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Internal Cation Concentrations and Resistant Ability to the Carbonyl Cyanide m-Chlorophenylhydrazone of the Halotolerant Bacterium *Brevibacterium* sp. and *Escherichia coli*

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ABSTRACT

Internal cation concentrations of the halotolerant bacterium, *Brevibacterium* sp., were investigated with the cells grown in complex medium. With increasing external NaCl concentrations, the amount of Na^+ ion in the cells increased, while intracellular concentrations of K^+ ion remained almost constant (about 300 mM). The same tendency was observed when NaCl was replaced with KCl, although Na^+ ion was very low (about 15 mM). On the contrary, intracellular concentrations of Mg^{2+} and Ca^{2+} decreased slightly with the increase in NaCl or KCl concentrations in the medium. When RbCl concentrations in the medium increased to more than 100 mM, Rb^+ ions in the cells increased about one hundred times, 550–600 mM, while K^+ ions decreased to one-tenth of their initial value. Internal cation concentrations of *Escherichia coli* were mainly occupied by Mg^{2+} and K^+ ions. Total cation concentrations in *E. coli* were low, about 200 mM, which was less than half of those in *Brevibacterium* sp.

When 50 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP), a proton conductor, was added to the growing cell suspension of *Brevibacterium* sp., the total cation concentration in the cells was reduced by half. At the same time, membrane potentials, efflux rates of K^+ ions from the cells, cell turbidities, and viable cell numbers of *Brevibacterium* sp. were decreased almost linearly in proportion to the incubation time with CCCP. The CCCP effect on these properties for *E. coli* was dependent upon the presence of glucose in the medium. In the presence of more than 30 mM glucose, the lethal effect of CCCP was quite remarkable. The intracellular cation concentrations, membrane potentials, and K^+ efflux rates of the cells were also sensitive to CCCP exposure, although the cell turbidities were unaffected. In contrast, CCCP did not cause any significant changes for *E. coli* in the absence of glucose. The CCCP effect for both strains was discussed from the interrelation between viability and internal cation concentrations.

INTRODUCTION

Internal cation concentrations in bacteria depend upon the species, concentrations and kinds of salts in the medium, and culture age. *Halobacterium* species which are extreme halophiles belonging to a member of the archaebacterial kingdom accumulate K^+, but not Na^+ as the major ion in the cytoplasm (Tindall and Trüper, 1986). Halobacteria use K^+ ions to regulate an osmotic balance. *Ectothiorhodospira halochloris*, an extremely halophilic eubacterium, produces betaine as an osmoregulatory solute without accumulating high concentrations of Na^+ in the cells (Galinski and Trüper, 1982). On the other hand, slightly and moderately halophilic micro-organisms have the tendency to increase the internal Na^+ concentrations in proportion to the NaCl concentration in the medium (Weisser and Trüper, 1985). Internal K^+ concentrations of these halophiles, however, was little affected by the external NaCl concentrations, except for some bacteria (Weisser and Trüper, 1985). In this respect, it seems to be of interest to quantitatively examine the changes of cation concentrations in the cells grown in the presence of salts other than NaCl. Furthermore, the culture age of halophilic micro-organisms influenced the amounts of cations in the cells (Kushner, 1988).

Concerning the energetic systems of halophilic bacteria which inhabit Na^+-rich environments and require Na^+ ions for growth, the importance of Na^+/H^+ antiporter via H^+ pump (Hamaide et al., 1985) and respiration-dependent primary Na^+ pump at alkaline external pH (Tokuda and Unemoto, 1982) has been pointed out. The former system is observed with a wide variety of cells and organisms (Krulwich, 1983), some of which have been known to possess the ability to grow in the absence of proton motive force (PMF), as reported by Kinoshita et al. (1984). The latter system is unique and only detected for the halophilic bacteria. To study the energy transducing systems inherent to a bacterium, it might be inevitable to establish the dissipation state of proton circulation across the membrane by a proton conductor such as carbonyl cyanide m-chlorophenylhydrazone (CCCP). Thus, we intended in this work to analyze the intracellular cation concentrations, especially Na^+ or K^+ ions, of the halotolerant bacterium *Brevibacterium* sp. and *Escherichia coli* in the absence and presence of CCCP. In addition, an attempt was made to examine the influence of PMF on the viability of both strains. Furthermore, the effect of CCCP on the release of K^+ ions from the cells at an alkaline external pH will be discussed.

MATERIALS AND METHODS

Organisms and growth

A halotolerant bacterium, *Brevibacterium* sp. (JCM 6894) isolated from a seawater sample, and *Escherichia coli* (ATCC 11775) were used in this work. The organisms were grown in a sterile complex medium which contained (g/l) Bacto-peptone (Difco), 5.0; Bacto-yeast extract (Difco), 1.0; $CaCl_2 \cdot 2H_2O$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.7; NH_4Cl, 1.0; K_2HPO_4, 0.035; KH_2PO_4, 0.015. Glucose medium was prepared by the addition of glucose, sterilized separately, to the complex medium. Amounts of glucose or salts added to the medium are given in the text. The pH of every medium was adjusted to 7.5 with tetramethyl ammonium hydroxide (TMAH), unless otherwise stated. Growth was measured as turbidity at 650 nm using a Shimadzu double-beam spectrophotometer model UV-200S.

The proton conductor, CCCP, was dissolved in dimethyl sulfoxide (DMSO) and added to the cultures at a final concentration of 50 μM. Numbers of viable cells after incubation together with CCCP were enumerated by counting the colonies formed on the complex medium containing 1.5% agar (3 days at 37°C). Protein content in the cells was assayed by a biuret method employing crystalline bovine serum albumin as a standard. For the viability and intracellular protein contents, triplicate determinations were made and averaged.

Determination of intracellular cation concentrations and K^+ efflux rates

The internal volume of *Brevibacterium* sp. was determined according to the procedures described by Rottenberg (1979). A 10 μ*l* solution of radiolabeled compounds (100 μCi ^3H_2O and 10 μCi ^{14}C-carboxylinulin) was added to 1 ml of a cell suspension containing about 2 mg protein. The mixture was incubated for 1 h and then centrifuged. After dissolving both supernatant and pellet fractions in 1 M $HClO_4$ and mixing them with scintillation liquid, they were assayed for ^3H and ^{14}C radioactivities in a Packard Model 2200CA liquid scintillation counter. 3.1 μl/mg protein was used as the cell volume of *E. coli* (Larsen et al., 1987). The internal cation concentrations in both strains were determined, as described previously (Nagata et al., 1991).

Efflux rates of K^+ ions from the cells were monitored in a diethanolamine (DEA)–HCl solution, pH 7–11 (50 mM), at 25°C by using a K^+ electrode (K-135, TOA Electronics Ltd., Japan) with a reference calomel electrode (HS-305DS).

Chemicals

^{14}C-carboxylinulin and ^3H_2O were obtained from Amersham International Plc., Buckinghamshire, England. CCCP was purchased from Sigma, St Louis, MO, USA. Other reagents were of analytical grade obtained from Nakarai Chemicals Ltd., Kyoto, Japan.

RESULTS

Internal cation concentrations in *Brevibacterium* sp.

Internal water space (IWS) in *Brevibacterium* sp. (inulin impermeable space) was determined with the cells grown in the complex medium (pH 7.5) containing 0–2 M NaCl or KCl. In the absence of salts, IWS tended to decrease a little with the alkalinization of medium pH; IWS was 5.5±0.1, 5.1±0.1, and 4.9±0.3 μl/mg protein at pH 7.5, 8.5, and 9.2, respectively. In contrast, IWS was almost constant under the acidification from 7.5 to 5.5. IWS decreased sharply with an increase in the external NaCl or KCl concentrations up to 0.5 M and then remained constant; e.g., 4.2±0.1 and 4.0±0.4 μl/mg protein for 0.5 and 2 M NaCl, respectively. These values were employed for the calculations of internal cation concentrations in *Brevibacterium* sp. described below.

Without the supplement of the salts, main cations detected in the cells at the stationary phase of growth were K^+ and Mg^{2+}. Both cations were highly concentrated in comparison with the cations in the broth (Fig. 1). In proportion to the increase in the external NaCl concentrations, the amount of Na^+ ions in the cells increased almost linearly (Fig. 1a). Up to 0.5 M extracellular NaCl concentrations, on the other hand, intracellular K^+ concentrations in-

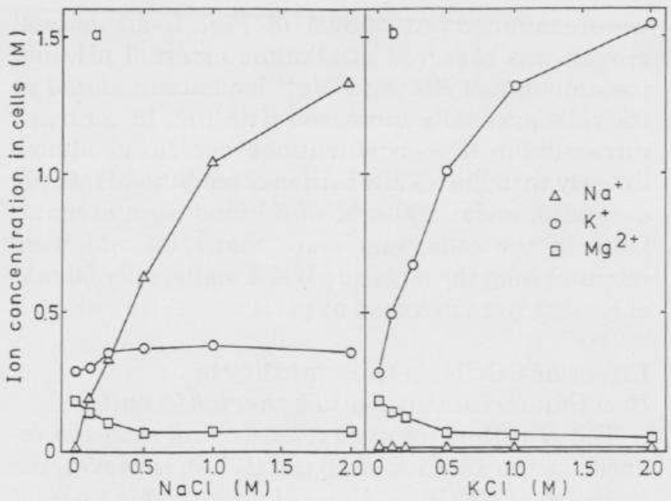


Fig. 1. Variations of the internal cation concentrations in *Brevibacterium* sp. as a function of the external NaCl or KCl concentrations. Incubation of the cells was carried out at 30°C in the complex medium (pH 7.5) containing 0–2 M NaCl (a) or KCl (b). At the stationary phases of growth, cells were harvested and washed. Analyses of the intracellular concentrations of Na^+, K^+, and Mg^{2+} ions were performed as described under "Materials and Methods".

creased a little, while those of Mg^{2+} ions decreased slightly; above 0.5 M, the concentrations of both cations were almost constant. Ca^{2+} concentrations in the cells were below 15 mM and tended to decrease a little with the increase in the NaCl concentrations (data not shown). As a result, total concentrations of the intracellular cations increased with the increase in the external NaCl concentrations.

When the KCl concentrations outside the cells were increased, on the other hand, the cells accumulated K^+ ions efficiently, but they did not accumulate Na^+, Mg^{2+}, and Ca^{2+} ions (Fig. 1b).

An optimal growth of *Brevibacterium* sp. was observed at pH 9.5–9.6 (Nagata, 1988). In this connection, pH effect on the intracellular ion concentrations was examined. As shown in Fig. 2, the internal K^+ concentrations in *Brevibacterium* sp. were 300 mM or more at pH values lower than 7. With the increase of medium pH, internal K^+ concentrations decreased and were less than 150 mM at pH higher than 9. In contrast, intracellular concentrations of Mg^{2+}, Na^+, and Ca^{2+} were little dependent on the external pH.

Effect of external concentrations of different kinds of salts on the internal cation concentrations in *Brevibacterium* sp.

The change in internal cation concentrations in *Brevibacterium* sp. was examined in response to the addition of external salts of different kinds (100 mM) in the medium. Regardless of the salt species added other than Na^+, intracellular Na^+ concentrations were 7–13 mM. When Na^+ ions were added to the medium, however, the concentration of Na^+ ions in the cells reached about 200 mM (Table 1). The amount of K^+ ions in the cells remained about 300 mM, irrespective of addition of salt except KCl or RbCl. By adding KCl to the medium, the effective accumulation of K^+ ions took place, while a remarkable decrease in K^+ ions in the cells was observed on the addition of RbCl. Intracellular concentrations of both Mg^{2+} and Ca^{2+} ions were little affected by the addition of monovalent cations, but the presence of divalent ones caused their distinct change.

Thus, a relationship of intracellular concentrations of Rb^+ and K^+ to external RbCl concentrations in the medium was quantitatively examined. In proportion to the increase in the external RbCl concen-

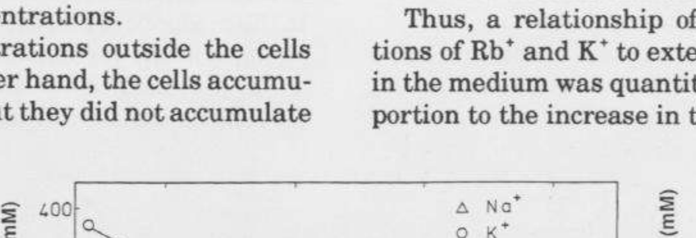


Fig. 2. pH dependence on the internal cation concentrations in *Brevibacterium* sp. Incubation of the cells was carried out at 30°C in the complex medium with pH 5.4–9.4 adjusted by the addition of HCl or TMAH. At the stationary phases of growth, cells were harvested and washed. Analyses of the intracellular concentrations of Na^+, K^+, Mg^{2+} and Ca^{2+} ions were performed as described under "Materials and Methods".

TABLE 1

Internal cation concentrations of *Brevibacterium* sp. grown in the complex medium (pH 7.5) supplemented with the salt (100 mM) indicated[a]

Salt	Intracellular concentration (mM)					Cell yield[b]
	Na^+	K^+	Mg^{2+}	Ca^{2+}	Total	
None	14	288	180	14	496	213
NaCl	192	298	140	18	648	259
KCl	8	493	142	17	660	245
RbCl	11	35	100	13	159	268
ChCl[c]	10	315	206	16	547	352
$MgCl_2$	7	366	473	8	854	343
$CaCl_2$	13	417	62	163	655	156
$NaNO_3$	181	276	134	14	605	359
NaSCN	204	266	144	12	626	304
Na_2SO_4[d]	138	327	160	13	638	392

[a] Cells were harvested after 24 h at stationary phase of growth.
[b] Cell protein concentration (mg) obtained from 1 l of the culture.
[c] Choline chloride.
[d] 50 mM Na_2SO_4 was used to make the equivalency of Na^+ concentration.

trations, the amount of Rb^+ ions in the cells increased, but those of K^+ and Mg^{2+} ions decreased (see Fig. 3). Eight mM was a critical concentration of external RbCl that exchanges the main cation for Rb^+. Below this critical concentration, the main cation was K^+ ion as usual.

In the medium with the critical concentration, 8 mM, the variations of growth and intracellular Rb^+, K^+, and Mg^{2+} concentrations with the external pH were examined. As shown in Fig. 4, an optimal growth was observed at alkaline external pH, and the amounts of Rb^+ and Mg^{2+} ions accumulated in the cells gradually increased with pH. In contrast, intracellular K^+ concentrations decreased almost linearly with the alkalinization of medium pH. At pH around 5, about 200 mM of K^+ ions were accumulated in the cells, and more than 100 mM were released from the cells at pH 8. Finally, only 50 mM of K^+ ions were detected at pH 9.

Effect of CCCP on the viability of *Brevibacterium* sp. and *Escherichia coli*

The growth rates of *Brevibacterium* sp. were reduced by the addition of 50 μM CCCP. However, the exposure of CCCP to *E. coli* had no effect at least during 48 h incubation. The changes in the turbidities for both strains were independent of the further addition of glucose to the culture medium (data not shown).

In order to quantitatively elucidate the effect of CCCP exposure, changes of the percent survival for both strains were directly obtained by counting the viable cell numbers (Fig. 5). Viability of *E. coli* grown in the medium was almost unchanged during 48 h incubation together with CCCP. Therefore, neither viability nor turbidities of *E. coli* were affected by CCCP when it was grown in the medium. In contrast, colony counts of *E. coli* sharply dropped at 8–12 h exposure to CCCP in the glucose medium. Over 24 h exposure, the survival percentage resulted in less than 1%. Such an abrupt decrease of viability in the presence of glucose was inconsistent with little changes in the turbidities after addition of CCCP.

On the other hand, the variations of the survival percentage for *Brevibacterium* sp. were decreased

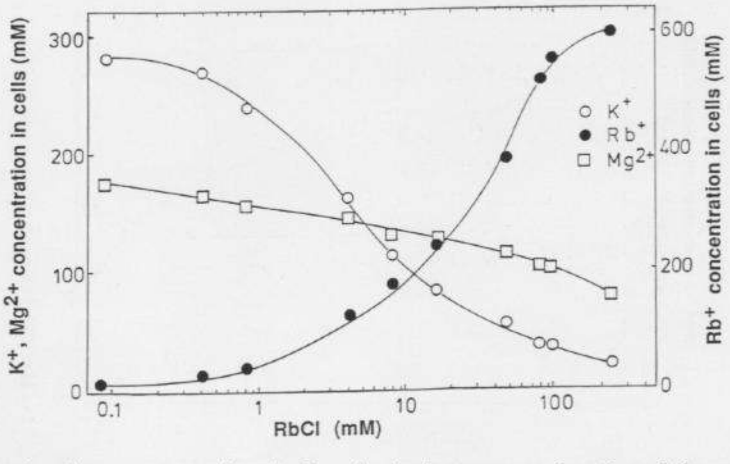
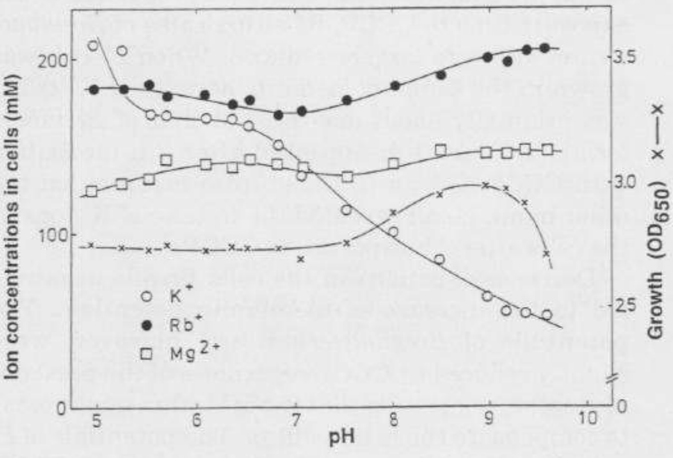


Fig. 3. Variations of the internal cation concentrations in *Brevibacterium* sp. as a function of the external RbCl concentrations. Incubation of the cells was carried out at 30°C in the complex medium (pH 7.5) containing various concentrations of RbCl. At the stationary phases of growth, cells were harvested and washed. Analyses of the intracellular concentrations of K^+, Rb^+ and Mg^{2+} ions were performed as described under "Materials and Methods".



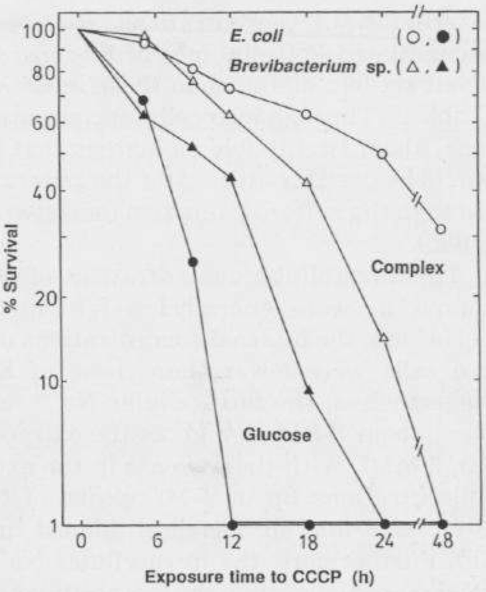


Fig. 4 (*above left*). **pH dependence on the internal cation concentrations in *Brevibacterium* sp. Incubation of the cells was carried out at 30°C in the complex medium containing 1 g/l of RbCl. pH of each medium was adjusted to be 4.9–9.5 by the addition of HCl or TMAH. After 24 h incubation, the growing cell suspension was turbidimetrically monitored at 650 nm (OD_{650}) for growth (x), and then harvested and washed. Analyses of the intracellular concentrations of K^+, Rb^+ and Mg^{2+} ions were performed as described under "Materials and Methods".**

Fig. 5 (*above right*). **Percent survival of *Brevibacterium* sp. and *Escherichia coli* as a function of exposure time to CCCP. Incubations of *Brevibacterium* sp. (triangle) and *E. coli* (circle) were carried out at 30°C for 24 h in the complex medium (open symbols) or glucose medium (closed symbols), to which 50 μM CCCP was added. Viability of both strains was obtained as described under "Materials and Methods".**

almost linearly, which was in good accordance with those of the turbidities. That is, the viability was lost in proportion to the exposure time to CCCP. This was independent of the presence of glucose.

Effect of CCCP on the internal cation concentrations

The variations of internal cation concentrations of *Brevibacterium* sp. and *E. coli* were obtained as a function of the exposure time to CCCP. As shown in Table 2, total cation concentrations in the former strain sharply decreased after 2 h incubation with CCCP, irrespective of whether the medium contained glucose or not. Especially, decrease of K^+ ions in the cells was remarkable. In contrast, *E. coli* grown in the medium maintained almost the total cations in the cells even after 5 h exposure to CCCP, although they were reduced a little in the glucose-containing medium.

DISCUSSION

From the result shown in Fig. 1, it is apparent that *Brevibacterium* sp. accumulated the external K^+ ions of the complex medium in the cytoplasm (2.5–2.8 mM). Independent of the increase in the

TABLE 2

Variations of internal cation concentrations (mM) of *Brevibacterium* sp. and *Escherichia coli* as a function of exposure time to CCCP (50 μM)*

Medium	Cation	*Brevibacterium* sp.			*Escherichia coli*		
		0 h	2 h	5 h	0 h	2 h	5 h
Complex	Na^+	14	15	37	12	24	47
	K^+	288	86	39	57	23	22
	Mg^{2+}	180	164	174	97	89	81
	Ca^{2+}	14	11	5	22	24	25
	Total	496	276	255	188	160	175
Glucose	Na^+	2	9	16	9	11	28
	K^+	305	90	46	51	19	8
	Mg^{2+}	142	147	144	91	82	83
	Ca^{2+}	10	8	8	13	12	8
	Total	459	254	214	164	124	127

*50 μM CCCP was added to the growing cells incubated for 24 h.

external NaCl concentrations, *Brevibacterium* sp. accumulated 300–400 mM of K^+ ions, which were about six-fold higher than those in *Escherichia coli* (Table 2). The high intracellular concentrations of K^+ ions, about twenty fold as high as that of Na^+ ions, would be possibly utilized for the generation of PMF through the buffering function indicated by Kushner (1985).

The intracellular concentrations of cations other than K^+ ion were generally low. Without the addition of Na^+ ion, the internal concentrations of Na^+ ion in the cells were lower than those of K^+ or Mg^{2+}. Nevertheless, the intracellular Na^+ concentrations were about 3-fold as high as the extracellular ones (ca. 5 mM). With the increase in the external NaCl concentrations up to 2 M, uptake of Na^+ ions by *Brevibacterium* sp. increased almost linearly (Fig. 1a). Furthermore, the intracellular Na^+ concentrations were higher than the extracellular ones at least up to 1 M NaCl outside the cells. Taking these observations into consideration, it is inferred that *Brevibacterium* sp. does not actively carry out the Na^+ extrusion based on the Na^+/H^+ antiporter (Krulwich, 1983) or Na^+ pump (Tokuda and Unemoto, 1982). By the addition of 1–2 M NaCl or KCl to the medium, the growth rates of *Brevibacterium* sp. were reduced somewhat during the first 24 h incubation. Moreover, the growth was insufficient in the presence of more than 3 M NaCl or KCl (Nagata, 1988). Furthermore, the internal Na^+ or K^+ concentrations of *Brevibacterium* sp. increased with the increase of external NaCl or KCl concentrations, respectively. Such growth response to the salt stress may be closely related to the occurrence of biosynthesis of compatible solutes which maintains an osmotic balance of the cell cytoplasm with the outside medium (Larsen et al., 1987).

The presence of glucose in the culture stimulated the lethal effect of CCCP, especially for *E. coli*. To ascertain whether *E. coli* grown in the presence of glucose was really killed by addition of CCCP, the internal protein contents in the cells were examined with the exposure time. Up to 5 h, they were almost unchanged, indicating that the cell lysis did not occur. In addition, growth recovered after a short lag period (about 10 h), when a cell suspension of *E. coli* incubated in the presence of CCCP and glucose for 48 h was inoculated into the freshly prepared medium. These facts suggest that *E. coli* grown in the presence of both glucose and CCCP is in a syncopic state losing an essential cellular function and hovering between life and death.

The cell damage by CCCP seems to be related to the remarkable extrusion of K^+ ions from the cells (Table 2). Thus, the efflux rates of K^+ ions from the cells were directly measured in the presence of the membrane permeable amine, DEA, at alkaline external pH (Nagata et al., 1991). In proportion to the exposure time to CCCP, K^+ efflux rates of *Brevibacterium* sp. were largely reduced. When *E. coli* was grown in the complex medium, activity of K^+ efflux was originally about one-third of that of *Brevibacterium* sp., but it disappeared after 2 h incubation with CCCP. Grown in the glucose medium, on the other hand, *E.coli* revealed the uptake of K^+ ions in the cells after 2 h exposure to CCCP.

Decrease of cations in the cells (inside negative) led to the increase of membrane potentials. The potentials of *Brevibacterium* sp., however, were slightly reduced by CCCP regardless of the presence of glucose, suggesting that the H^+ influx might occur to compensate the cation efflux. The potentials of *E. coli* originally were ca. −160 mV, but became −50 mV after 2 h incubation with CCCP in the glucose medium, although they were little changed in the absence of glucose. This indicates that when *E. coli* was grown in the glucose medium, the potentials and ΔpH will be reduced by the H^+ influx, since the external environment was acidic ($pH_O = 4.5$). Consequently, the generation of PMF will be disrupted. In contrast, the internal cation concentrations and the potentials of *E. coli* in the absence of glucose were unaffected by the CCCP exposure, which seems to be consistent with the fact that the cells were tolerant to CCCP.

CONCLUSION

Growth of *Brevibacterium* sp. was dependent upon the proton circulation across the membrane, which was verified from the reductions of membrane potentials in proportion to the exposure time to CCCP. In addition, an optimal growth of this strain at an alkaline external pH was attributed to the K^+ extrusion from the cells by the coupling with the influx of membrane-permeable amine. On the other hand, the culture of *E.coli* exhibited the syncopic state hovering between life and death when it was exposed to CCCP in the presence of more than 30 mM glucose.

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