Mitigation of Hyper KCl Stress at 42°C with Externally Existing Sodium Glutamate to a Halotolerant Brevibacterium sp. JCM 6894

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Halotolerant Brevibacterium sp. JCM 6894 grew at 37°C in the presence of 2.3 M KCl, while the growth was repressed with the same concentration of NaCl. When resting cells, \(10^{7.4} \pm 0.1\) (CFU·mL\(^{-1}\)), prepared from cells grown in the absence of salts at 30°C, were exposed to 3.3 M NaCl for 36 h at 42°C, reduction of the number of resting cells was maintained within a 1-log cycle in the presence of proline, betaine, or ectoine (50 mM). In the presence of 3.3 M KCl, the most functional osmoprotectant was sodium glutamate (50 mM), and the value was \(10^{7.2} \pm 0.1\) (CFU·mL\(^{-1}\)) when exposed for 72 h at 42°C. In the absence of osmoprotectants, the value was reduced to four orders of magnitude in each experimental condition. The number of resting cells, \(10^{6.8} \pm 0.1\) (CFU·mL\(^{-1}\)), prepared from grown cells pre-adapted to 2.3 M KCl at 37°C, was hardly reduced when exposed to 3.3 M KCl in the presence of sodium glutamate more than 50 mM for 72 h at 42°C. Those results indicate that the isolate can sense the difference in hyper KCl stress as opposed to hyper NaCl stress, and different kinds of osmoadaptation systems can function to cope with each hyper salt stress.

Key words: Marine halotolerant Brevibacterium sp. / Glutamate / Proline / Survivability / Hyper KCl Stress.

INTRODUCTION

Halophilic and halotolerant bacteria inhabit seawater. Halophilic bacteria require Na\(^+\) for growth, while halotolerant bacteria can grow in a wide range of NaCl concentrations, including without the addition of NaCl (Larsen, 1986). An osmoadaptation system is essential to preventing the movement of water molecules to the outside from the cytoplasm by accumulating osmoprotectants. In general, betaine, proline, ectoine, and glutamate, etc. function as osmoprotectants. Halophilic Vibrio parahaemolyticus (Naughton et al., 2009) and V. cholerae (Pflughoft et al., 2003) synthesize ectoine and transport betaine externally existing into the cytoplasm at higher concentrations of NaCl. Ectoine was the most accumulated osmoprotectant of Brevibacterium sp. JCM 6894 when grown in a medium containing 2 M NaCl at 30°C (Nagata et al., 1996).

Transporters located in cell membranes play an important role in growing and surviving the strain at high concentrations of NaCl in the presence of osmoprotectants. Some transporters possess a Na\(^+\)-binding site. For example, the betaine transporter, BetP, of soil bacterium Corynebacterium glutamicum (Krämer and Morbach, 2004; Perez et al., 2014; Ressl et al., 2009) and the proline transporter, PutP, of Escherichia coli (Bracher et al., 2016) need Na\(^+\). Glutamic acid is transported coupled with Na\(^+\) and H\(^+\) by symporters such as GltTBs of Bacillus stearothermophilus and GltTBc of Bacillus caldotenax (Tolner et al., 1992).

Excess amounts of Na\(^+\) in the cytoplasm are toxic to bacteria. On the other hand, K\(^+\) abundantly existing in the cytoplasm helps maintain the osmotic balance across the membrane, the activities of many enzymes, and the intracellular pH, as well as reducing the negative charge of DNA. High-affinity K\(^+\)-transporters, such as KdpFABC of E. coli (Csonka and Epstein, 1996) and KtrAB of Bacillus subtilis (Gundlach et al., 2017), can bind and transport K\(^+\) into the cytoplasm efficiently, even with considerably lower concentrations of K\(^+\) existing externally. High concentrations of KCl added externally give bacteria the same cationic and osmotic stresses chemically as those given by the same concentrations...
of NaCl. However, the adaptation and tolerance mechanisms of bacteria under hyper KCl stress are different from those under hyper NaCl stress (Yin and Mimura, 2020). We believe that studying survivability under hyper KCl stress will contribute to a thorough understanding of bacterial adaptation to salt stress.

Marine Brevibacterium sp. JCM 6894 can grow in wide ranges of KCl as well as NaCl concentrations. In this study, we examined the mitigation of hyper NaCl or KCl stress with each osmoprotectant existing externally to the strain with exposure to 30, 37, and 42°C.

MATERIALS AND METHODS

Strain and growth conditions

Halotolerant Brevibacterium sp. JCM 6894 that was isolated from seawater (Nagata, 1988) was used in the experiments. The strain was pre-incubated at 30°C for 28 h in the medium containing 5 g Bacto Peptone (L⁻¹) (BD Diagnostics-Diagnostic Systems, Maryland, USA) and 1 g yeast extract (L⁻¹) (BD Diagnostics-Diagnostic Systems, Maryland, USA) in 50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)–tetramethylammonium hydroxide (TMAH) buffer, pH 7.5. Incubation was started with the addition of cell suspension (50 μL) to the medium (50 mL)—at 30°C or 37°C—which contained no salts, 2.3 M NaCl, 2.3 M KCl, 2.6 M NaCl, or 2.6 M KCl. Turbidity in the medium was measured at a given time with a photometer (BioPhotometer #6131, Eppendorf AG, Hamburg, Germany) at 600 nm.

Preparation of resting cells

The strain was grown for 28 h at 30°C, or 18 h at 37°C, in the medium without salts added. The strain was also grown for 189 h in the medium containing 2.3 M NaCl at 30°C, 2.3 M KCl at 30°C, 2.3 M KCl at 37°C, and 2.6 M KCl at 30°C. Cells that had reached the early stationary phase of growth were harvested by centrifugation (10,000 × g, 5 min) at room temperature and washed twice with 50 mM HEPES-TMAH buffer, pH 7.5, with or without the same concentration of NaCl or KCl as in the growth medium. A cell pellet was then resuspended in the same buffer. Resting cells thus obtained were used for the surviving experiments. The cell pellet was also resuspended in distilled water for measuring the free amino acids pooled in the cytoplasm as well as the content of cell proteins.

Surviving experiments under hyper salt stress

Surviving experiments were carried out at 30°C, 37°C, and 42°C by adding resting cells to 50 mM HEPES-TMAH buffer, pH 7.5, containing various concentrations of NaCl or KCl to obtain a one-hundredth dilution. The experiments were also carried out in the presence of each osmoprotectant in 3.3 M NaCl at 42°C or 3.3 M KCl at 42°C.

Enumeration of the surviving cells

Resting cells (100 μL) were pipetted out at a given time and diluted serially with 50 mM HEPES-TMAH buffer, pH 7.5, containing the same concentration of NaCl or KCl as in the washing buffer. Each sample was then spread onto an agar plate containing the same amounts of nutrients used for the growth experiments and 1.5% agar in seawater. After incubation for 5 d at 30°C, colonies on the plates were counted. The data are shown as colony-forming units (CFU)-mL⁻¹.

Measurement of free amino acids pooled in cytoplasm

Six percent trichloroacetic acid (2 mL) was added to the cell suspension (2 mL), and the sample was kept at 4°C for 1 d. After centrifugation (10,000 × g, 5 min), the supernatant was collected and kept in a tube with a screw cap until use. Samples thus obtained were analyzed with an amino acid analyzer (L-8900, Hitachi High-Technologies Corporation, Tokyo, Japan) in the Global Facility Center, Hokkaido University.

Cell protein assay

The protein content for each sample was assayed by bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) as directed in the instruction manual. Bovine serum albumin was used as a standard.

RESULTS AND DISCUSSION

Usefulness of resting cells for studying osmoadaptation under hyper salt stress in the presence of an osmoprotectant

Brevibacterium sp. JCM 6894 can grow in the absence or presence of high concentrations of KCl as well as NaCl (Nagata, 1988), indicating that survivability at hyper KCl stress can be comparable to that at hyper NaCl by using resting cells prepared from cells grown in the absence of salts. In addition to that, a highly functional osmoprotectant can be determined by examining survivability. Resting cells are also available to determine the functional osmoprotectant in relation to the growth with non-salt stress and high concentrations of NaCl or KCl with exposure to temperatures higher than that in which the strain can grow.

Growth at hyper salt stress with exposure to 37°C

Growth of the strain was examined in the absence or presence of high concentrations of NaCl or KCl at 30°C (Fig. 1A) and 37°C (Fig. 1B). Growth in the presence
of 2.6 M KCl was better than that in the presence of 2.6 M NaCl at 30°C (Fig. 1A). It took more than 170 h for the strain to reach the early stationary phase in the presence of 2.3 M KCl at 37°C (Fig. 1B). Growth at 2.3 M NaCl was strongly repressed.

The strain is a halotolerant bacterium, which can grow in the absence or presence of high concentrations of NaCl (Larsen, 1986; Nagata, 1988). Previously published data showed that the strain grew in the presence of up to 3 M NaCl or KCl at 30°C, and growth was slightly better with the addition of NaCl than that with the same concentration of KCl (Nagata, 1988). The results obtained from the present experiments at 30°C were inconsistent with the previously published data for growth at hyper salt stress. The difference might depend on small amounts of inorganic salts such as CaCl₂, MgSO₄, NH₄Cl, K₂HPO₄, and KH₂PO₄ externally added to the nutrient medium (Nagata, 1988). The strain grew well at 37°C in the presence of 2.3 M KCl, but not at 2.3 M NaCl, indicating that hyper KCl stress functions to mitigate temperature stress.

Changes in survivability of resting cells after exposure to hyper salt stress at 30, 37, and 42°C (grown in the absence of salts at 30°C)

The initial number of resting cells, $10^{6.8} ± 0.1$ (CFU·mL⁻¹), was maintained within a 7-log order of magnitude after 120 h at 30°C in the absence of salts (Fig. 2A). The number of surviving cells was reduced to $10^{4.2} ± 0.3$ (CFU·mL⁻¹) after 72 h at 37°C. At 42°C, it was reduced exponentially to less than $10^{2.9}$ (CFU·mL⁻¹) within 24 h. The values showed more than $10^{3.0}$ (CFU·mL⁻¹) even when the exposure time was prolonged to 120 h at 37°C and 48 h at 42°C in the presence of 3.3 M NaCl (Fig. 2B). The values for 120 h at 37°C and 120 h at 42°C were $10^{4.2} ± 0.3$ (CFU·mL⁻¹) and $10^{2.9} ± 0.7$ (CFU·mL⁻¹), respectively, in the presence of 3.3 M KCl (Fig. 2C).

The strain increases heat resistance for a short period, such as within 30 min, by exposure to high concentrations of NaCl or KCl existing externally (Mimura and Nagata, 1998). In the present study, it was found that hyper KCl stress rather than hyper NaCl stress could mitigate heat stress to the strain for a prolonged period (Fig. 1B and Fig. 2).

Changes in the survivability of resting cells after exposure to 3.3 M KCl at 37 and 42°C (grown in the presence of 2.3 M KCl at 37°C)

The initial number of resting cells, $10^{6.8} ± 0.1$ (CFU·mL⁻¹), was reduced gradually to $10^{5.1} ± 0.1$ (CFU·mL⁻¹) for 240 h at 37°C in the presence of 2.3 M KCl (Fig. 3), indicating that the concentration of KCl osmotically damages the strain, although the strain could grow at 2.3 M KCl at 37°C in the nutrient medium. The value was reduced
to 10^{3.4 \pm 0.1} \text{ (CFU}\cdot\text{mL}^{-1})\) when exposed to 3.3 M KCl for 240 h at 37ºC. It was 10^{3.4 \pm 0.1} \text{ (CFU}\cdot\text{mL}^{-1}) after 120 h at 42ºC.

The time of exposure required to obtain a 3-log cycle of reduction at 37ºC was 120 h longer for resting cells prepared from pre-adapted cells grown in the presence of 2.3 M KCl at 37ºC than for resting cells prepared from cells grown in the absence of salts at 30ºC.

Similar results were observed at 42ºC (see Fig. 2C).

The strain grown with 2.3 M KCl seemed to synthesize stress-induced proteins that contribute to an increase in survivability (Hantke et al., 2019; Lindquist, 1992).

Changes in free amino acids accumulated in cytoplasm when grown in the absence and presence of hyper salt stress at 30 and 37ºC

Concentrations and compositions of free amino acids accumulated in cytoplasm were measured when grown in the absence (Table 1) and presence of hyper NaCl or KCl stress at 30 and 37ºC (Table 2).

The value of the total content, 517.4 (nmol mg-protein^{-1}), was less than 50% for the strain grown at 37ºC in comparison with that at 30ºC (Table 1). Glutamic acid, hydroxyproline, and proline were major free amino acids when grown at 30ºC without salts added externally. The occupation ratios of glutamic acid were more than 70% of the total content at 30 and 37ºC. At 37ºC, the total content was reduced to less than half that at 30ºC.

The strain might utilize free amino acids preferentially to synthesize stress-induced proteins when grown at
Changes in the survivability of resting cells after exposure to 3.3 M NaCl or KCl in the presence of each osmoprotectant at 42°C

The initial number of resting cells—$10^{7.4 \pm 0.1}$ (CFU·mL$^{-1}$)—that were prepared from cells grown without salts added to the nutrient medium at 30°C was reduced to four orders of magnitude after exposure to 3.3 M NaCl alone for 36 h at 42°C (Table 3). The number of surviving cells was maintained within the reduction of a 1-log cycle when 50 mM of proline, betaine, or ectoine existed externally. A 2-log cycle reduction from the initial number of resting cells was observed in the presence of 50 mM sodium glutamate. The reduction was a 3-log cycle in the presence of 3.3 M KCl alone for 72 h at 42°C. That was hardly reduced by the presence of 50 mM potassium glutamate. The number of surviving cells for potassium glutamate was a 2-log cycle lower than that for sodium glutamate. As for proline, the reduction was a 2-log cycle. Betaine, hydroxyproline, or ectoine at 50 mM did not mitigate hyper KCl stress to the strain. When the exposure time was prolonged to 120 h, the most functional osmoprotectant was sodium glutamate.

TABLE 1. Free amino acids pooled in the cytoplasm of cells grown without salts externally added.

| Amino acid$^a$ | 30°C for 28 h | 37°C for 18 h |
|----------------|---------------|---------------|
|                | nmol mg$^{-1}$ | Occupation ratio (%)$^c$ | mM$^d$ | nmol mg$^{-1}$ | Occupation ratio (%)$^c$ | mM$^d$ |
| Asp            | 10.9          | 1.0           | 2.0    | 6.4           | 1.2           | 1.2 |
| Thr            | 14.1          | 1.3           | 2.6    | 6.3           | 1.2           | 1.1 |
| Ser            | 24.3          | 2.3           | 4.4    | 5.5           | 1.1           | 1.0 |
| Glu            | 797.5         | 75.3          | 145.0  | 400.8         | 77.5          | 72.9 |
| Gln            | 29.9          | 2.8           | 5.4    | 33.0          | 6.4           | 6.0 |
| Lys            | 3.9           | 0.4           | 0.7    | 6.0           | 1.2           | 1.1 |
| Hyp            | 93.8          | 8.9           | 17.1   | 25.1          | 4.8           | 4.6 |
| Pro            | 51.7          | 4.9           | 9.4    | 20.8          | 4.0           | 3.8 |
| Total$^b$      | 1026.1        | 96.9          | 186.6  | 503.9         | 97.4          | 91.7 |
|                | 1059.1        |               |        | 517.4         |               |      |

$^a$ When the occupation ratio of an free amino acid was less than 1.0% in the sample, the data is not shown here.

$^b$ The content of free amino acids (nmol mg·protein$^{-1}$) in this Table is shown above, and the value of total free amino acids detected is shown below.

$^c$ The occupation ratio was calculated by the following equation: (Content of each free amino acid / Total content of free amino acids) × 100.

$^d$ Internal water space of 5.5 (μL mg·protein$^{-1}$) was used to estimate each amino acid concentration (mM) in the cytoplasm (Nagata et al., 1991).
**TABLE 2.** Free amino acids pooled in the cytoplasm of cells grown with hyper salt stress.

| Amino acid | 2.3 M NaCl at 30°C | 2.3 M KCl at 30°C | 2.3 M KCl at 37°C | 2.6 M KCl at 30°C |
|------------|---------------------|-------------------|-------------------|------------------|
|            | nmol mg·protein⁻¹ | Occupation ratio (%) | mM | Occupation ratio (%) | mM | Occupation ratio (%) | mM | Occupation ratio (%) | mM |
| Asp        | 47.5 5.0 11.9       | 29.8 5.2 7.5       | 35.1 5.7 8.8       | 35.0 5.1 8.8 |
| Glu        | 660.7 69.3 165.2    | 444.7 78.0 111.2   | 347.3 56.0 86.8    | 496.2 72.2 124.1 |
| Gln        | 37.1 3.9 9.3        | 14.9 2.6 3.7       | 23.6 3.8 5.9       | 23.6 3.4 5.9 |
| Ala        | 17.6 1.8 4.4        | 7.4 1.3 1.9        | 11.1 1.8 2.8       | 8.7 1.3 2.2 |
| Trp        | 10.0 1.0 2.5        | 8.0 1.4 2.0        | 5.6 0.9 1.4        | 10.8 1.6 2.7 |
| Lys        | 6.5 0.7 1.6         | 6.1 1.1 1.5        | 7.6 1.2 1.9        | 6.0 0.9 1.5 |
| Hyp        | 121.8 12.8 30.5     | 29.1 5.1 7.3       | 147.9 23.8 37.0    | 69.5 10.1 17.4 |
| Pro        | 4.7 0.5 1.2         | 2.8 0.5 0.7        | 6.4 1.0 1.6        | 4.4 0.6 1.1 |
| γ-ABA      | 17.1 1.8 4.3        | - - -              | - - -              | - - - |
| Total      | 923.0 96.80 230.90  | 542.8 95.2 135.8   | 584.6 94.2 146.2   | 654.2 95.2 163.7 |

- **a** When the occupation ratio of an free amino acid was less than 1.0% in the sample, the data is not shown here.
- **b** The content of free amino acids (nmol mg·protein⁻¹) in this Table is shown above, and the concentration of total free amino acids detected is shown below.
- **c** The occupation ratio was calculated by the following equation: (Content of each free amino acid / Total content of free amino acids) × 100.
- **d** Internal water space of 4.0 (μL mg·protein⁻¹), that was obtained from cells grown with 2.0 M NaCl at 30°C (Nagata et al., 1991), was used to estimate each amino acid concentration (mM) in the cytoplasm when cells were grown with 2.3 M NaCl at 30°C and more than 2.3 M KCl at 30 and 37°C.
The strain seems to express constitutively the Na$^+$-coupled transporters for proline, betaine, and/or ectoine, such as PutP for proline uptake (Bracher et al., 2016; Olkhova et al., 2011), BetP for betaine uptake (Güler et al., 2016; Koshy et al., 2013; Krämer and Morbach, 2004; Perez et al., 2014; Ressl et al., 2009), and TeaABC for ectoine uptake (Grammann et al., 2002; Marinelli et al., 2011). The constitutive expression of those transporters is thought to play an important role in the strain’s adaptation to rapidly changing salt stress. A Na$^+$ transported into the cytoplasm with each osmoprotectant seems to be exchanged with external H$^+$ via the Na$^+$/H$^+$ antiporter (Hunte et al., 2005).

A transport system without coupling Na$^+$ is necessary for transporting proline into the cytoplasm when the strain is exposed to hyper KCl stress in the absence of Na$^+$. As a possible transport system, the strain might possess an ATP-binding cassette (ABC) transporter, such as the OpuF transporters of Bacillus infantis and Bacillus panaciterrae, to transport proline and/or other amino acids (Teichmann et al., 2018). Mitigation by externally existing sodium glutamate was higher than that of potassium glutamate to the strain at 3.3 M KCl, indicating that glutamic acid is transported coupled with Na$^+$. Externally existing betaine did not mitigate hyper KCl stress. That means that the coupling ion with betaine is not K$^+$ but Na$^+$ when the strain transports betaine into the cytoplasm.

The concentration dependence of each externally existing osmoprotectant on survivability with 3.3 M KCl for 72 h at 42°C was examined by using resting cells prepared from pre-adapted cells grown in the presence of 2.3 M KCl at 37°C (Fig. 4). The number of surviving cells at 3.3 M KCl alone was reduced to $10^{5.7} ± 0.2$ (CFU·mL$^{-1}$) from $10^{6.8} ± 0.1$ (CFU·mL$^{-1}$). The value was increased with an increase in the concentrations of sodium glutamate and showed the maximum value—$10^{6.8} ± 0.1$ (CFU·mL$^{-1}$)—at 50 mM. The value barely changed up to 200 mM. In the presence of proline, the maximum value of $10^{6.5} ± 0.1$ (CFU·mL$^{-1}$) was obtained at 100 mM. Mitigating hyper KCl stress to resting cells was hardly observed in concentrations of ectoine up to 200 mM. A 2-log cycle of the number of surviving cells was reduced in the presence of 1 mM betaine, and the values were hardly changed in concentrations up to 200 mM.

Externally existing sodium glutamate was found to be the most functional osmoprotectant examined thus far for mitigating hyper KCl stress at 42°C on the strain regardless of growth conditions (see Table 3 and Fig. 4). On the other hand, externally existing proline, betaine, and ectoine (50 mM) functioned better than

### Table 3. Effect of each osmoprotectant externally existing on the survivability of Brevibacterium sp. after exposure to hyper salt stress at 42°C.

| Osmoprotectant (50 mM) | Resting cells$^a$ were exposed to |
|------------------------|----------------------------------|
|                        | 3.3 M NaCl for 36 h at 42°C | 3.3 M NaCl for 72 h at 42°C | 3.3 M KCl for 120 h at 42°C |
| None                   | $10^{4.6} ± 0.2$ | $10^{4.0} ± 0.5$ | $10^{3.9} ± 0.7$ |
| Sodium L-Glutamate     | $10^{5.2} ± 0.2$ | $10^{7.2} ± 0.1$ | $10^{5.6} ± 0.1$ |
| Potassium L-Glutamate  | $10^{5.6} ± 0.3$ | $10^{5.1} ± 0.2$ | $10^{3.9} ± 0.5$ |
| L-Proline              | $10^{7.1} ± 0.2$ | $10^{6.5} ± 0.1$ | $10^{4.2} ± 0.6$ |
| L-Hydroxyproline       | $10^{6.1} ± 0.3$ | $10^{4.0} ± 0.3$ | $10^{2.5} ± 0.4$ |
| Betaine                | $10^{6.8} ± 0.1$ | $10^{3.8} ± 0.7$ | $10^{2.7} ± 0.7$ |
| Ectoine                | $10^{6.8} ± 0.2$ | $10^{4.7} ± 0.7$ | $10^{2.4} ± 0.4$ |

$^a$ Resting cells, that were prepared from the cells grown in the absence of salts for 28 h at 30°C, were used in the experiments. The initial number of resting cells was $10^{7.4} ± 0.1$ (CFU·mL$^{-1}$).

$^b$ Surviving experiment was carried out three times independently, and the data are shown as the averaged value ± SD.
sodium glutamate to mitigate hyper NaCl stress at 42°C. These results indicate that the strain can recognize differences in each hyper salt stress and adapt to it by using different kinds of osmoadaptation systems. As for the uptake of sodium glutamate into the cytoplasm at hyper KCl stress, the strain might possess a binding protein-dependent transport system belonging to ABC transporters (Kronemeyer et al., 1995). In the presence of more than 1 mM betaine, survivability was weakened when resting cells, which were prepared from pre-adapted cells grown at 2.3 M KCl, were used in the experiments. Changes in survivalability were examined after exposure to 3.3 M KCl for 72 h at 42°C in the presence of betaine (open circles), sodium glutamate (open triangles), proline (open squares), hydroxyproline (open diamonds), and ectoine (cross marks). Each experiment was carried out three times independently, and the average value ± SD is shown here.

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