^{23}Na NMR spectroscopy of free Na^+ in the halotolerant bacterium *Brevibacterium* sp. and *Escherichia coli*

Shinichi Nagata,¹ Kyoko Adachi,² Katsuhisa Shirai³ and Hiroshi Sano²

Author for correspondence: Shinichi Nagata. Tel: +81 78 431 6342. Fax: +81 78 431 6364.

¹ Research Institute of Marine Cargo Transportation, Kobe University of Mercantile Marine, Fukae, Higashinada-ku, Kobe 658 Japan

² Marine Biotechnology Institute Co. Ltd, 1900, Sodeshicho, Shimizu, Shizuoka 424 Japan

³ Idemitsu kosan Co. Ltd, 1280 Kamiizumi, Sodegauracho, Chiba 299-02 Japan

^{23}Na NMR spectroscopy was used to determine free Na^+ concentrations in a halotolerant bacterium, *Brevibacterium* sp., and *Escherichia coli*. The internal Na^+ concentration of both strains depended little on the growth phases and was unchanged after 5 d storage at 2 °C. In *Brevibacterium* sp. the level of intracellular sodium increased gradually at higher extracellular NaCl concentrations in both the presence and absence of yeast extract in the growth medium. *E. coli* cells accumulated a higher concentration of free Na^+ than those of *Brevibacterium* sp. The change of Na^+ concentration in both strains was inverse to that of growth rate. When appropriate amounts of osmoprotectants (proline, glycine betaine, or γ -aminobutyrate) were added with the NaCl, internal free Na^+ levels in *Brevibacterium* sp. were lowered, but those of *E. coli* were unchanged. While addition of KCl to medium containing NaCl increased the intracellular level of free Na^+ , the total sodium concentration in the cells remained unchanged, indicating that sodium that had been bound or attached was made free in the cytosol. In *Brevibacterium* sp. grown in the presence of 0.5 M NaCl, free and bound sodium concentrations in the cytosol were estimated to be 0.14 and 0.23 $\mu\text{mol (mg protein)}^{-1}$, respectively. As a result, visibility by ^{23}Na NMR was 38%.

Keywords: ^{23}Na NMR, sodium ions, halotolerance, *Brevibacterium* sp., *Escherichia coli*

INTRODUCTION

Halophilic archaeobacteria require high NaCl concentrations for growth, but they accumulate K^+ rather than Na^+ in the cells to balance the external NaCl concentration (Imhoff, 1993). The exception is some methanogenic archaeobacteria, which produce compatible solutes (Sowers *et al.*, 1990; Lai *et al.*, 1991). In the eubacterial halophiles, production and accumulation of organic osmolytes are the main means of osmotic adaptation (Imhoff, 1986; Csonka, 1989; Bernard *et al.*, 1993; Galinski, 1993), although some anaerobic species accumulate K^+ (Oren, 1986; Rengpipat *et al.*, 1988). This is consistent with K^+ or compatible solutes supporting the activity of enzymes and protein synthesis in the cells, with Na^+ often being inhibitory (Kushner, 1986; Kamekura, 1986; Nagata, 1993).

Intracellular ion concentrations have been analysed by inductively coupled plasma emission (Rengpipat *et al.*,

1988), flame photometry (Oren, 1986) or atomic absorption (Shindler *et al.*, 1977; Unemoto & Hayashi, 1979; Nagata *et al.*, 1991). However, these techniques do not allow the cellular free cation concentrations to be determined. The use of ^{23}Na NMR spectroscopy in the presence of paramagnetic shift reagents enables differentiation between the free Na^+ in the cytosol from ions that are combined or bound with cellular components (Gupta & Gupta, 1982; Ogino *et al.*, 1983; Castle *et al.*, 1986; Bental *et al.*, 1988). Gilboa *et al.* (1991) used ^{23}Na NMR spectroscopy to study the moderately halophilic eubacterium *Vibrio costicola*, reporting an internal free Na^+ concentration of 0.07–0.14 M which was independent of the external NaCl concentration (0.6–2 M).

In the present study, ^{23}Na NMR was used to measure internal Na^+ concentrations in the halotolerant bacterium *Brevibacterium* sp., and non-halophilic *Escherichia coli* for comparison, grown in media with different NaCl concentrations. In addition, estimates were made of the levels of sodium bound to cellular components in the cytosol, i.e. the fraction invisible by ^{23}Na NMR spectroscopy (Hutchison & Shapiro, 1991). Because *Brevibacterium* sp.

Abbreviations: GABA, γ -aminobutyrate; GB, glycine betaine.

also grows in high concentrations of KCl (Nagata, 1988), we examined whether K^+ or other osmolytes affected the internal free Na^+ concentration in the cells.

METHODS

Growth of cells. *Brevibacterium* sp. (strain JCM 6894), isolated from seawater, and *E. coli* ATCC 11775 were grown aerobically as described by Nagata (1988) in complex or chemically defined medium containing NaCl, KCl or organic osmolytes as indicated. Unless otherwise specified, each culture was harvested (8000 g, 10 min) at the stationary phase of growth (after 24 h incubation at 30 °C), except for the growth of *Brevibacterium* sp. in more than 1 M NaCl or KCl (48 h incubation). Since the intracellular free Na^+ concentration was essentially independent of the growth phase (discussed below), analyses were carried out for the stationary phase cells; thus the cells were maintained in a physiologically invariable state, especially in higher NaCl concentrations, during NMR measurement. *V. costicola* NCMB 701 (from Dr H. Tokuda, University of Tokyo) was grown aerobically in proteose peptone plus 0.3% (w/v) tryptone (PPT) medium (Gilboa *et al.*, 1991) and harvested at the exponential growth phase for comparison. Bacterial pellets, collected by centrifugation, were diluted to 5–10 (mg cell protein) ml^{-1} in the same growth medium, and were used for the analyses of free Na^+ concentration in the cells by NMR measurement. We manipulated lower concentrations of cell suspension (10–20-fold concentrated from the culture) to avoid the reduction of Na^+ efflux activity due to an anoxic state (Shindler *et al.*, 1977) and to increase the visibility of intracellular Na^+ by ^{23}Na NMR measurements (Castle *et al.*, 1986). Viable cell numbers were obtained from colony counts on agar medium incubated at 30 °C for 3 d.

Shift reagent. The shift reagent, 0.025 M dysprosium triphosphate [$Dy(PPP)^{7-}_2$], was prepared by mixing $DyCl_3 \cdot 6H_2O$ with a solution of $Na_5P_3O_{10}$ in D_2O to give a 1:4 molar ratio. For the examination of cell washing effects, $K_5P_3O_{10}$ was used instead of $Na_5P_3O_{10}$.

Analytical procedure for ^{23}Na NMR spectroscopy. The concentrated cell suspensions were kept at 2 °C (maximum 1 d). Just before the NMR measurement, 2 ml bacterial suspension was centrifuged at 8000 g (2 °C, 5 min) and the supernatant of known volume was removed. A sample of 0.4 ml shift reagent, containing Na^+ at a concentration equal to that in the medium and prepared by dilution of a solution of 0.025 M $DyCl_3$ /0.1 M $Na_5P_3O_{10}$, was added to the pellets when the Na^+ concentration in the medium was below 0.5 M. When the concentration was over 0.5 M, shift reagent containing 0.5 M Na^+ (0.025 M $DyCl_3$ /0.1 M $Na_5P_3O_{10}$ solution) was used. This was because useful resolution was not obtained at the high concentration of the shift reagent. Samples thus prepared were sufficiently aerated to prevent anaerobiosis during NMR measurements.

^{23}Na NMR spectra were taken on a Varian UNITY 500 spectrometer at 132.218 MHz with a 5 mm tube, operated with 90° pulses and 0.2 s repetition at 20 °C. A D_2O solution of 0.005 M NaCl was used as the external standard. The calibration curve of Na^+ showed a linear correlation in the concentration ranges 0.0005–1.0 M.

Visibility of Na^+ associated with the cells was estimated by NMR as follows. Cell suspensions after 1–3 washes with distilled water (or 50 mM Tris/HCl buffer, pH 7.5, containing 0.5 M KCl) were divided into two samples. One sample was used for the analyses of both intracellular (free) and extracellular sodium concentration. The other sample was treated with TCA at

95–98 °C (Nagata *et al.*, 1991) and used for the measurement of total sodium concentration.

Calculation of intracellular Na^+ concentration. Internal free Na^+ concentration was calculated based on the cell volumes determined by Nagata *et al.* (1991) and Larsen *et al.* (1987). That of *E. coli* was also expressed relative to dry weight. Protein concentration in cells was determined by a BCA Protein Assay Reagent (Pierce Chemical) using BSA as a standard.

RESULTS

^{23}Na NMR spectra of *Brevibacterium* sp. and *V. costicola*

To examine the influence of temperature on ion leakage during centrifugation (Gilboa *et al.*, 1991), the intracellular Na^+ concentration was evaluated by ^{23}Na NMR for cells harvested at 2 °C and room temperature. Since there was no difference between them, centrifugation was routinely carried out at 2 °C.

A typical ^{23}Na NMR spectrum of *Brevibacterium* sp. grown in 0.5 M NaCl is shown in Fig. 1. To examine the reliability of the NMR measurements of internal free Na^+ concentrations, we analysed the spectra of *V. costicola*, which showed internal Na^+ concentrations of 128, 128, 72 or 106 mM when cells were grown in PPT medium containing 0.8, 1.0, 1.6 or 2.0 M NaCl, respectively. Since there was little difference between our results and those of Gilboa *et al.* (1991), we concluded that our protocol was satisfactory for sodium analyses in bacterial cells.

Free Na^+ concentration in *Brevibacterium* sp. and *E. coli*

Intracellular free Na^+ concentration was less than 50 mM in cells grown in 0.1–1 M NaCl (Fig. 2a). A gradual in-

Fig. 1. ^{23}Na NMR spectrum of *Brevibacterium* sp. grown in complex medium containing 0.5 M NaCl. The measurements were carried out 0.5 M Na^+ and 0.017 M $Dy(PPP)^{7-}_2$. The chemical shift was referenced to a 0.005 M NaCl solution.

Fig. 2. Intracellular free Na^+ concentration in *Brevibacterium* sp. (a) and *E. coli* (b) as a function of external NaCl concentration. Both strains were grown in complex medium containing various concentrations of NaCl. Concentrated cell suspensions, after harvesting during the stationary phase of growth, were used for the determination of intracellular free Na^+ concentration (●) by ^{23}Na NMR. Cell growth yields (○) and growth rates (×) are also shown.

crease in the internal Na^+ level was seen in the NaCl concentration ranges from 1 to 2 M. Internal Na^+ levels increased rapidly when the NaCl concentration exceeded 2 M, ending at about 170 mM in cells grown in medium containing 2.5 M NaCl. Conversely, the growth yield of *Brevibacterium* sp. showed a maximum at 0.5–1.0 M

NaCl and then decreased with elevated osmolarity. The alteration of the growth rate was in inverse proportion to the intracellular Na^+ level in the external NaCl concentration ranges examined (Fig. 2a).

Internal free Na^+ concentration was also examined in wild-type *E. coli* grown in 0.1–0.8 M NaCl. When the external NaCl concentration was increased from 0.1 to 0.7 M, the level of intracellular Na^+ increased almost linearly (Fig. 2b). *E. coli* cells grown in the presence of 0.7 M NaCl contained 236 mM free Na^+ , which was about sixfold higher than *Brevibacterium* sp. grown under the same conditions (Fig. 2a, b). In 0.8 M NaCl, the level of intracellular Na^+ increased to 368 mM or $0.26 \mu\text{mol Na}^+$ (mg dry wt) $^{-1}$. When *E. coli* was grown in medium containing 0.1, 0.3, 0.5 or 0.7 M NaCl, the values were 0.13, 0.18, 0.23 or $0.25 \mu\text{mol Na}^+$ (mg dry wt) $^{-1}$, respectively. It is of interest that changes in the internal free Na^+ concentration in *E. coli* [mM and $\mu\text{mol Na}^+$ (mg dry wt) $^{-1}$] were converse to those of both growth rate and cell growth yield in 0.1–0.8 M NaCl (Fig. 2b).

Effect of growth phase and storage on free Na^+ concentration

The effect of the growth phase on the internal free Na^+ concentration in *Brevibacterium* sp. was examined with cells grown in medium containing 0.5–2 M NaCl. With 0.5 or 1 M NaCl, the concentration of internal free Na^+ was affected little by incubation time (12–48 h) at 30 °C (Table 1). In 2 M NaCl the concentration of internal free Na^+ (100–118 mM) was almost constant from mid-exponential (30–33 h) to stationary phase (48 h), and then increased with incubation time (see also Fig. 3). The free Na^+ concentration in *E. coli* grown in 0.1–0.7 M NaCl was constant during the 12–48 h incubation period from early exponential to late stationary growth phases (data not shown). Thus, it is reasonable to conclude that the level of internal free Na^+ in both strains was constant, at least from exponential to stationary phases, regardless of the NaCl concentration in the medium.

Table 1. Change in internal free Na^+ concentration in *Brevibacterium* sp. cells as a function of incubation time

Results are means $\pm$ SD (no. of experiments given in parentheses).

Incubation time (h)	Concn of NaCl in culture (M)...	Internal free Na^+ concn (mM)		
		0.5	1.0	2.0
12		34.1 (1)		
24		35.5 $\pm$ 4.7 (5)	43.4 (1)	50.8 $\pm$ 9.3 (3)
48		44.9 (1)	48.0 $\pm$ 6.1 (4)	117.7 $\pm$ 18.8 (6)
72				215.2 $\pm$ 21.1 (2)
96				330.7 (1)
120				357.3 (1)

Fig. 3. Internal free Na^+ concentration of *Brevibacterium* sp. as a function of incubation temperature and storage time. *Brevibacterium* sp. was grown in complex medium containing 2 M NaCl for 1–5 d. The concentrated cell suspensions ($5\text{--}10\text{ mg cell protein ml}^{-1}$) after harvesting were used immediately ($\circ$), or after storage at 2°C or 30°C for the NMR measurements to obtain the intracellular free Na^+ concentration. Cell suspensions for storage were taken from cells grown for either 24 h or 48 h. $\triangle$, Cells taken from 24 h culture and stored at 2°C ; $\blacktriangle$, cells taken from 24 h culture and stored at 30°C ; $\square$, cells taken from 48 h culture and stored at 2°C ; $\blacksquare$, cells taken from 48 h culture and stored at 30°C .

Table 2. Effect of compatible solutes on the internal free Na^+ concentration and growth yield of *Brevibacterium* sp.

Brevibacterium sp. was grown in the presence of 2 M NaCl with and without compatible solute and harvested at the stationary phase of growth. Results are means $\pm$ SD. The number of measurements is given in parentheses.

Solute	Concn (mM)	Concn of free Na^+ (mM)	Growth yield [(mg protein) l^{-1}]
None		117.7 ± 18.8 (6)	281
Proline	1	50.4 ± 5.8 (4)	284
	10	113.3 ± 56.9 (4)	430
GB	1	86.5 ± 15.8 (4)	333
	10	112.6 ± 44.9 (4)	470
GABA	5	74.0 ± 28.4 (4)	389
	50	97.2 ± 31.7 (4)	461

The alteration of the level of intracellular free Na^+ of concentrated cell suspensions manipulated for the NMR measurement was followed as a function of the storage time at different temperatures. At 2°C , the intracellular Na^+ concentration in *Brevibacterium* sp. changed little

during 5–6 d storage (Fig. 3). Large increases were observed when cells were stored at 30°C . To investigate these differences, we examined the total and cell-associated sodium concentration in the cell suspensions stored at 30°C for 5 d. These analyses showed that levels of cellular sodium did not change significantly, from 694 mM to 699 mM after 5 d storage, indicating that intracellular Na^+ which had been combined or bound with cellular components became free during storage at the elevated temperature. Like *Brevibacterium* sp., the intracellular Na^+ concentration in *E. coli* was constant for 5 d when the concentrated cell suspension was stored at 2°C . At 30°C storage, however, the level of cellular sodium was reduced to about half and disappeared after 5–6 h and 3 d, respectively (data not shown). This implies that the storage of the concentrated *E. coli* cell suspensions at high temperature stimulated in part the efflux of free Na^+ from the cells, since the extracellular Na^+ concentration increased.

Effect of compatible solutes on free Na^+ concentration

Glycine betaine (GB) or a related precursor from yeast extract is transported in methanogens when grown in high salt concentrations (Robertson *et al.*, 1990). In fact, NMR analysis of yeast extract showed the presence of GB, trehalose or glutamate with 0.14, 0.19 or 0.70 mmol (g yeast extract) $^{-1}$, respectively. Since these osmoprotectants were only at trace levels in Bactopeptone, the free Na^+ concentration in *Brevibacterium* sp. was analysed and compared to cells grown in medium without yeast extract. Growth in such medium with 0.5, 1.0, 1.5 or 2.0 M NaCl resulted in 35.9, 38.7, 49.8 or 64.4 mM cellular Na^+ , respectively. When *Brevibacterium* sp. was grown in chemically defined medium containing glutamate as a sole carbon source, the level of cellular Na^+ was almost the same as in cells from yeast-extract-free medium (data not shown). In both media with high concentrations of NaCl, the levels of cellular Na^+ were reduced a little compared to that in the complex medium. Conversely, Na^+ levels in *E. coli* showed insignificant differences in the presence or absence of yeast extract (data not shown).

Free Na^+ levels in both strains were compared when they were grown in the presence or absence of compatible solutes such as proline, GB or γ -aminobutyrate (GABA). In the presence of 1 mM proline together with 2 M NaCl, the free Na^+ concentration in *Brevibacterium* sp. cells was lowered to about half that without proline (Table 2). Addition of 1 mM GB or 5 mM GABA to the medium brought about a slightly lesser reduction in the free Na^+ level in the cells. Insignificant changes in the concentration of cellular Na^+ were seen for cells grown in the presence of 10 mM each of proline and GB, or 50 mM GABA, though it is necessary to take large standard deviations into consideration. For comparison, we examined the effect of compatible solutes when *Brevibacterium* sp. was grown in the chemically defined medium with 2 M NaCl; the concentration of cellular Na^+ was 70.2 mM for cells grown in 2 M NaCl, lower

Table 3. Effect of compatible solutes on the internal free Na^+ concentration in *E. coli*

E. coli was grown in the presence of 0.5 or 0.7 M NaCl with and without compatible solute and harvested at the stationary phase of growth. Results are the means $\pm$ SD (no. of experiments is given in parentheses).

Solute	Concn (mM)	Concn of NaCl in culture (M)...	Internal free Na^+ concn (mM)	
			0.5	0.7
None			185.5 $\pm$ 39.5 (15)	236.1 $\pm$ 28.9 (8)
Proline	1		227.2 $\pm$ 15.9 (5)	264.8 $\pm$ 1.5 (2)
	10		157.9 $\pm$ 32.6 (5)	224.1 $\pm$ 2.2 (3)
GB	1		228.1 $\pm$ 8.3 (5)	168.7 $\pm$ 13.2 (2)
	10		216.1 $\pm$ 41.5 (6)	178.5 $\pm$ 0.2 (2)
GABA	5		238.6 $\pm$ 8.4 (3)	251.6 (1)
	50		296.0 $\pm$ 15.3 (3)	274.1 (1)

Fig. 4. Total and free sodium concentration in *Brevibacterium* sp. grown in the presence of 2 M salts (NaCl+KCl). *Brevibacterium* sp. cells were harvested at the stationary phase of growth and resuspended in the same growth medium for the analyses of free Na^+ concentration (○). Total sodium concentrations (●) were measured in terms of ^{23}Na NMR spectroscopy for the samples manipulated as described in Methods. For a reference, total sodium concentrations of *Brevibacterium* sp. grown in the presence of only NaCl were also obtained by the same manner as described above (▲).

than that of the complex medium. In the presence of 1 mM proline or GB the cellular Na^+ concentration was little changed.

The influence of the compatible solutes added externally was not as remarkable on the internal free Na^+ concentration in *E. coli* (Table 3). Except for 10 mM proline, the presence of every osmolyte with 0.5 M NaCl led to the increase of cellular Na^+ concentration regardless of the

concentration of the osmolyte. In 0.7 M NaCl, the intracellular Na^+ concentration also increased with the addition of compatible solutes except for GB. It seems reasonable to conclude that the addition of the compatible solutes had little effect on the internal free Na^+ concentration in *E. coli*, although they might support cell growth.

Effect of K^+ on free Na^+ concentration

The internal free Na^+ concentration in *Brevibacterium* sp. was determined quantitatively as a function of both Na^+ and K^+ concentration in the medium. The free and total sodium concentration was estimated in *Brevibacterium* sp. grown in the medium where the sum of NaCl and KCl concentrations was set at 2 M. The intracellular free Na^+ concentration increased in proportion to an increase of the NaCl/KCl ratio up to 1 (Fig. 4). The free Na^+ concentration in *Brevibacterium* sp. showed a maximum at 1–1.5 M NaCl and decreased as the external NaCl concentration increased from 1.5 to 2 M. On the other hand, the total sodium concentration of *Brevibacterium* sp. continued to increase in proportion to the increase in the NaCl concentration, irrespective of whether KCl was present. Total sodium was always higher in the presence of both NaCl and KCl than with NaCl only, which might imply that the co-presence of K^+ with Na^+ added externally induced the association of Na^+ with the cell. Thus, the difference between free and total sodium concentration, which was largest in the absence of KCl, was attributed to the presence of sodium fractions combined or bound to the cellular structure and attached to the cell envelope which were invisible by ^{23}Na NMR spectroscopy (Castle *et al.*, 1986).

To ascertain the role of K^+ in influencing the level of cell-associated Na^+ , changes in the total and free sodium concentration in *Brevibacterium* sp. were also traced. When grown in 0.5 M NaCl-containing media where KCl concentrations were varied between 0 and 2 M, the total cellular sodium concentration increased linearly up to

Fig. 5. Total and free sodium concentration in *Brevibacterium* sp. grown in the presence of 0.5 M NaCl together with various concentrations of KCl. Analyses of total (●) and free (○) sodium concentration were carried out by the same method as for Fig. 4.

1.5 M, although the latter was almost constant (Fig. 5). In > 1.5 M KCl, the level of total sodium was unchanged at about 180 mM, while that of free Na^+ decreased abruptly. As a whole, the difference between the levels of total and free sodium was the largest and the smallest when grown in the absence and presence of 2 M KCl,

respectively. This is consistent with the other results (see Fig. 4). Therefore, it is clear that the addition of 0.1–1.5 M KCl to the NaCl-containing medium promoted not sodium accumulation, but the conversion of the state of the sodium in the cytosol, from bound (or otherwise associated) to free, since the total sodium concentration was little changed. Furthermore, it is plausible that the threefold higher concentration of KCl might exclude Na from the cells without affecting the intracellular free Na^+ concentration.

Analyses of the concentration of bound Na^+

Quantitative analyses of Na^+ bound to cellular structures or components were carried out by the measurement of both free and extracellular Na after washing the cells. Viable cell numbers were hardly affected by three washes (8000 g, 10 min, 2 °C) with distilled water (Table 4), indicating that the cell envelope of *Brevibacterium* sp. was sufficiently rigid to protect against osmotic changes in the external environment. This characteristic of *Brevibacterium* sp. enabled us to estimate the sodium content of the water among the cells and attached to the cell surface. Hence, the total concentration of sodium in the cell suspension was regarded as the sum of the following different states of sodium that are free, bound or combined to the cellular structures, attached to the cell surface and present in water (solvent) between the cells. By use of the shift reagent, the solvent and the weakly attached sodium fractions were separated from others before washing the cells. By washing the cell pellets with distilled water, the solvent fraction was sufficiently excluded and the tightly attached Na fraction gradually left the cell surface by the aid of the shift reagent after resuspension in distilled water. Thus, the extracellular Na content was almost constant after 2–3 washes of the cell pellets, at about $1.8 \mu\text{mol (mg protein)}^{-1}$ (Table 4). The free Na^+ concentration in the cells was

Table 4. Variation in internal free, extracellular and bound sodium concentration of *Brevibacterium* sp. cells during washing procedures

The culture of *Brevibacterium* sp. grown in the presence of 0.5 M NaCl was harvested at the stationary phase of growth, washed with distilled water and mixed with the shift reagent, 0.0125 M $\text{DyCl}_3/0.05$ M $\text{K}_5\text{P}_3\text{O}_{10}$. Where the washing was by 50 mM Tris/HCl buffer (pH 7.5) containing 0.5 M KCl, values are given in parentheses. The amount of bound Na^+ was calculated from the difference between total sodium, measured after cell disruption by 25% TCA treatment, and the sum of intracellular and extracellular sodium concentrations obtained from the ordinary method using shift reagent. Visibility (%) = $[\text{intracellular free Na}^+ / (\text{intracellular free Na}^+ + \text{bound Na}^+)] \times 100$.

No. of washings	Concn [$\mu\text{mol (mg protein)}^{-1}$] of:			Visibility (%)	Viability (c.f.u. ml^{-1})
	Free Na^+	Extracellular Na^+	Bound Na^+		
0	0.13	6.85	—	—	5.91×10^9
1	0.13 (0.04)	1.86 (0.89)	0.26 (—)	33.3 (—)	5.73×10^9 (5.90×10^9)
3	0.14 (0.01)	1.77 (0.14)	0.23 (0.02)	37.8 (—)	4.98×10^9 (5.33×10^9)

$0.13 \mu\text{mol (mg protein)}^{-1}$, or about 35 mM (see Table 1), before washing, and was almost unchanged even after three washes, at $0.14 \mu\text{mol (mg protein)}^{-1}$. As a consequence, visibility by ^{23}Na NMR was 38 %, which seems to be reasonable from the limit of pulsed-NMR with excessive deadtimes between excitation and detection (Hutchison & Shapiro, 1991).

DISCUSSION

The free Na^+ concentration in the halotolerant *Brevibacterium* sp. was quite low regardless of the growth medium, corresponding to 6 and 3 % of the external NaCl concentration when cells were grown in medium containing 2 M NaCl with and without yeast extract, respectively. Such low ratios of the intracellular to extracellular Na^+ concentration in *Brevibacterium* sp. are in agreement with those of *V. costicola* (Gilboa *et al.*, 1991) and the halotolerant alga *Dunaliella salina* (Bental *et al.*, 1988). Since halophilic eubacteria such as *Brevibacterium* sp. and *V. costicola* possess the ability to grow in a wide range of NaCl concentrations, they might, in general, maintain a Na^+ concentration low enough not to interfere with enzyme- and protein-synthesizing activities, and to utilize efficiently the ion gradient across the membrane (Kushner, 1988; Nagata, 1993).

Low concentrations of cellular free Na^+ in halophilic micro-organisms might be ascribed to their active Na^+ efflux systems through the Na^+ pump or Na^+/H^+ antiporter (Tokuda & Unemoto, 1982; Hamaide *et al.*, 1985). This is consistent with the fact that the alteration of the internal Na^+ concentration in *Brevibacterium* sp. was in inverse proportion to that of the growth rates. When a concentrated cell suspension of *Brevibacterium* sp. was stored at 30 °C, the internal Na^+ level increased significantly, which implies that the intracellular Na^+ ions were shifted to a free state from a bound or combined state, and that little was effluxed from the cells due to the occurrence of an anaerobic state under high-temperature storage, as in *V. costicola* (Shindler *et al.*, 1977).

The free Na^+ concentration in *E. coli* was higher than that of the halophilic bacteria or alga mentioned above. Such a difference tended to increase at higher external NaCl concentrations. For example, when grown in 0.8 M NaCl, the free Na^+ concentration in *E. coli* was more than 350 mM, while that of *Brevibacterium* sp. was at most 12 % of this figure (43 mM). Conversely, cell growth yields and growth rates of *E. coli* lessened at the elevated NaCl concentrations, which indicates that the halotolerance of individual cells might be closely related to their internal free Na^+ concentration.

Until the ^{23}Na NMR method was applied to the Na^+ analyses in cells, analyses were restricted to the cell-associated ions, and the bound or attached sodium content was not distinguished (Ogino *et al.*, 1983; Gilboa *et al.*, 1991). According to our analyses, the concentrations of free and bound sodium in the cytosol were 0.13–0.14 and 0.23–0.26 $\mu\text{mol (mg protein)}^{-1}$, respectively. The 33–38 % visibility of Na^+ signals by NMR was slightly lower than

that in the yeast *Saccharomyces cerevisiae* (Ogino *et al.*, 1983) or the alga *D. salina* (Bental *et al.*, 1988), which was about 40 %. This difference might partly occur from the presence of Na remaining attached after several washes.

Addition of K^+ into the NaCl-containing medium brought about an increase of free Na^+ concentration in *Brevibacterium* sp. (Figs 4 and 5). The total sodium concentration associated with the cells was independent of KCl concentration from 0 to 1.5 M (Fig. 5), which implies that Na bound to cellular components was partly converted to free Na^+ . The alteration of the sodium state in the cytosol might be caused by the accumulation of K^+ ions in the cells and/or ions attached to the cell envelope, because they actually increased in proportion to the increase of the external KCl concentration (determined by atomic absorption spectrophotometry). The cell-associated K^+ concentration in *Brevibacterium* sp. remained almost constant (about 300 mM) at 0.1–2 M external NaCl concentration (Nagata *et al.*, 1991), as in *V. costicola* (Shindler *et al.*, 1977) and *V. alginolyticus* (Unemoto & Hayashi, 1979). K^+ ions thus accumulated or attached may function to stimulate the conversion of the state of Na in the cells, although the physiological significance of this is still unclear.

It is of interest that the internal free Na^+ concentration in *Brevibacterium* sp. grown in the yeast extract-free and defined medium (1–2 M NaCl) was lower than that in complex medium. Since exogenously supplied osmoprotectant prevents accumulation of both inorganic and endogenous organic solutes, quantitative analyses should be done for both compatible solutes and free ions (Na^+ and K^+) in cells grown under different osmolarities. According to the recent study with *E. coli* (McLaggan *et al.*, 1994), intracellular K^+ in the free state, which balances the charge of small anions such as glutamate, was 44 and 52 % of total K^+ at low and high osmolarities, respectively. In this connection, it seems to be necessary to clarify the essential role of both free Na^+ and K^+ ions in *Brevibacterium* sp., since they surely play an important role in maintaining cell activity under different osmolarities.

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