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Supplementation Effects of Hydroxyectoine on Proline Uptake of Downshocked *Brevibacterium* sp. JCM 6894

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Downshock treatment of the halotolerant *Brevibacterium* sp. JCM 6894 was a prerequisite for proline uptake which is a function for cell survival. Hydroxyectoine served as an effective stimulator for the proline uptake and cell survival of the downshocked cells of this strain. Duration of osmotic downshock, downshock strength, and the kinds of osmolyte affected the efficient rate of growth (ERG) and the uptake of proline. A shorter duration of osmotic downshock, that is ≤1 h, induced higher ERG values and stimulated proline uptake, compared with those of non-downshocked cells when incubated with hydroxyectoine and proline. These activities, however, were gradually suppressed with the prolongation of the duration of osmotic downshock. When the cells grown in KCl-containing medium were subjected to downshock treatment for 1 h, their activities were enhanced more remarkably than those of cells grown in NaCl-containing medium.

[**Key words:** proline, hydroxyectoine, ectoine, *Brevibacterium* sp. JCM 6894, osmotic downshock]

The accumulation of compatible solutes is a prerequisite for the adaptation of most microorganisms to osmotic stress brought about by ions and/or solutes. The mechanisms for coping with high external salinity are salt-in or salt-out mechanisms (1, 2). To maintain the osmotic changes in external environments, microorganisms sense and respond to multiple environmental factors through signal transduction pathways in terms of the activation or repression of specific target genes (3–5). The genes and enzymes responsible for the modulation of osmoregulatory solute levels have been identified in diverse bacteria (4, 6–8). To cope with hyperosmotic conditions, microorganisms accumulate large quantities of a particular group of organic osmolytes (1, 8, 9) and release them when they are exposed to hypoosmotic conditions (10, 11).

Ectoine and its hydroxyl derivative, hydroxyectoine, are well known as ubiquitous compatible solutes serving mainly as osmoprotectants for halophilic and halotolerant bacteria under high osmolarity conditions. Hydroxyectoine is a protein-protecting agent which has properties superior to those of ectoine in a number of applications (12). In our previous reports, ectoine was shown to be released from the cell cytoplasm via hypoosmotic shock and reutilized as an organic carbon and for energy reservoirs by accumulating it in the cell cytoplasm (13–16). To date, however, there has been little knowledge regarding the functions of hydroxyectoine on the physiological characteristics of downshocked cells when various types of substrate are present in the medium.

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Recently, we have investigated some notable characteristics of the halotolerant *Brevibacterium* sp. JCM 6894 on the fate of ectoine for cell survival when the cells were subjected to osmotic downshock from 2 and 0.7 M to 0 M NaCl, and incubated with ectoine alone, in which ectoine showed dual functions as an osmoprotectant and a metabolic substrate (15, 16). Furthermore, acidic amino acid glutamate promoted the cell survival of downshocked *Brevibacterium* sp. JCM 6894 cells as described previously, in which the presence of ectoine led to the suppression of glutamate utilization in the absence of NaCl (17). Therefore, it seems valuable to examine how ectoine and hydroxyectoine affect the uptake of substrates and cell survival in the presence of typical carbon and nitrogen sources for downshocked cells of the strain JCM 6894, because this strain can synthesize both ectoine and hydroxyectoine in the cell cytoplasm and utilize them as osmoprotectants. Moreover, we also tried to elucidate the effects of duration of osmotic downshock, downshock strength and the kinds of osmolyte on the cell activities mentioned above. Our present findings indicate that both hydroxyectoine and ectoine were slightly metabolized by the downshocked cells in the presence of proline, in contrast to the non-downshocked cells. For downshocked cells, hydroxyectoine served as an efficient stimulator for the uptake of proline which played an important role in cell proliferation.

MATERIALS AND METHODS

Media and growth conditions A halotolerant strain *Brevibacterium* sp. JCM 6894 used in this study was grown aerobically at 30°C in a nutrient medium ($g \cdot l^{-1}$): Bacto peptone (Difco Laboratories, Detroit, MI, USA), 5.0; Bacto yeast extract (Difco), 3.0;

KCl or NaCl (0.5–2 M as a final concentration). Medium pH was adjusted to 7.2 with NaOH before autoclaving. Cell growth rate was determined by measuring turbidity at 650 nm (OD_{650}) using a Beckman DU 640 spectrophotometer (Beckman Instruments, Fullerton, CA, USA). If the OD_{650} was above 1.0, the samples were diluted 3–5-fold with their respective sterile growth medium and measured, because linearity was observed at $OD_{650}<1$.

Osmotic downshock treatment The procedure for osmotic downshock was slightly modified from that described previously (16); the cells were grown in 30 ml of nutrient medium in a 300-ml flask until the late exponential phase of growth and centrifuged (12,000×*g*, 10 min, 4°C). The pellets were washed twice with 10 mM KP_i buffer (pH 7.2) containing the same concentrations of KCl or NaCl as those in the medium. Subsequently, the cells were subjected to hypoosmotic shift by suspending and mixing in deionized water (pH 7.0) using a vortex. The cell suspensions were dipped in a water bath (30°C) without shaking for an appropriate time. To prepare the downshocked cells, cell density was adjusted to $OD_{650}\approx 3$. The *t* h-DS_{KCl} and DS_{NaCl} cells were referred to as the cells grown in the presence of 0.7 M each KCl and NaCl, respectively, which were subsequently subjected to osmotic downshock for *t* h ($t=0-5$). The 0 h-DS_{KCl} and 0 h-DS_{NaCl} cells, wherein the procedure of hypoosmotic shift for the preparation of downshocked cells was omitted, were referred to as non-downshocked cells grown in the presence of 0.7 M each KCl and NaCl, respectively.

Both downshocked and non-downshocked cells were centrifuged and suspended in deionized water (pH 7.0) to adjust cell density ($OD_{650}\approx 1$). Six milliliters of the cell suspension was transferred into each test tube (18×180 mm). Immediately after adding 10 mM substrate and 1 mM each hydroxyectoine or ectoine into the cell suspensions, incubation was started at 30°C with reciprocal shaking (150 strokes·min^{-1}). Hydroxyectoine, ectoine, sugars and amino acids used in this study were sterilized separately by filtration through a 0.2-μm membrane filter (Toyo Roshi Kaisha, Tokyo).

HPLC analyses of hydroxyectoine and ectoine At a designated incubation time, an aliquot of 0.2 ml of the cell suspensions was sampled and washed once with the same volume of 10 mM KP_i buffer (pH 7.2) containing the same concentrations of KCl or NaCl as those in the nutrient media. Both supernatant and pellet fractions, the latter being extracted with 80% ethanol as described previously (7), were used for the determination of extracellular and intracellular concentrations of hydroxyectoine or ectoine.

Hydroxyectoine and ectoine were detected using a high-performance liquid chromatography (HPLC) system (L-5020; Hitachi, Tokyo) with a Bio-Sil C_8 90-5S reversed phase column (Bio-Rad Laboratories, Hercules, CA, USA) as described previously (18). Five microliters of samples were eluted with 50 mM KP_i buffer at pH 6.0 with a flow rate of 1 ml/min at 35°C and detected by UV absorbance at 210 nm. The identification and quantification of hydroxyectoine as well as ectoine were carried out using original samples.

To demonstrate the supplementation effects of hydroxyectoine and ectoine on cell survival, efficient rate of growth (ERG) at 48 h of incubation was conceptualized and calculated using

$$\text{ERG (HE)} = OD_{CS+HE}/OD_{CS} \quad \text{or}$$
$$\text{ERG (EC)} = OD_{CS+EC}/OD_{CS}$$

where OD_{CS+HE} or OD_{CS+EC} denotes the turbidity at 650 nm in the presence of 10 mM carbon source with 1 mM each hydroxyectoine or ectoine, respectively; OD_{CS} denotes the turbidity in the presence of 10 mM carbon source alone.

Analysis of proline At a designated incubation time, an aliquot of 0.5 ml of the cell suspensions was sampled and centrifuged and the supernatant was recovered, which was used for the analyses of extracellular concentrations of proline. The identification and quantification of proline were slightly modified from that described by Whatmore *et al*. (19).

Chemicals Hydroxyectoine and ectoine with >97% purities were provided by Dr. E.A. Galinski, Universität Bonn, Germany and Dainippon Pharmaceutical, Tokyo, respectively. The other chemicals used were of reagent or higher grade.

RESULTS AND DISCUSSION

Supplementation effects of hydroxyectoine and proline on downshocked cells

1 h-DS_{NaCl} cells In relation to the previous observations on the stimulation of downshocked cells in the presence of an acidic amino acid, glutamate (17), we prepared the same downshocked cells of *Brevibacterium* sp. JCM 6894 (1 h-DS_{NaCl} cells) to compare and examine their physiological functions, taking the supplementation effects of ectoine and hydroxyectoine into consideration. As shown in Fig. 1, the turbidities of the cells incubated in the presence of glutamate alone strikingly increased after 6 h of incubation, in which we observed the maximal value of OD_{650} at 1.66 after 48 h of incubation. With the supplementation of ectoine, however, cell turbidity was lower than that in the presence of glutamate alone. Thus, we tried to examine the supplementation effects of hydroxyectoine, where we observed a higher growth inhibition than that of ectoine supplemented to the medium containing glutamate (Fig. 1).

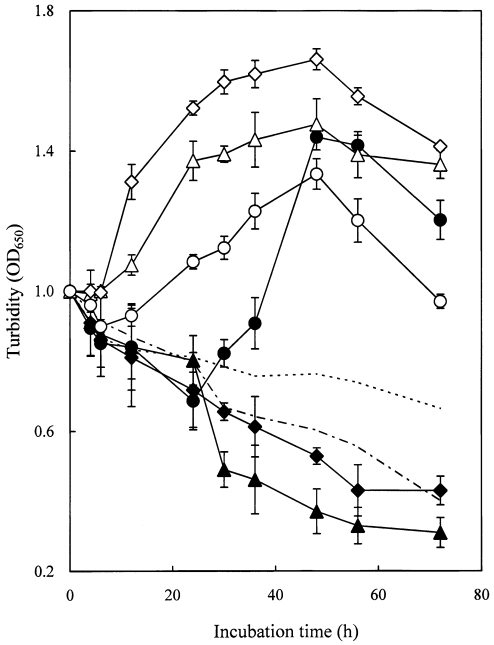

FIG. 1. Cell survival of 1 h-DS_{NaCl} cells of *Brevibacterium* sp. JCM 6894. Strain JCM 6894 was grown in a medium containing 0.7 M NaCl at 30°C for 24 h. After centrifugation and washing of the cells, they were subjected to osmotic downshock by incubating in deionized water at 30°C for 1 h. The downshocked cells were incubated with 10 mM glutamate (open symbols) or 10 mM proline (closed symbols) at 30°C with 1 mM hydroxyectoine (circles), 1 mM ectoine (triangles) or without supplementation (diamonds). The dotted or dash dotted line indicates turbidity changes in the presence of hydroxyectoine with proline or glutamate for non-downshocked cells, respectively. Cell growth was determined by measuring the OD_{650}. The values are the means±SD from three independent experiments.