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Intracellular Changes in Ions and Organic Solutes in Halotolerant *Brevibacterium* sp. Strain JCM 6894 after Exposure to Hyperosmotic Shock

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In the present study we aimed to observe the intracellular responses when there was a hyperosmotic shock with a large shift in ionic strength in nutrient-rich and nutrient-poor external environments in order to clarify the availability of substrates. To do this, we used the halotolerant organism *Brevibacterium* sp. strain JCM 6894, which is able to grow in the presence of a wide range of salt concentrations. Hyperosmotic shock was induced by transferring cells in the late exponential phase of growth in a complex medium containing 0.5 M NaCl into either old or fresh culture medium containing 2 M NaCl. Changes in the growth rate, in the pH of the medium, and in the internal cation or organic solute concentrations in the cytosol after an upshock were analyzed as a function of incubation time. The cells exhibited very different responses to upshocks in fresh culture medium and in old culture medium; in fresh culture medium, growth was stimulated and the medium became more acidic, whereas the old culture medium repressed growth and the medium became more alkaline. The intracellular free Na^+ concentrations remained low (80 nmol mg of protein^{-1}) after an upshock in fresh culture medium, although they quickly increased twofold in the old culture medium. In contrast, K^+ ions immediately accumulated in the cells in fresh culture medium, whereas K^+ ions were taken up quite slowly in old culture medium. Furthermore, the cells placed in fresh culture medium transiently accumulated alanine and glutamine in response to the upshock, but the cells placed in old culture medium did not. Growth of the *Brevibacterium* strain at higher levels of salinity was supported by ectoine synthesis but was not observed after the shift to high-osmolarity conditions in the old culture. In the fresh culture, however, ectoine was vigorously synthesized in cells for more than 5 h after the upshock; the concentration of ectoine in cells was more than 3,500 nmol mg of protein^{-1} at 10 h, which corresponded to a ninefold increase compared to the concentration before the shock. These findings are consistent with the results of an analysis of the extracellular medium composition before and after the upshock.

When organisms are confronted with extreme changes in the external environment, such as changes in temperature, pH, and pressure, some responses in the cell cytosol are required for survival. One of these responses, the response to an increase in osmotic pressure, has been studied extensively in gram-negative enteric bacteria (4). In general, a hyperosmotic shock immediately induces a decrease in the cytoplasmic volume via water efflux or plasmolysis. After a short time lag, K^+ uptake occurs (20), which stimulates glutamate synthesis (2, 6, 14). Later, trehalose is synthesized inside the cells (11), or either glycine betaine or proline, if present, is taken up from the medium by the ProU system activated in cells exposed to osmotic shock (3). Whatmore et al. (24) observed K^+-dependent proline synthesis in *Bacillus subtilis*, a gram-positive bacterium, induced by a shift to high osmolarity.

Halophilic eubacteria are known to accumulate various compatible solutes when they are grown in the presence of high external osmolarity. The kinds of solutes accumulated in the cells can be divided into the following two groups: charged amino acids, such as glutamate; and polar molecules with no net charge, such as glycine betaines and ectoines (9). Since the advent of nuclear magnetic resonance (NMR) analyses of organic solutes in cells, accumulation of ectoine has been reported for a variety of halophilic gram-positive eubacteria (10), including *Nocardiopsis*, *Brevibacterium*, and *Marinococcus* species, and gram-negative eubacteria, including *Halomonas* (5, 25), *Pseudomonas* (21), and *Vibrio* (19) species.

Brevibacterium sp. strain JCM 6894, which was isolated from seawater at a depth of 5 m (Aburatsubo, Japan), is a gram-positive eubacterium, in contrast to the majority of the bacteria in marine environments, which are gram negative. The halotolerant nature of this strain made it possible to examine the intracellular changes at a wide range of NaCl concentrations, which showed that in JCM 6894 cells the Na^+ concentration remains low and the K^+ concentration is almost constant regardless of the external salinity (16, 17). In addition, this strain synthesizes ectoine as a major osmolyte at high levels of external salinity (18), and the amount of ectoine that accumulates in the cells increases almost linearly up to an NaCl concentration of 2 M, indicating that the halotolerant nature of this strain may be closely related to its ability to synthesize ectoine. Among the members of the genus *Brevibacterium* accumulation of ectoine in cells at high levels of salinity so far has been reported mainly for *Brevibacterium linens* (1, 7, 13), but the mode of accumulation in this organism is somewhat different from that in strain JCM 6894.

Results of analyses of internal changes after hyperosmotic shock have been reported for nonhalophilic bacteria, notably *Escherichia coli* (14, 20) and *Salmonella typhimurium* (4), but

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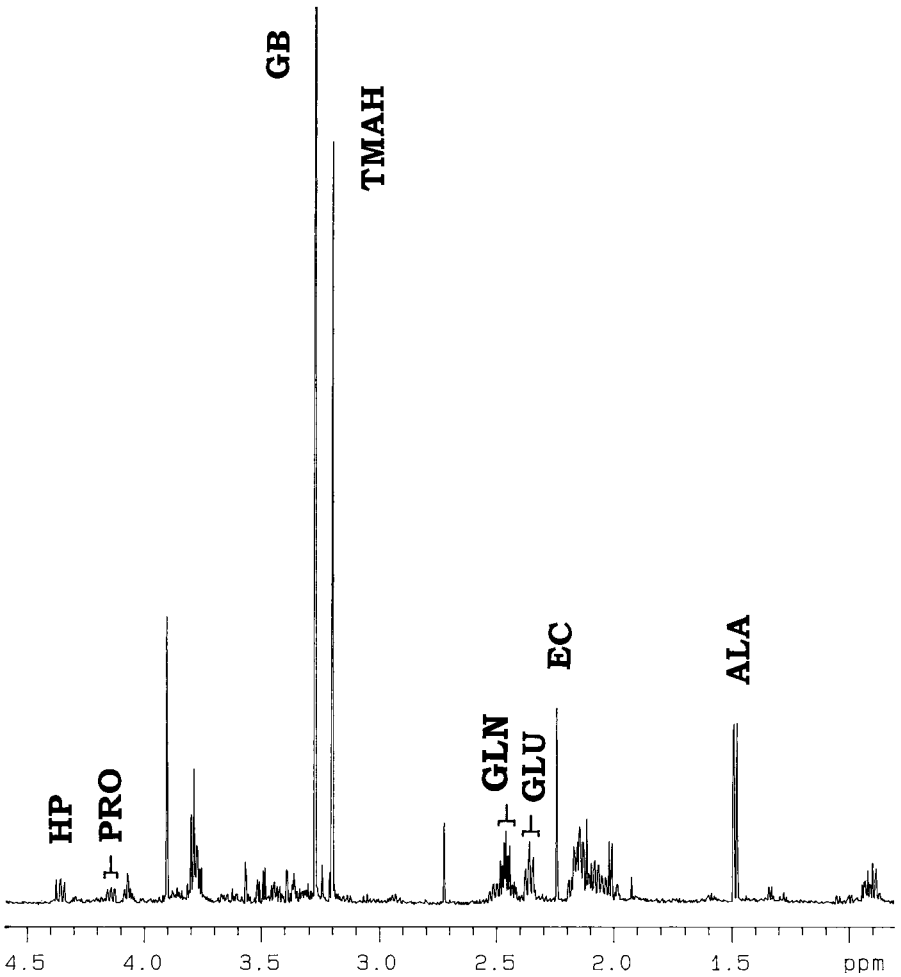

FIG. 1. ^{1}H NMR spectrum of ethanol extract of *Brevibacterium* sp. strain JCM 6894 cells upshocked in fresh medium containing 2 M NaCl for 1.5 h at 30°C. Signals were assigned to alanine (ALA), ectoine (EC), glutamate (GLU), glutamine (GLN), TMAH, glycine betaine (GB), proline (PRO), and hydroxyproline (HP).

the previous studies focused on the transient responses (responses after 30 to 60 min) of the cells to small osmotic changes (0 to 0.5 M) with no consideration of the medium conditions. Thus, it was of interest to examine the physiological changes in *Brevibacterium* sp. after a hyperosmotic shock consisting of a change in ionic strength under both nutrient-rich and nutrient-poor growth conditions. In the present study, the intracellular concentrations of ionic and nonionic solutes were determined by ^{1}H and ^{13}C NMR, by ^{23}Na NMR, and by atomic absorption analysis as a function of a long incubation time (24 h) when the osmolarity of the medium was suddenly changed from 0.5 to 2 M by adding NaCl or KCl. Taking into consideration the effect of culture conditions, we paid special attention to ectoine synthesis in the cell cytosol after the upshock, since this process is the key response which enables *Brevibacterium* sp. to grow under high-salinity conditions (18). The extracellular medium compositions before and after the upshock were also analyzed to provide a better understanding of the intracellular responses (22, 23).

MATERIALS AND METHODS

Media and growth conditions. *Brevibacterium* sp. strain JCM 6894 was grown aerobically at 30°C in a complex medium containing (per liter) 5 g of Bacto Peptone (Difco Laboratories, Detroit, Mich.), 1 g of Bacto Yeast Extract (Difco), 0.7 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of NH_4Cl, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.035 g of K_2HPO_4, and 0.015 g of KH_2PO_4 (15). Unless otherwise noted, the medium contained 0.5 M NaCl and had an initial pH of 7.5. The pH was adjusted with tetramethylammonium hydroxide (TMAH). After growth to the late exponential phase, the cells were harvested by centrifugation (8,000 × *g*, 10 min, 4°C) and washed with 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]–TMAH buffer (pH 7.2) containing 0.5 M NaCl. Growth was determined by measuring turbidity at 650 nm with a model DU 640 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Hyperosmotic shock experiments. Cultures were osmotically upshocked from 0.5 to 2 M NaCl by using the following two methods: (i) enough sterile solid NaCl was added to a growing culture containing 0.5 M NaCl so that the final NaCl concentration was 2 M; and (ii) after cells were washed, they were inoculated into freshly prepared medium containing 2 M NaCl. In this paper, these two methods are referred to as upshock in old cultures and upshock in fresh cultures, respectively. The same procedures were used for osmotic upshock with KCl instead of NaCl. Intracellular solutes were extracted by using procedures described previously (17). Ethanol extracts were used for the NMR analyses, as well as for high-performance liquid chromatography performed with a model L-5020 instrument (Hitachi, Tokyo, Japan) equipped with an RSpak NN814 column (Shodex, Tokyo, Japan).

NMR analyses of solutes. NMR spectra were obtained with a Varian UNITY 500 spectrometer by using a 5-mm indirect probe as described elsewhere (18). The acquisition parameters used for ^{1}H (500-MHz) NMR were 45° pulse width, 4-s pulse repetition time, and 32 to 128 transients depending on the solute concentration. Resonances were identified as belonging to a particular solute based on the results of a series of standard two-dimensional NMR analyses. The concentration of solutes was determined by integrating the area under the isolated peak of each solute in the ^{1}H NMR spectrum and comparing it with the peak area of *N*-methylglycine [δ = 2.75 ppm for $-NH(CH_3)$] which was the internal standard used. Measurements were determined at least three times, and data were expressed as averages ± standard deviations. The chemical shifts of the peaks used for integration were the same as those reported previously (18).

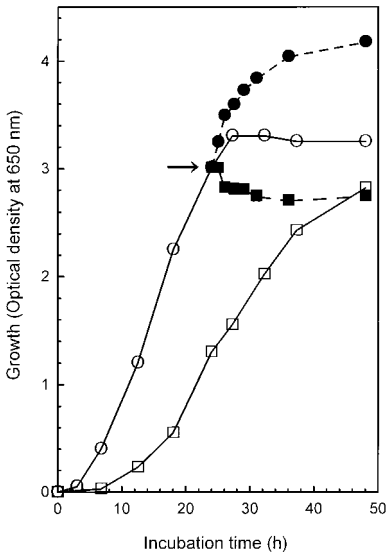

FIG. 2. Growth of *Brevibacterium* sp. strain JCM 6894 before and after osmotic upshock from 0.5 to 2 M NaCl. Cells were grown aerobically at 30°C in a complex medium containing 0.5 M NaCl (○) or 2 M NaCl (□). After 24 h of incubation, the cells were subjected to hyperosmotic shock (arrow) in old culture medium (■) or fresh culture medium (●). Cell growth was determined by measuring the OD_{650}.

For alanine and glutamine, the peaks at 1.49 and 2.45 ppm of the methyl group were used, respectively. A representative ^1H NMR spectrum of ethanol extracts of cells which were upshocked from 0.5 to 2 M NaCl and incubated for 1.5 h at 30°C is shown in Fig. 1.

^1H NMR measurements were also carried out with ethanol extracts of the extracellular medium, which were manipulated as follows: after centrifugation supernatants from old and fresh cultures were concentrated by evaporation at 50°C under a vacuum, and the concentrates were extracted with 99.5% ethanol after NaCl was removed by filtration.

Na^+ and K^+ concentrations in the cells. Internal free Na^+ concentrations in the *Brevibacterium* sp. strain were measured by ^{23}Na NMR with the Varian UNITY 500 spectrometer by using the membrane-impermeable shift reagent dysprosium triphosphate (17). The concentrations of K^+ in the cells were determined by using atomic absorption spectrometry (Nippon Jarrell Ash AA-11, Kyoto, Japan), as described elsewhere (16).

Calculation of intracellular solute concentrations. Internal solute concentrations in the *Brevibacterium* sp. strain were expressed as nanomoles per milligram of protein; the protein concentration in the cells was determined by using a bicinchoninic acid protein assay reagent (Pierce Chemical, Rockford, Ill.) with bovine serum albumin as the standard.

RESULTS

Response of growth and pH. Hyperosmotic upshock to 2 M NaCl for *Brevibacterium* sp. cells grown in the presence of 0.5 M NaCl was carried out under both old and fresh culture conditions. As shown in Fig. 2, the optical density at 650 nm (OD_{650}) of the *Brevibacterium* sp. cells decreased immediately after the upshock in the old culture and then became stationary for a few hours. Growth of this culture reached the same level as growth of cells grown for 48 h in the presence of 2 M NaCl from the beginning. The cell viability decreased only slightly from $3.15 \times 10^8 \pm 0.18 \times 10^8$ CFU/ml (before the shock) to $2.88 \times 10^8 \pm 0.21 \times 10^8$ CFU/ml (24 h after the shock); thus, the survivability of the cells upshocked in old culture was essentially maintained. On the other hand, growth was stimulated when the cells were transferred to freshly prepared medium containing 2 M NaCl. The OD_{650} increased from 3.0 to 3.8 during incubation for a few hours after the upshock (cell viability also increased [data not shown]) and then continued to increase slightly.

The changes in the pH of the media following the upshock from 0.5 to 2 M NaCl for both old and fresh culture media were measured. The pH of the old culture quickly changed from neutral to alkaline and then gradually increased to more than 8 during incubation (Fig. 3). In contrast, rapid acidification was observed (from pH 7.6 to 6.7) for cells in the fresh culture subjected to the upshock. Thereafter, the pH of the medium gradually increased and stabilized around 7.3 to 7.4.

Response of Na^+ concentrations. The internal free Na^+ concentration in the cells upshocked from 0.5 to 2 M NaCl in old culture medium increased from 80 to 190 nmol mg of protein^{-1} after 10 min of incubation (Fig. 4a). After a gradual decrease, the Na^+ concentration increased again almost linearly and was more than 400 nmol mg of protein^{-1} after 5 h of incubation (Fig. 5). In fresh medium, the intracellular Na^+ concentration was unchanged, (80 nmol mg of protein^{-1}) for 30 min after the upshock (Fig. 4b). Even after 5 h of incubation, the Na^+ concentration was less than 30% (120 nmol mg of protein^{-1}) of the Na^+ concentration in old medium.

The change in the free Na^+ concentration in the cells was also analyzed when the upshock was induced by adding K^+ instead of Na^+. The response of the Na^+ concentration was quite different from the responses observed when NaCl was added (see above); irrespective of whether the upshock procedure was conducted in fresh or old medium, the internal Na^+ concentrations in the *Brevibacterium* sp. strain rapidly decreased from 80 to 20 nmol mg of protein^{-1} and then remained constant at less than one-half the original concentration for 5 h (Fig. 5).

Response of K^+ concentrations. The internal K^+ concentration in the cells upshocked by NaCl in the old culture gradually increased from 1,530 nmol mg of protein^{-1} before the shock to 1,800 nmol mg of protein^{-1} at 1 h after the shock (Fig. 6). In contrast, a large amount of K^+ quickly accumulated in the *Brevibacterium* sp. cells when they were upshocked in fresh medium; within 10 min after the upshock, the K^+ levels were more than 2,800 nmol mg of protein^{-1}. Then, a slow release of K^+ from the cells was observed during the next few hours of incubation.

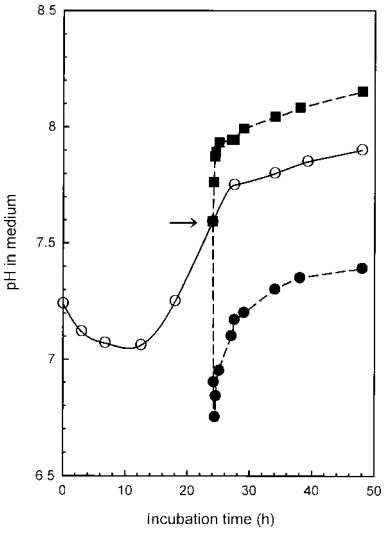

FIG. 3. Changes in the pH of the medium accompanying the growth of *Brevibacterium* sp. strain JCM 6894 as a function of the incubation time before and after osmotic upshock. Cells were grown aerobically at 30°C in a complex medium containing 0.5 M NaCl (○). After 24 h of incubation, the cells were subjected to hyperosmotic shock (arrow) in old culture medium (■) or fresh culture medium (●).

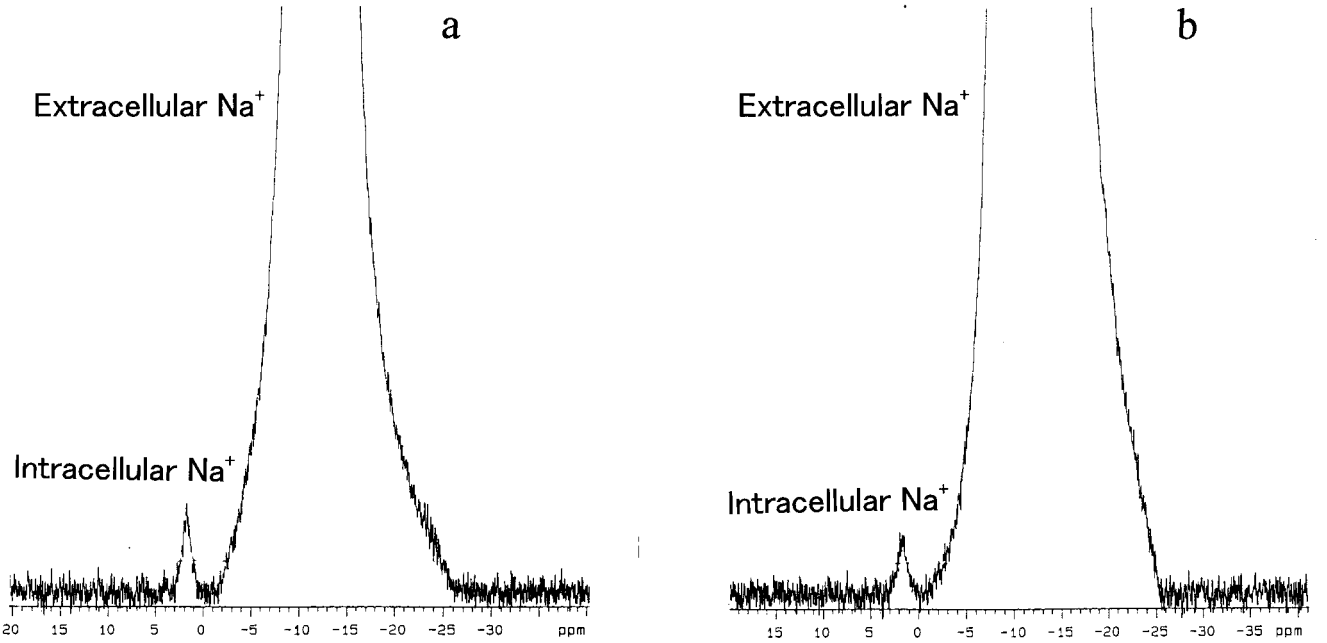

FIG. 4. ^{23}Na NMR spectra of *Brevibacterium* sp. cultures subjected to hyperosmotic shock (0.5 to 2 M NaCl) in old (a) and fresh (b) media (incubation time, 30 min). Measurements were determined in the presence of 0.5 M Na^+ and 0.017 M dysprosium triphosphate and the chemical shifts were referenced to a 0.005 M NaCl solution.

When KCl was used as the osmoticum for the upshock instead of NaCl, the JCM 6894 culture in fresh medium accumulated K^+ quite rapidly; at 20 to 30 min after the shock, the concentration of intracellular K^+ was more than 4,500 nmol mg of protein^{-1}. Upshock of the growing culture induced a similar accumulation pattern, but the amount of K^+ in the cells was about 80% of the amount of K^+ in the cells in fresh medium. After 30 min of incubation after the upshock, internal K^+ was slowly released from the cells in both media.

Responses of compatible solutes. Intracellular organic solutes that accumulated in the cells before and after osmotic upshock were quantitatively analyzed by ^{1}H and ^{13}C NMR spectroscopy, as well as by high-performance liquid chromatography. Because *Brevibacterium* sp. strain JCM 6894 synthesizes ectoine as a major compatible solute when it is grown in the presence of 2 M NaCl (18), we expected that initiation of ectoine synthesis would occur quickly after the shift to high

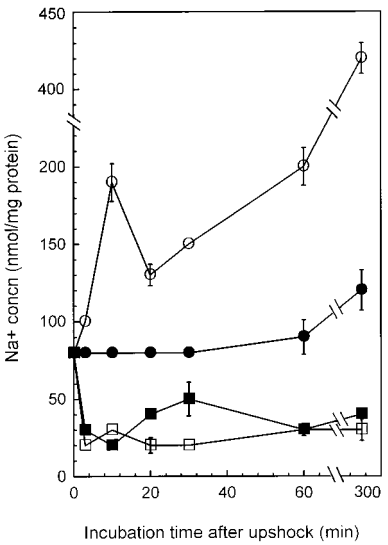

FIG. 5. Internal free Na^+ concentrations in *Brevibacterium* sp. strain JCM 6894 cells after osmotic upshock from 0.5 to 2 M NaCl. Cells were grown aerobically at 30°C in a complex medium for 24 h and inoculated into old culture medium (open symbols) or fresh culture medium (solid symbols) with a total salts concentration of 2 M achieved by adding either 1.5 M NaCl (circles) or KCl (squares). Details concerning Na^+ measurements are given in Materials and Methods.

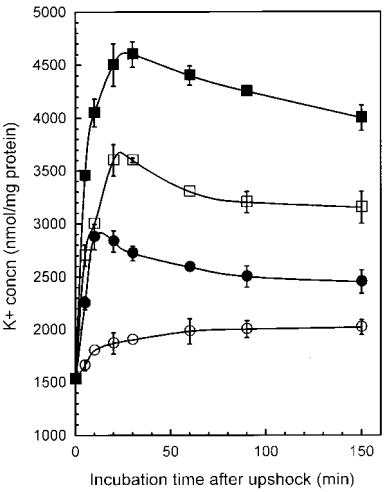

FIG. 6. Internal K^+ concentrations in *Brevibacterium* sp. strain JCM 6894 cells after osmotic upshock from 0.5 to 2 M NaCl. Cells were grown aerobically at 30°C in a complex medium for 24 h and inoculated into old culture medium (open symbols) or fresh culture medium (solid symbols) with a total salts concentration achieved by adding either 1.5 M NaCl (circles) or KCl (squares). Details concerning K^+ measurements are given in Materials and Methods.

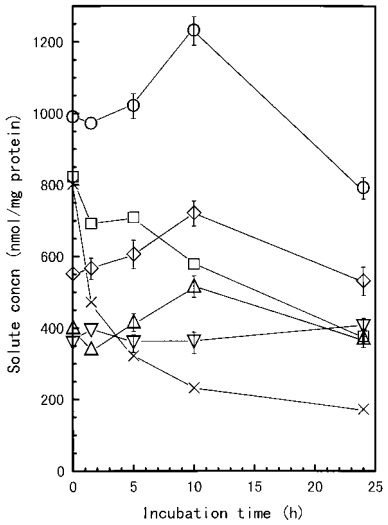

FIG. 7. Changes in organic solute concentrations in *Brevibacterium* sp. strain JCM 6894 cells after osmotic upshock in old cultures. Cells were grown aerobically at 30°C in a complex medium until the late exponential phase of growth and inoculated into old culture medium containing 2 M NaCl. The procedures used for cell extraction and NMR measurement are described in Materials and Methods. The following solutes were identified and quantified by ^{1}H NMR: ectoine (△), glutamine (▽), glutamate (○), glycine betaine (◇), TMAH (×), and hydroxyproline (□). The values are the averages ± standard deviations from three independent experiments.

osmolarity. The ectoine levels in the cells, however, decreased a little initially and then increased only slightly, as shown in Fig. 7. A similar response was observed for both glutamate and glycine betaine levels, which increased slightly for 10 h after the shock and then gradually decreased. The concentration of TMAH, which was approximately 500 nmol mg of protein^{-1} before the upshock, decreased abruptly to approximately 100 nmol mg of protein^{-1}; this corresponded to a release of more than 70% of the TMAH from the cells (Fig. 7). Neither alanine nor hydroxyectoine was detected in the cells (detection limit, 3 nmol mg of protein^{-1}).

A notable increase in the synthesis of ectoine was observed in the cells upshocked in fresh medium; after a short time lag, the ectoine levels in the cells markedly increased to 2,500 and ~3,500 nmol mg of protein^{-1} at 5 and 10 h after the upshock, respectively (Fig. 8a). The glutamine levels in the cells increased almost linearly for 5 h, as did the levels of ectoine; then the levels rapidly decreased with increased incubation time. Although an approximately twofold increase in the glycine betaine levels was also observed at 1.5 h after the shock, the glutamate levels were unchanged. In contrast to the drastic release of TMAH from the cells subjected to upshock in the old culture, the TMAH concentration increased almost twofold after the upshock in the fresh culture. Surprisingly, alanine was first detected in the cells at 1.5 h after the shock and then disappeared (Fig. 8b). As in the old culture, accumulation of hydroxyproline was also observed in this culture. Hydroxyectoine was detected in the cells incubated for 10 to 24 h after the upshock, and the levels tended to increase with increased incubation time. Similar patterns of accumulation of these compatible solutes were observed when KCl instead of NaCl was used as the osmoticum for the cells upshocked in both media (data not shown).

Response of medium composition. To better understand the intracellular changes after the upshock, we attempted to measure the changes in medium composition. Thus, supernatant

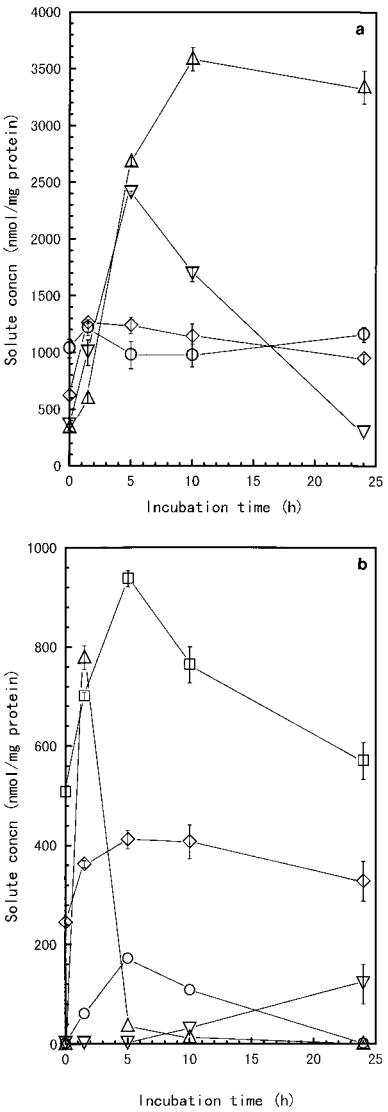

FIG. 8. Changes in organic solute concentrations in *Brevibacterium* sp. strain JCM 6894 cells after osmotic upshock in fresh cultures. Cells were grown aerobically at 30°C in a complex medium until the late exponential phase of growth and inoculated into freshly prepared medium containing 2 M NaCl. The procedures used for cell extraction and NMR measurement are described in Materials and Methods. The following solutes were identified and quantified by ^{1}H NMR: ectoine (△), glutamine (▽), glutamate (○), and glycine betaine (◇) (a); and TMAH (□), hydroxyproline (◇), alanine (△), hydroxyectoine (▽), and trehalose (○) (b). The values are the averages ± standard deviations from three independent experiments.

fractions obtained from cultures before and after the upshock were analyzed by using ^{1}H NMR spectroscopy. Representative peaks for the fresh and old cultures before the shock were assigned (Fig. 9) and quantified, as shown in Table 1. A comparison of the two cultures before the shock showed that most of the amino acids present in the fresh medium were utilized during cell growth; the only exceptions were arginine and the cyclic amino acid 2-pyrrolidone-5-carboxylic acid (PCA). In old cultures before and after the shock we detected only limited amounts of the nutrients originally present. In the cells subjected to hyperosmotic shock in fresh medium, major amino acids were utilized at least for 5 h. The concentration of TMAH present in the fresh culture decreased from 0.51 to 0.41

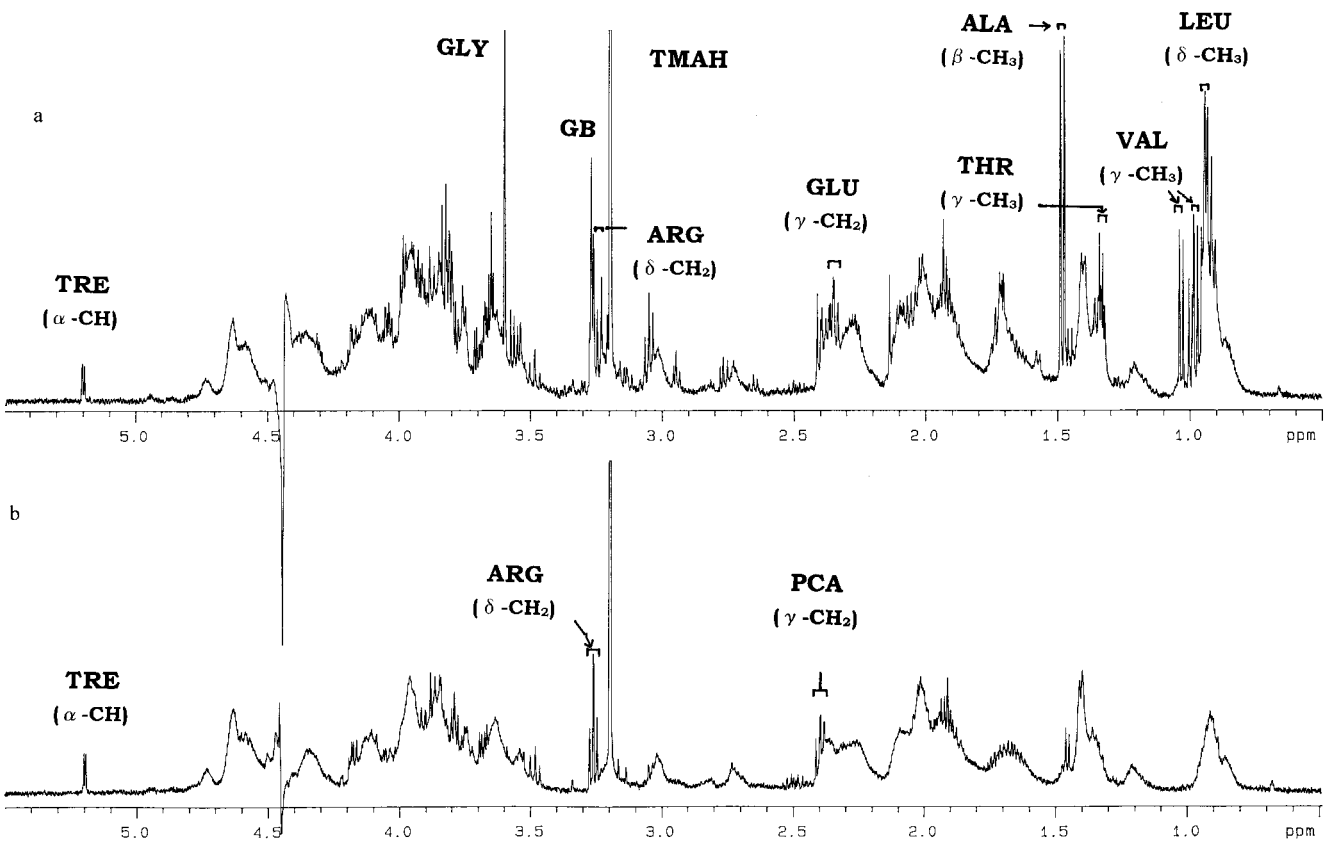

FIG. 9. ^{1}H NMR spectra of cell-free supernatants of fresh (a) and old (b) culture media before upshock. Cells were grown aerobically at 30°C in 0.5 M NaCl-containing medium until the late exponential phase of growth, and then a supernatant fraction of the old culture medium was obtained by centrifugation. Signals were assigned to leucine (LEU), valine (VAL), threonine (THR), alanine (ALA), glutamate (GLU), TMAH, glycine betaine (GB), glycine (GLY), trehalose (TRE), PCA, and arginine (ARG).

mM during cell growth for 24 h, probably due to accumulation of TMAH in the cells. As expected from the results obtained for TMAH release and uptake by cells when they were upshocked in old and fresh cultures, the extracellular concentration of TMAH increased and decreased by 0.06 and 0.08 mM at 5 h after the upshock, respectively. When the cells were upshocked in the old culture containing yeast extract or peptone, the extracellular concentration of TMAH decreased, as it did in cells upshocked in the fresh culture, indicating that strain JCM 6894 possesses a cotransporter system for TMAH and nutrients (data not shown). Furthermore, we observed reductions in PCA levels after the upshock in both media.

TABLE 1. Extracellular solute concentrations in fresh and old cultures before upshock and after upshock after 5 h of incubation[a]

Solute	Extracellular concn (mM)			
	Fresh culture		Old culture	
	Before shock	After shock	Before shock	After shock
TMAH	0.51	0.43	0.41	0.47
Trehalose	0.07	0.06	0.07	0.07
Glycine betaine	0.12	ND[b]	ND	ND
Aspartate	0.28	ND	ND	ND
Threonine	0.24	0.07	ND	ND
Glutamate	0.50	ND	ND	ND
Glycine	0.55	ND	ND	ND
Alanine	1.00	ND	ND	ND
Valine	0.50	0.13	ND	ND
Leucine	0.82	ND	ND	ND
Arginine	0.48	0.31	0.37	0.28
PCA	0.27	0.09	0.25	0.10

[a] The errors in solute levels were ± 0.02 mM.
[b] ND, not detected. The detection limit was <0.01 mM.

DISCUSSION

When the *Brevibacterium* sp. cells in an old culture were subjected to an upshock caused by the addition of NaCl, the concentrations of the main solutes in the cells before the shock, such as K^+, glutamate, and glycine betaine, did not differ significantly during the subsequent 24 h of incubation. These solutes also accumulated in nonhalophilic bacteria at an early stage after an upshock (6). As determined from the growth curve (Fig. 2), accumulation of these compatible solutes in strain JCM 6894 could not support sufficient cell growth, although the acute depletion of available nutrients in the old culture (Fig. 8b) had to be taken into consideration. Cell survival without cell multiplication, however, was helped by accumulation of these solutes for at least 24 h, since viability changed little.

In contrast, the *Brevibacterium* sp. cells grown in fresh medium accumulated K^+ quite rapidly; the rate of uptake of K^+ was fivefold higher in fresh medium than in old medium. At the same time, the alanine levels in the cells increased remark-

ably for 1.5 h after the upshock. The variations in the alanine levels in the cells suggest that the cells rapidly take up alanine from the medium and digest it in the cytosol, since the NMR analysis of cell-free supernatants of a fresh culture after the upshock revealed that the alanine that was present in the fresh medium before the shock disappeared (Table 1). The same was true for aspartate and leucine. The fact that glutamine almost constantly accumulated in the cells upshocked in fresh medium is consistent with the extracellular composition analytical results, which showed that the glutamine that was originally present was not detected 5 h after the upshock. Thus, a series of cellular responses which were not observed in the old culture suggest that strain JCM 6894 accumulates K^+ as an instantaneous adaptation for sudden changes in osmotic pressure and then utilizes alanine and glutamine as compatible solutes in the subsequent stage. It is likely that in face of the hyperosmotic shock, the *Brevibacterium* strain initiated the necessary preparations for growth through these responses. After the transient accumulations, ectoine synthesis was initiated in the cells. Since ectoine has a potent osmoprotective ability for nonhalophilic bacteria that cannot synthesize ectoine at higher salinity values (12, 22), the relationship between accumulation of ectoine and growth rate indicates that the synthesis of ectoine is essential for the growth of strain JCM 6894 when it is suddenly exposed to high osmolarity.

A striking contrast was observed between the responses of intracellular K^+ and intracellular Na^+ to the osmotic changes in the fresh culture; after the upshock, the internal K^+ concentrations in the cells increased rapidly, whereas the internal Na^+ concentrations were unchanged. Similar increases in the intracellular K^+ concentration after upshock were observed for *Escherichia coli* (6, 14, 20), *Bacillus subtilis* (24), and the soil bacterium *Rhizobium fredii* (8). In contrast, the Na^+ concentration was controlled at low levels (1/35th of the K^+ concentration), which suggests that strain JCM 6894 excluded Na^+ to maintain low concentrations of Na^+ even in an environment containing large amounts of Na^+. The fact that Na^+ entered the cells when they were suspended in the old culture indicates that the cellular activity of growing cells was greatly reduced under the severe high-osmolarity and poor-nutrient conditions (Table 1).

The alkalinization and acidification of the medium after upshock of the cells incubated in old and fresh media might be explained in terms of TMAH release and uptake, respectively. In addition, because strain JCM 6894 exhibited quite rapid uptake of K^+ after the shock (Fig. 6), it is likely that the instant acidification observed in fresh medium was induced by H^+ efflux via a K^+-H^+ antiporter (16). Unfortunately, it is not yet clear why *Brevibacterium* sp. strain JCM 6894 quickly accumulated and digested both alanine and glutamine when the fresh culture was upshocked (Fig. 8) but also utilized PCA after the upshock in both cultures. In connection with these cellular activities, further comparative studies are in progress to clarify the quick responses of cells to a variety of changes in external environments.

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