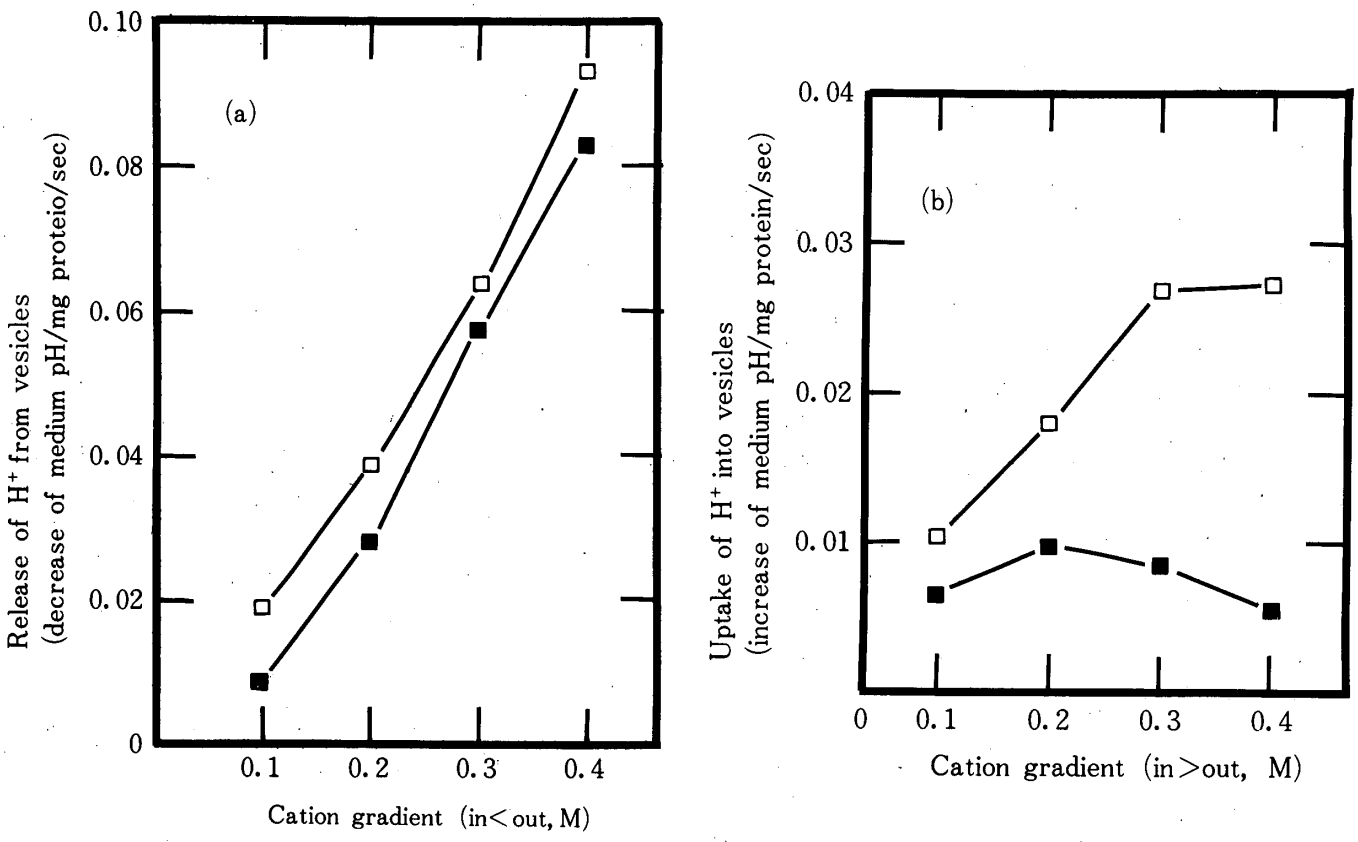

Fig. 1 Effect of Na^+ -or K^+ -gradient on H^+ -movement across the membrane.
Cation (Na^+ or K^+) gradient was fomed from outside to inside (a) or from inside to outside (b) of the vesicles. Assay mixture (total 12 ml, at 37°C) contained 2.5 mM $MgCl_2$, 0.4 M salts (NaCl and choline-Cl for □, KCl and choline-Cl for ■), and 0.4 Choline-Cl loaded vesicles (3 mg protein) for (a) or 0.4 M salt (NaCl, □ ; KCl, ■) loaded vesicles for (b). Other condition is written in MATERIALS AND METHODS.

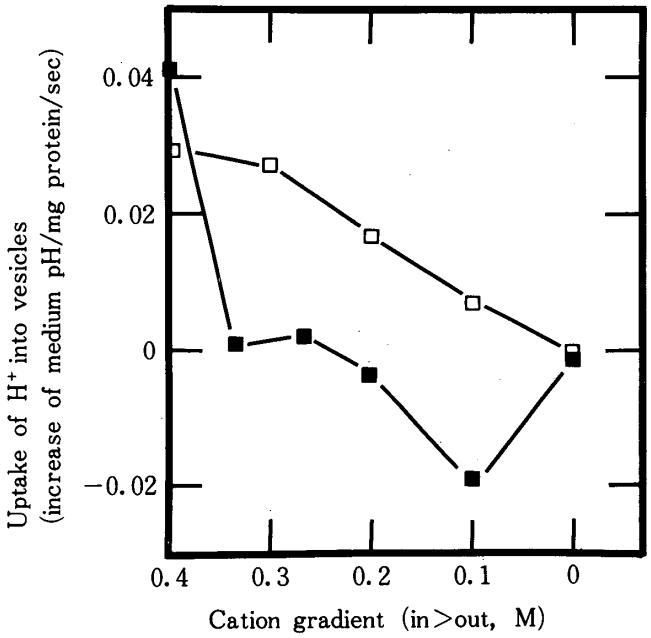

Fig. 2 Effect of K^+ -or Na^+ -gradient (in<out) on Na^+ -gradient (in > out) stimulated H^+-uptake activity.

Assay mixture (total 12 ml, at 37°C) contained 2.5 mM $MgCl_2$, 0.4 M salts (NaCl and choline-Cl, □ ; KCl and choline-Cl, ■), and 0.4 M NaCl-loaded vesicles (3 mg protein).

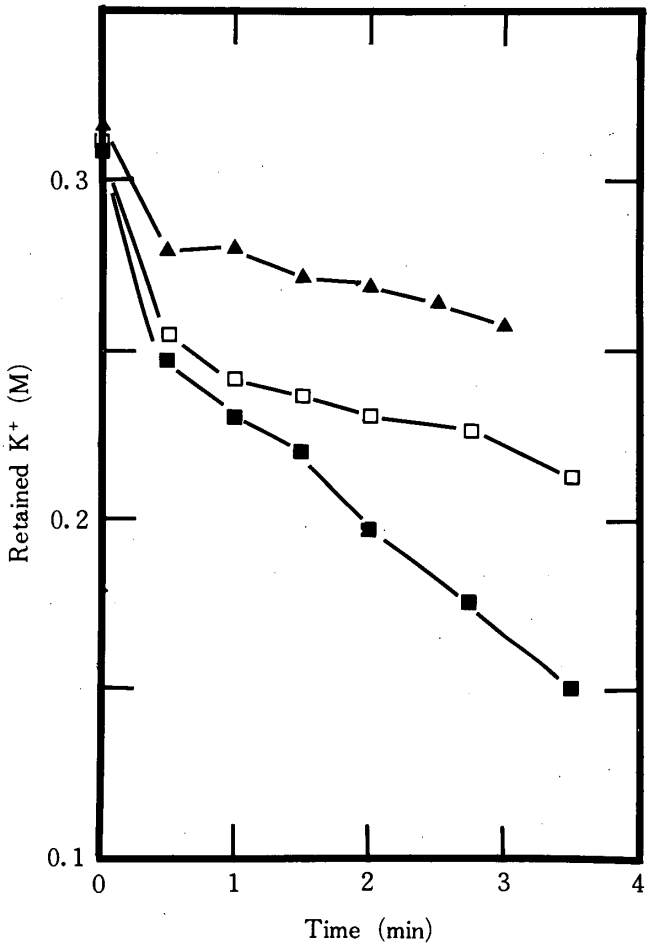

Fig. 3 Effect of H^+ -gradient on K^+ -release from K^+ -loaded vesicles.

Assay mixture (total 6 ml, at 37°C) contained 25 mM Tris/HCl (pH 6.2, ■ ; pH 7.5, □ ; pH 8.9, ▲), 2.5 mM $MgCl_2$, 0.4 M choline-Cl, and 0.4 M KCl-loaded vesicles (3 mg protein, prepared at pH 7.5).

of cation gradient (Fig. la). Rb^+ -gradient showed similar pattern as that of K^+ (data not shown). Li^+ -gradient was not effective as drive force (data not shown). The opposite K^+ -or Na^+ -gradient (in>out) caused uptake of H^+ into memberane vesicles (*e. g.* medium pH increased, Fig. 1b).

Although the cation dependency pattern of H^+ -release from vesicles was similar (Fig. la), that of H^+ -uptake was significantly different between Na^+ -and K^+ -dependent activity (Fig. 1b). Then Na^+ -gradient (0.4 M, in>out) was gradually abolished by imposing opposite Na^+ -or K^+ -gradient to clarify whether they shared a common antiporter or not. As shown in Fig. 2, decrease pattern of H^+ -uptake activity driven by net Na^+ -gradient was different between the counter ion gradient (Na^+ or K^+). H^+ was released according to the net magnitude of the Na^+ -gradient for the abolishment with Na^+ -gradient. But for the abolishment which K^+ -gradient, this was not the case. Namely, K^+ -gradient (in<out) seemed to cause H^+ -release even in the presence of reverse Na^+ -gradient, suggesting that Na^+ -and K^+ -dependent H^+ -antiport systems were different.

2. Effect of H^+ -gradient on K^+ -release from vesicles.

In above experiments, movement of H^+ across the membrane was monitored. Then effect of H^+ -gradient on K^+ -release from pre-loaded membrane vesicles was examined by monitoring K^+ -concentration of the vesicles using membrane filter method and flame-photometry. Although we tried to measure Na^+ -concentration of the vesicles, contamination of Na^+ from buffer-washed membrane filter hindered the measure-

ment.

When H^+-gradient (in, pH 7.4 < out, pH 6.2) was imposed, K^+-release activity was stimulated, and reverse H^+-gradient (in, pH 7.5 > out, pH 8.9) decreased the activity comparing with that of without H^+-gradient (in = out, pH 7.5) (Fig. 3). The H^+-gradient stimulation on K^+-release activity was abolished in the presence of protonophore (FCCP), suggesting direct coupling of H^+-and K^+-movement (Fig. 4a).

When H^+-gradient (in < out) stimulated K^+-release activity was measured in the presence of 1 mM NEM, the activity was slightly inhibited. Then, prior to the assay, K^+-loaded vesicles were incubated at 0 °C for 5 min with 10 mM, the activity was significantly inhibited (Fig. 4b). This result suggested that some SH-residue(s) played a significant role in H^+/K^+-translocation.

3. Effect of ATP on K^+-release activity.

As the presence of K^+-pumping ATPase was suggested in the previous paper (ANDO et al, 1983b), we also examined K^+-efflux from vesicles in which 2 mM Mg-ATP was loaded with 0.4 M KCl. As shown in Fig. 5, the loaded ATP decreased K^+-efflux, while with the ATP-loaded vesicles, which treated with 10 mM DCCD at 0°C for 5 min befor measurement of K^+-efflux, the activity was similar to that without ATP. 10 mM is enough concentration to inhibit ATPase activity of membrane at 100% (unpublished data).

DISCUSSION

Mandel et al (MANDEL et al, 1980) suggested the existence of cation/proton antiporters in memberane vesicles of *Bacillus alcalophilus*. They examined the movement of proton with cation-loaded vesicles using

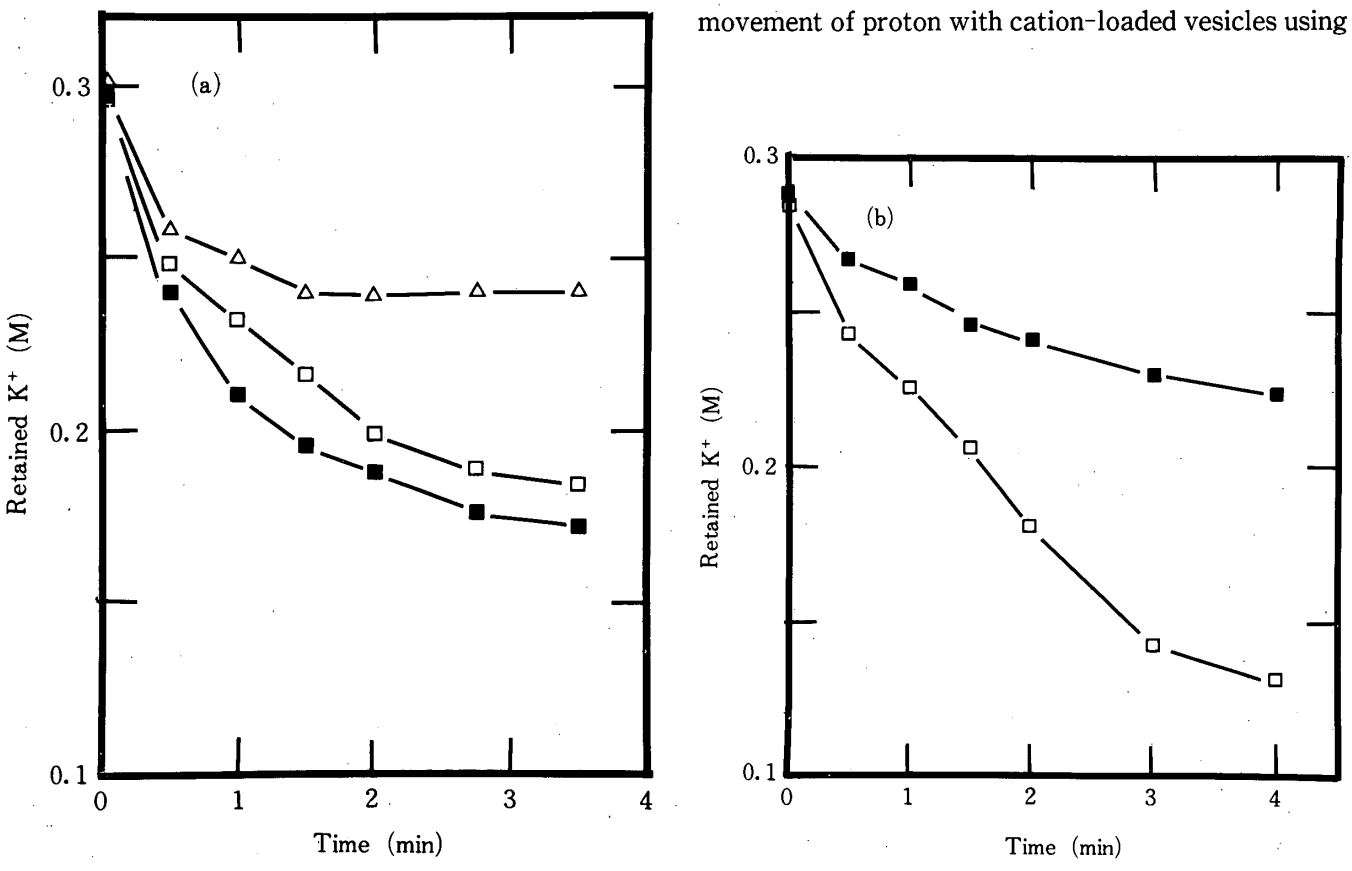

Fig. 4 Effect of FCCP or NEM on H^+-gradient stimulated K^+-release activity.
Assay mixture is the same as that of Fig. 3 in which pH 6.2 was used as extravesicular pH. For (a), with (■) or without (□) FCCP (2 μg/ml). For (b), with (□, △) or without (■) 1 mM NEM. and at △, KCl-loaded vesicles were pre-treated with 10 mM NEM at 0°C for 5 min and diluted to 10 fold with the assay mixture.

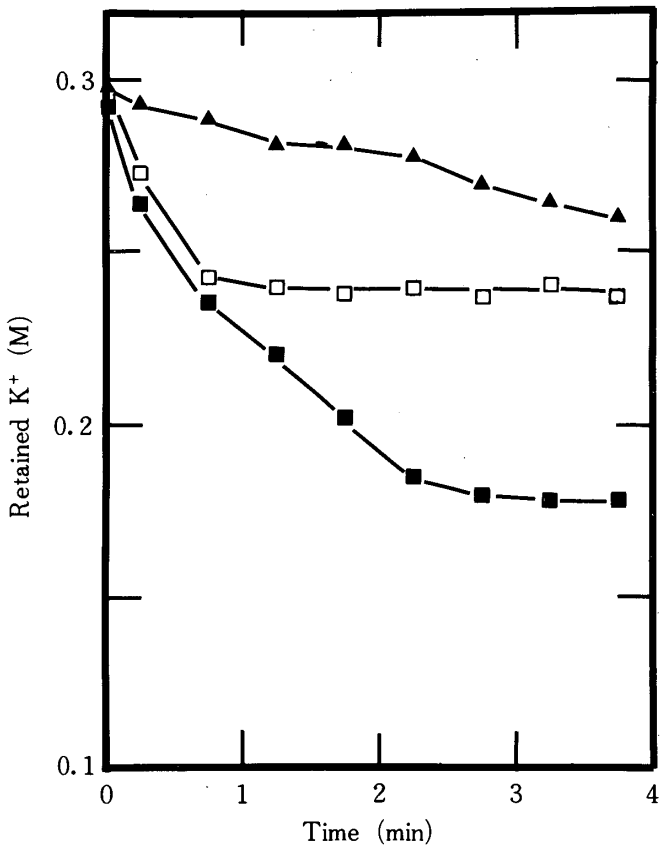

Fig. 5 Effect of ATP of K^+-release from K^+-loaded vesicles.

Assay mixture (total 6 ml, at 37°C) contained 25 mM Tris/HCl (pH 6.2, □, ■ ; pH 7.5, ▲), 2.5 mM $MgCl_2$, 0.4 M choline-Cl, and 2 mM Mg-ATP and 0.4 M KCl loaded vesicles (3 mg protein, prepared at pH 7.5). The loaded vesicles were pre-treated with (■, ▲) or without (□) 10 mM DCCD at 0°C for 5 min and diluted to 10 fold with the assy mixture.

respiratory chain as a primary proton pump. The data presented here showed more clearly the existence of K^+/H^+-antiporter as well as Na^+/H^+-antiporter in alkalophilic *Bacillus* sp. A-007. In alkalophiles, Na^+/H^+-antiporter seems to be responsible for the maintenance of intracellular pH, while the role of K^+/H^+-antiporter is not clearly understood (KITADA et al, 1982, KTADA & HORIKOSHI, 1985). In other alkalophile, the generation of membrane potential is idependent on the presence of Na^+ and K^+ (MATSUKURA & IMAE, 1983).

In this paper, existence of K^+-pumping ATPase was also suggested. This confirmed the previous relults in which K^+-stimulated ATPase and Na^+-and K^+-gradient stimulated ATPase activity was observed (ANDO et al, 1983a & 1983b). In other alkalophile, ATP synthesis was driven by membrane potential (diffusion potential of K^+) or proton gradient. So, it was concluded that the ATPase was H^+-ATPase (MATSUKURA & IMAE, 1985),

Na^+-pumping ATPase, which pumps Na^+ outside of the cell, is reported with *Streptococcus faecalis* (KAKINUMA & HAROLD, 1985). This Na^+-ATPase requires external K^+ for the exchanger of Na^+. For the further examination of these possioility, ATP synthesis experiment in which Na^+-and K^+-gradients are used as drive-forces, is necessary.

In some bacteria like alkalophiles, it is plausible to think that most (or some) part of $\Delta\bar{\mu}H$ in chemiosmotic theory is replaced by $\Delta\bar{\mu}Na$ (SKLACHEV, 1984). So, to examine cation/proton antiporter mechanisms is more important for understanding biochemical nature of alkalophiles.

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好アルカリ性 *Bacillus* 膜小胞のK^+/H^+-アンチポーター

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日本摘要

好アルカリ性 *Bacillus* (sp. A-007) 膜小胞においてK^+もしくはNa^+濃度勾配依存性のH^+の動きが認められた．また，H^+濃度勾配依存性のK^+の放出も観察された．このK^+放出活性はNEMにより阻害された．さらに，膜小胞内にATPを添加することにより，H^+濃度勾配依存性K^+放出活性が低下し，このATP効果はDCCDを加える事により無効化した．