



Mitigation of Hyper KCl Stress with Externally Existing Osmoprotectants to a Halophilic *Vibrio* sp. and a Halotolerant *Brevibacterium* sp. JCM 6894

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Doctoral Dissertation

Mitigation of Hyper KCl Stress with Externally Existing
Osmoprotectants to a Halophilic *Vibrio* sp. and a Halotolerant
Brevibacterium sp. JCM 6894

(好塩性細菌 *Vibrio* sp. と耐塩性細菌 *Brevibacterium* sp. JCM 6894
に対する浸透圧保護物質の共存による高 KCl ストレスの緩和)

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Chapter 1

General Introduction

Environmental bacteria are exposed to stresses such as changes in salinity, temperature, and pH, etc. When such environmental changes occurred transiently, bacteria would be injured and dead. Therefore, environmental stress adaptation is essential for bacteria to survive in the habitat.

Larsen (1986) proposed classification of bacteria into two categories, 'requirement for salt' and 'tolerance for salt' based on the growth in the presence of various concentrations of NaCl (Fig. 1.1). Salt means NaCl. Halophilic bacteria were divided into four groups. Non-halophilic bacteria are stimulated the growth by the addition of approximately 1% NaCl (w/v) comparing with that without the addition of NaCl. Slightly halophilic bacteria can not grow without the addition of NaCl. Moderately and extremely halophilic bacteria can grow in the range of approximately 5-20% and more than 20% NaCl, respectively. Halotolerant bacteria were divided into four groups including non-halotolerant bacteria, that grow in the absence as well as the presence of approximately 1% NaCl. Slightly halotolerant bacteria can grow in the presence of up to 6-8% NaCl. Moderately and extremely halotolerant bacteria can grow in the absence and the presence of up to 18-20% NaCl and up to saturated concentration of NaCl, respectively. Osmoadaptation system(s) of halotolerant bacteria seems to be different from that of halophilic bacteria. Especially, activities of membrane-binding transporters in halotolerant bacteria, that function to take osmoprotectants existing externally into the cytoplasm, seem to be remained in the wide ranges of NaCl concentrations.

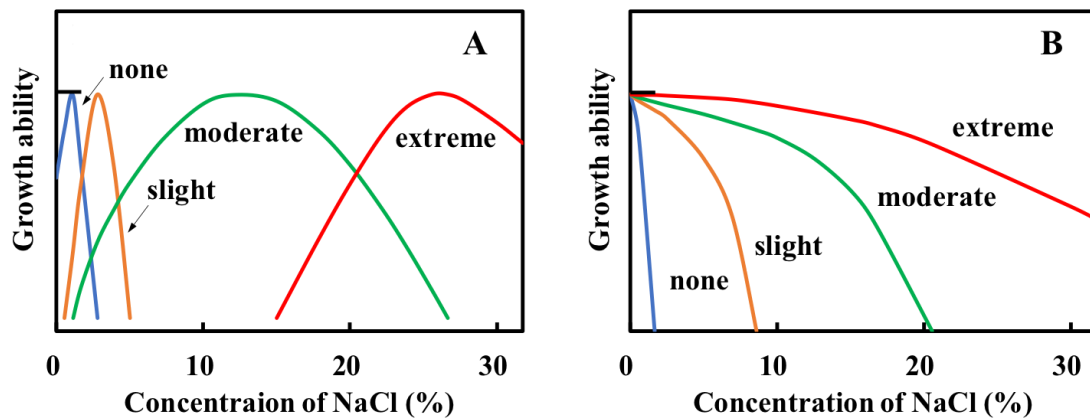


FIG. 1.1 Classification of bacteria based on the growth in the presence of NaCl. Bacteria classified into “requirement for NaCl” and “tolerance for NaCl” are shown in Fig. 1.1A and 1.1B, respectively. (Slightly modified from Larsen’s paper (1986))

Compounds that can stimulate the growth and the survival at high osmolarity are called osmoprotectants. Some osmoprotectants such as betaine, proline, and ectoine are zwitterionic solutes (Table 1.1).

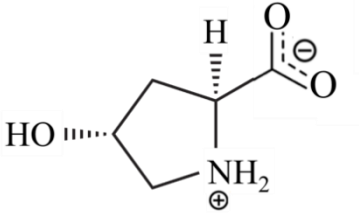
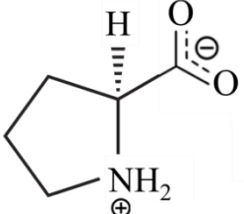
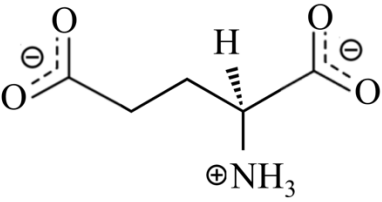
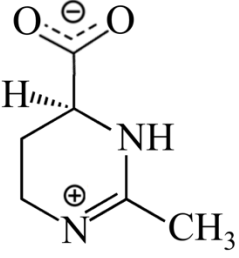
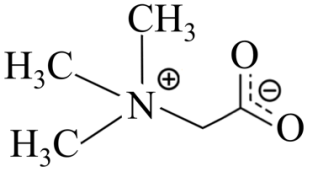
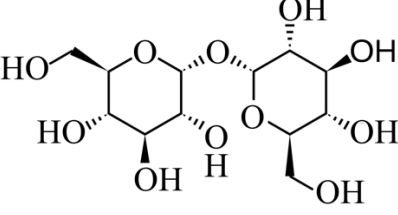
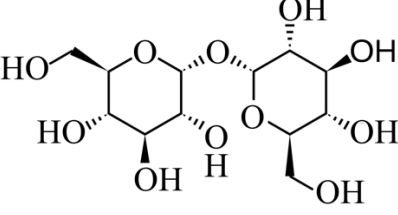
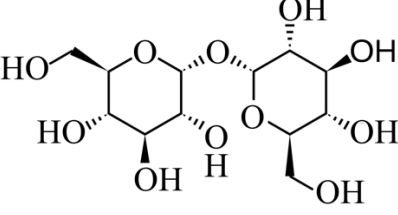
Escherichia coli K-12 accumulates large amounts of K^+ rapidly by uptake systems of Kdp, Trk, and Kup as the initial response to NaCl stress, and then the strain accumulates proline and betaine via ProP and ProU (Csonka and Epstein, 1996). Genus *Vibrio* belongs to slightly and moderately halophilic bacteria. *Vibrio cholerae* possesses two transporters, OpuD and PutP, to take proline and betaine into the cytoplasm (Kapfhammer et al., 2005). Extremely halophilic bacteria utilize K^+ to cope with hyper NaCl stress. For example, *Salinibacter ruber*, that was isolated from saltern crystallizer ponds (Antón et al., 2000), and halophilic archaea, *Halobacterium halobium* and *Halobacterium salinarum* (Martin et al., 1999; Oren et al., 2002), accumulate K^+ in the cytoplasm.

Genus Brevibacterium such as *B. casei*, *B. iodinum*, and *B. linens* are commonly isolated from milk, cheese, and human skin. A new species, *B. antiquum* (JCM 13317^T), was isolated from 1.8- to 3-million-year-old ancient permafrost sediments, the Kolyma Lowland, East Siberia, Russia (Gavrish et al., 2004). Coryneform bacteria are included genus *Brevibacterium* (Alves et al., 2002). Osmoadaptation of a glutamic acid producing *Corynebacterium glutamicum* has been carried out by three transporters, the proline and ectoine transporter, ProP (Peter et al., 1998), the ectoine, proline and betaine transporter, EctP (Peter et al., 1998), the betaine transporter, BetP (Schiller et al., 2004), and the betaine and ectoine transporter, LcoP (Steger et al., 2004).

I examined osmoadaptation of slightly halophilic and non-pathogenic *Vibrio* sp. September 1, that was isolated from a ship's ballast water (Mimura 2005), and moderately halotolerant *Brevibacterium* sp. JCM 6894, that was isolated from sea water (Nagata 1988), under hyper NaCl, or KCl, stress as well as with exposure to temperature upshift (Yin and Mimura 2020a, Yin and Mimura 2020b). I also examined mitigation of salt stress to the isolates in the presence of an osmoprotectant such as betaine, proline, ectoine, or glutamate, etc.

Hyper KCl stress adaptation of marine bacteria has not yet been studied well. Halophilic archaeon, *Haloarcula marismortui*, can adapt to the concentrations of KCl ranging from 8 to 720 mM for the growth (Jensen et al., 2015). Further understanding of the osmoadaptation system(s) of marine bacteria is expected by studying the hyper KCl stress adaptation.

TABLE 1.1 Compatible solutes used in this study.

Type	Structure		
Electrolytes	 L-Hydroxyproline  L-Proline  L-Glutamate  Ectoine  Betaine <tr><td>Nonelectrolyte</td><td>  Trehalose </td></tr>	Nonelectrolyte	 Trehalose
Nonelectrolyte	 Trehalose		

References

- Alves, A., Santos, O., Henriques, I., and Correia, A. (2002) Evaluation of methods for molecular typing and identification of members of the genus *Brevibacterium* and other related species. *FEMS Microbiol. Lett.*, 213, 205–211.
- Antón, J., Rosselló-Mora, R., Rodríguez-Valera, F., and Amann, R. (2000) Extremely halophilic Bacteria in crystallizer ponds from solar salterns. *Appl. Environ. Microbiol.*, 66, 3052–3057.
- Csonka, L. N., and Epstein, W. (1996) Osmoregulation. Chapter 77 in *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology (2 Volumes). 2nd Edition (Editor Frederick C. Neidhardt), ASM Press.
- Gavrish, E., Krauzova, V. I., Potekhina, N. V., Karasev, S. G., Plotnikova, E. G., Altynitseva, O. V., Korosteleva, L. A., and Evtushenko, L. I. (2004) Three new species of brevibacteria—*Brevibacterium antiquum* sp. nov., *Brevibacterium aurantiacum* sp. nov., and *Brevibacterium permense* sp. nov. (in Russian) *Mikrobiologiya*, 73, pp. 218–225.
- Jensen, M. W., Matlock, S. A., Reinheimer, C. H., Lawlor, C. J., Reinheimer, T. A., and Gorrell, A. (2015) Potassium stress growth characteristics and energetics in the haloarchaeon *Haloarcula marismortui*. *Extremophiles*, 19, 315–325.
- Kapfhammer, D., Karatan, E., Pflughoeft, K. J., and Watnick, P. I. (2005) Role for glycine betaine transport in *Vibrio cholerae* osmoadaptation and biofilm formation within microbial communities. *Appl. Environ. Microbiol.*, 71, 3840–3847.
- Larsen, H. (1986) Halophilic and halotolerant microorganisms - an overview and historical perspective. *FEMS Microbiol. Rev.*, 39, 3–7.

- Martin, D. D., Ciulla, R. A., and Roberts, M. F. (1999) Osmoadaptation in archaea. *Appl. Environ. Microbiol.*, 65, 1815–1825.
- Mimura, H., Katakura, R., and Ishida, H. (2005) Changes of microbial populations in a ship's ballast water and sediments on a voyage from Japan to Qatar. *Mar. Pollut. Bull.*, 50, 751–757.
- Nagata, S. (1988) Influence of salts and pH on the growth as well as NADH oxidase of the halotolerant bacterium A505. *Arch. Microbiol.*, 150, 302–308.
- Oren, A., Heldal, M., Norland, S., and Galinski, E. A. (2002) Intracellular ion and organic solute concentrations of the extremely halophilic bacterium *Salinibacter ruber*. *Extremophiles*, 6, 491–498.
- Peter, H., Weil, B., Burkovski, A., Krämer, R., and Morbach, S. (1998) *Corynebacterium glutamicum* is equipped with four secondary carriers for compatible solutes: identification, sequencing, and characterization of the proline/ectoine uptake system, ProP, and the ectoine/proline/glycine betaine carrier, EctP. *J. Bacteriol.*, 180, 6005–6012.
- Steger, R., Weinand, M., Krämer, R., and Morbach, S. (2004) LcoP, an osmoregulated betaine/ectoine uptake system from *Corynebacterium glutamicum*. *FEBS Lett.*, 573, 155–160.
- Schiller, D., Krämer, R., and Morbach, S. (2004) Cation specificity of osmosensing by the betaine carrier BetP of *Corynebacterium glutamicum*. *FEBS Lett.*, 563, 108–112.
- Yin, Y., and Mimura, H. (2020a). Changes in the survivability of marine *Vibrio* sp. under hyper KCl stress in the presence of betaine as well as with exposure to 37°C. *Biocontrol Sci.*, 25, 17–24.

Yin, Y. and Mimura, H. (2020b) Mitigation of hyper KCl stress at 42°C with externally existing sodium glutamate to a halotolerant *Brevibacterium* sp. JCM 6894. *Biocontrol Sci.*, 25, 139–147 (2020)..

Chapter 2

Changes in the Survivability of Marine *Vibrio* sp. under Hyper KCl Stress in the Presence of Betaine as Well as with Exposure to 37°C

Introduction

The stress adaptation of environmental bacteria is vitally important to life and survival. Osmoprotectants such as betaine, proline, ectoine, and trehalose are newly biosynthesized and/or transported into the cytoplasm during osmoadaptation to hyper salt stress. Pathogenic *Vibrio parahaemolyticus* (Naughton et al., 2009) and *V. cholerae* (Pflughoeft et al., 2003) synthesize ectoine and transport betaine into the cytoplasm at higher concentrations of NaCl. The bacterial transport systems of osmoprotectants possess the Na⁺-binding site. For example, the betaine transporter of *Corynebacterium glutamicum* (Perez et al., 2014; Ressler et al., 2009) and the proline transporter of *Escherichia coli* (Pirch et al., 2002) need Na⁺. Membrane-binding transporters from *Vibrio* species are shown in Fig. 2.1 (Herz et al., 2003; Kapfhammer et al., 2005; Lee et al., 2003; Nakamura et al., 1998; Quinn et al., 2012).

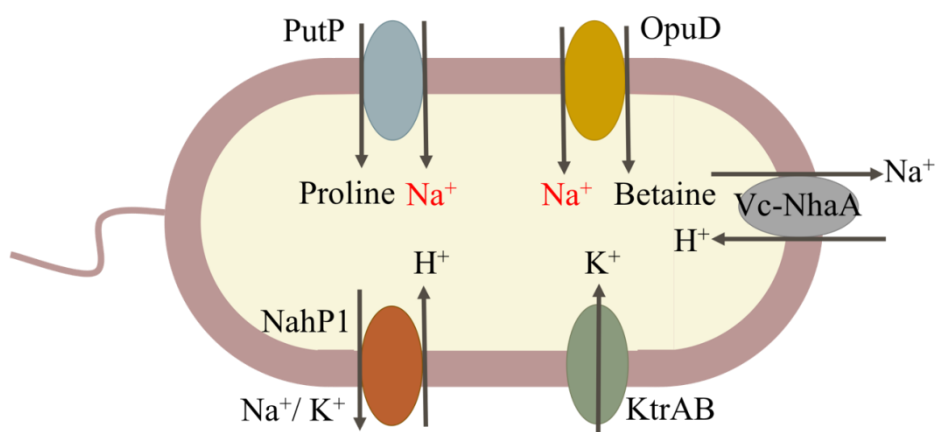


FIG. 2.1 Membrane-binding transporters of osmoprotectants in *Vibrio* species.

Excess amounts of Na^+ in the cytoplasm are toxic to bacterial cells. On the other hand, K^+ abundantly existing in the cytoplasm maintains the osmotic pressure, activities of many enzymes, and intracellular pH and reduces the negative charge of DNA as well. The high-affinity K^+ -transporter of *E. coli* and *Bacillus subtilis* can bind and transport K^+ into the cytoplasm efficiently, even at considerably lower concentrations of K^+ existed externally (Csonka and Epstein, 1996; Gundlach et al., 2017). High concentrations of KCl externally added give bacteria the same cationic and osmotic stresses chemically as those given by the same concentrations of NaCl. However, the adaptation and tolerant mechanisms of bacteria under hyper KCl stress seem to be different from those under hyper NaCl stress. We believe that studying survivability under hyper KCl stress will contribute to a thorough understanding of bacterial adaptation to salt stress.

Marine vibrios are slightly halophilic bacteria that require Na^+ for their growth (Larsen, 1986). In this study, we examined the function of each externally existing osmoprotectant to the survivability of marine *Vibrio* sp. under hyper salt stress.

Materials and Methods

Strain and growth conditions

Non-pathogenic *Vibrio* sp. September 1 was used in the experiments. The strain, which was isolated from a ship's ballast water, could grow at 37°C and metabolize sucrose (Mimura et al., 2005). The partial rDNA sequences were deposited in the DDBJ/GenBank/EMBL under accession number AB195981 (Mimura et al., 2005).

The isolate was pre-incubated for 12 h at 30°C in the medium containing 5 g Bacto Peptone (L^{-1}) (BD Diagnostics-Diagnostic Systems, Maryland, USA), 1 g yeast extract (L^{-1}) (BD Diagnostics-Diagnostic Systems, Maryland, USA), and 0.5 M NaCl dissolved in 50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) - tetramethylammonium hydroxide (TMAH) buffer, pH 7.5. Incubation was carried out at 30°C, or 37°C, with the addition of cell suspension to a growth medium containing various concentrations of NaCl and/or KCl to a one-thousandth dilution. Turbidity in the medium was measured at given time with a photometer (BioPhotometer #6131, Eppendorf AG, Hamburg, Germany) at 600 nm.

Preparation of resting cells

The isolate was grown for 8 h at 30°C in the presence of 0.5 M NaCl, or grown for 10 h at 37°C in the presence of 0.8 M KCl and 50 mM NaCl. Cells reached to the early stationary phase of growth were harvested by centrifugation ($10,000 \times g$, 5 min) at room temperature and washed twice with 50 mM HEPES-TMAH buffer, pH 7.5, containing the same concentrations of NaCl and/or KCl added to the growth medium. Cell pellet was then resuspended in the same buffer. Resting cells thus obtained were used for the surviving experiments. Cell pellet was also resuspended in distilled water for the measurement of free amino acids pooled in the cytoplasm as well as the content of cell proteins.

Surviving experiments under hyper salt stress

Resting cells were added to 50 mM HEPES-TMAH buffer, pH 7.5, containing various concentrations of NaCl at 30°C, or various concentrations of KCl and 50 mM

NaCl at 30°C, to give a one-hundredth dilution. Resting cells were also added to the buffer containing each osmoprotectant and 1.8 M NaCl at 30°C and 37°C, or each osmoprotectant, 1.2 M KCl and 50 mM NaCl at 30°C and 37°C.

Enumeration of the surviving cells

Resting cells (100 μ L) were pipetted out at given time and diluted serially with 50 mM HEPES-TMAH buffer, pH 7.5, containing the same concentrations of salts as added in the 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 2 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 $\times$ g, 5 min), supernatant was taken 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 SCIENTIFIC, Rockford, IL, USA) following the instruction manual. Bovine serum albumin was used as a standard.

Results and Discussion

Importance of hyper KCl stress experiments by using resting cells

Osmoadaptation of bacteria has been well studied focusing on the growth in a nutrients medium containing high concentrations of NaCl. In the medium, the strain can synthesize and/or transport osmoprotectants to cope with hyper NaCl stress during an adaptation period and exponential phase of growth (Csonka and Epstein, 1996; Ongagna-Yhombi and Boyd, 2013). On the other hand, survivability of resting cells under hyper salt stress primarily depends on chemical species and concentrations of free amino acids and their related compounds accumulated in the cytoplasm. The most functional osmoprotectant, that is farther required by the isolate to mitigate hyper salt stress, can be determined at 30°C as well as with exposure to 37°C by using resting cells.

Chemical properties of KCl are similar to those of NaCl. Nevertheless, bacteria recognize the structural difference of K^+ with Na^+ precisely and transport K^+ selectively into the cytoplasm at hyper NaCl stress (Csonka and Epstein, 1996). It seems interesting to study the differences of osmoadaptation responses at hyper KCl stress with those at hyper NaCl stress. The study will contribute to a thorough understanding of bacterial osmoadaptation.

Growth at hyper salt stress

Growth of the isolate was examined in the presence of NaCl up to 1.5 M at 37°C as well as in the presence of 0.5 M NaCl at 30°C (Fig. 2.2A). The isolate grew well at 0.5 M NaCl at 30°C and 37°C, and the values of doubling time (h) at the exponential phase of growth were 0.56 ± 0.02 h at 30°C and 0.46 ± 0.02 h at 37°C ($n = 3$). Growth could

reach the early stationary phase within 8 h of incubation in the presence of 1.2 M NaCl at 37°C, and the value was 0.59 ± 0.01 h. Growth was not observed in the presence of 1.5 M NaCl at 37°C. Growth experiment was also carried out in the presence of up to 0.8 M KCl with and without 50 mM NaCl at 37°C (Fig. 2.2B). The isolate could grow even in the presence of 0.8 M KCl with the addition of 50 mM NaCl at 37°C, and the value showed 0.60 ± 0.01 h.

Growth of the isolate was strongly repressed under hyper KCl stress. However, the growth drastically improved with the addition of a relatively small amount of NaCl, indicating that externally existing Na^+ plays an essential role in improving the growth of the isolate under hyper KCl stress, such as the coupling ion of the symporter with a nutrient across the membrane (Perez et al., 2014; Pirch et al., 2002; Ressler et al., 2009; Wilson and Ding, 2001).

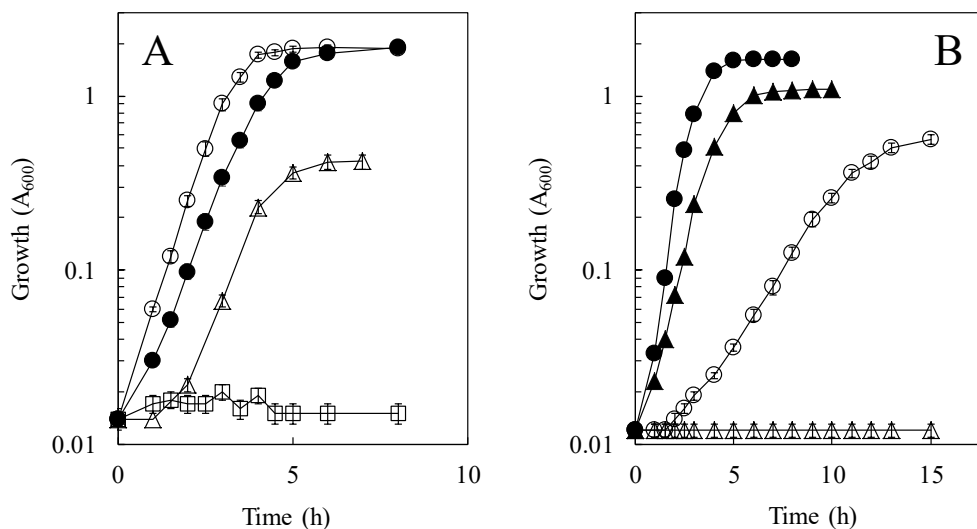


FIG. 2.2 Growth curves of *Vibrio* sp. September 1 under hyper salt stress.

Growth experiments were carried out in the presence of 0.5 M NaCl at 30°C (closed circles) and 37°C (open circles), 1.2 M NaCl at 37°C (open triangles), and 1.5 M NaCl at 37°C (open squares) (Fig. 2.2A). The experiments were also carried out at 37°C in the presence of 0.5 M KCl (open circles), 0.5 M KCl and 50 mM NaCl (closed circles), 0.8 M KCl (open triangles), and 0.8 M KCl and 50 mM NaCl (closed triangles) (Fig. 2.2B).

Each experiment was carried out three times independently, and the averaged value $\pm$ SD is shown here.

Changes in the survivability of resting *Vibrio* sp. cells after exposure to hyper salt stress

Survivability was examined at hyper salt stress. After 24 h at 30°C, the numbers of surviving cells at 1.2 M and 1.5 M NaCl alone were reduced to $10^{5.3 \pm 0.3}$ and $10^{3.3 \pm 0.3}$ (CFU·mL⁻¹), respectively, from the initial number of $10^{7.1 \pm 0.2}$ (CFU·mL⁻¹) (Fig. 2.3A). The values were reduced almost exponentially with exposure to 1.8 M NaCl, and it showed $10^{2.7 \pm 0.4}$ (CFU·mL⁻¹) for 12 h.

Changes in the number of surviving cells with exposure to KCl up to 1.2 M were examined in the presence of 50 mM NaCl at 30°C (Fig. 2.3B). The value was reduced slightly to $10^{6.7 \pm 0.2}$ (CFU·mL⁻¹) after 24 h at 0.5 M KCl. Reduction of the value was maintained within a 3-log cycle for 24 h at 0.8 M KCl. It was reduced exponentially to $10^{3.9 \pm 0.8}$ (CFU·mL⁻¹) for 5 h at 1.2 M KCl.

The extrusion of K⁺ from the cytoplasm is necessary to cope with the inflow of K⁺ into the cytoplasm when cells are exposed to hyper KCl stress. *V. parahaemolyticus* possesses at least three K⁺ extrusion systems, such as a potassium-specific K⁺/H⁺ antiporter system and two Na⁺/H⁺ antiporter systems that work as to pump out K⁺ as well as Na⁺ (Radchenko et al., 2006). The survivability of resting cells under hyper KCl stress was obviously weaker than that under hyper NaCl stress, indicating that the K⁺ regulatory system(s) is inducible, or the system(s) as constitutively expressed is inactivated at higher concentrations of externally existing K⁺.

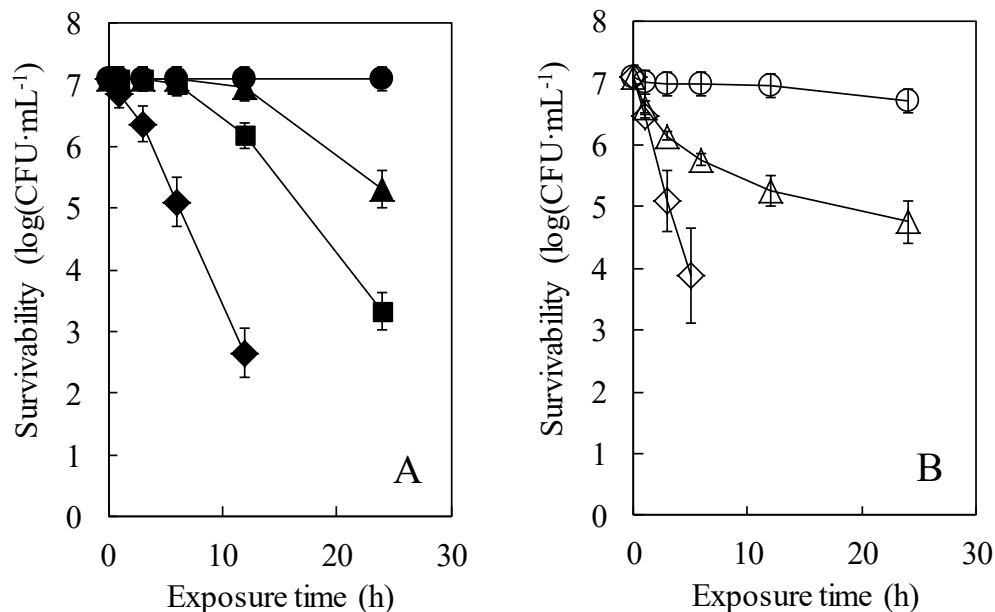


FIG. 2.3 Changes in the survivability of resting cells after exposure to hyper salt stress. Resting cells prepared from the cells grown for 8 h at 30°C in the presence of 0.5 M NaCl were used in the experiments. Changes in survivability were examined in the presence of 0.5 M NaCl (closed circles), 1.2 M NaCl (closed triangles), 1.5 M NaCl (closed squares), and 1.8 M NaCl (closed diamonds) at 30°C (Fig. 2.3A). Experiments were also carried out in the presence of 0.5 M KCl and 50 mM NaCl (open circles), 0.8 M KCl and 50 mM NaCl (open triangles), and 1.2 M KCl and 50 mM NaCl (open diamonds) at 30°C (Fig. 2.3B). Each experiment was carried out three times independently, and the average value $\pm$ SD is shown here.

Changes in free amino acids pooled in the cytoplasm of cells grown with hyper salt stress

Glutamate was the most abundant free amino acid when the isolate was grown in the presence of NaCl of up to 1.2 M (Table 2.1). The occupation ratios were 70.7% and 66.3% in the concentration of total free amino acids for the isolate grown at 30°C and 37°C, respectively, in the presence of 0.5 M NaCl. The high occupation ratio of 75.3% was maintained even when grown in the presence of 1.2 M NaCl at 37°C. The accumulation of glutamate as a major free amino acid is consistent with accumulations previously reported for a marine bioluminescent bacterium, *Beneckeia harveyi* (Makemson and Hastings, 1979), and a marine halotolerant *Brevibacterium* sp. JCM 6894 (Mimura et al., 1994).

The occupation ratio of glutamate was drastically reduced to less than 30.0% when grown at 0.8 M KCl and 50 mM NaCl. As for the other negatively charged aspartate, the occupation ratio was relatively higher than those at hyper NaCl stress. Negatively charged amino acid is quite important for neutralizing positively charged inorganic cations Na^+ and K^+ in the cytoplasm. In addition to the glutamate pooled, the aspartate pooled seems to play an important role in mitigating hyper KCl stress. The occupation ratios of α -amino adipic acid and citrulline at 0.8 M KCl and 50 mM NaCl were 3.6 times and 7.6 times higher than those at 1.2 M NaCl at 37°C, respectively. The compounds also seem to mitigate hyper KCl stress.

The concentration of total free amino acids pooled in the cytoplasm of the isolate grown in the presence of 0.5 M NaCl at 37°C was 726.5 (nmol mg $\cdot$ protein $^{-1}$), the value of which was 48.5% lower than that at 30°C. The isolate might use free amino acids primarily to synthesize heat shock proteins at a moderately high temperature (Koga et

al., 1996). As for adaptation to hyper KCl stress at 37°C, the concentration of total free amino acids, 444.1 (nmol mg·protein⁻¹), was obviously lower than that at hyper NaCl stress. Free amino acids are dissolved in the internal water volume in the cytoplasm, the value of which reduces with the increase in salt stress (Nagata et al., 1991). For example, the water volume of halotolerant *Brevibacterium* sp. JCM 6894 was 5.5 ± 0.1 (μL mg·protein⁻¹) at the stationary phase of growth in the absence of salts. The value reduced sharply to 4.0 ± 0.4 (μL mg·protein⁻¹) when grown at 2 M NaCl. The halophilic isolate used in the experiments might adapt to hyper KCl stress by reducing the water volume more than that at hyper NaCl stress.

TABLE 2.1 Free amino acids pooled in the cytoplasm of cells grown with hyper salt stress.

amino acids ^a	Cells were grown with					
	0.5 M NaCl at 30°C		0.5 M NaCl at 37°C		1.2 M NaCl at 37°C	
	nmol/mg protein	Occupation ratio (%) ^c	nmol/mg protein	Occupation ratio (%) ^c	nmol/mg protein	Occupation ratio (%) ^c
Asp	11.8	0.8	16.5	2.3	21.6	2.2
Asn	5.4	0.4	21.5	3.0	2.5	0.3
Glu	996.5	70.7	481.9	66.3	744.1	75.3
α -Aminoadipic acid	13.2	0.9	6.6	0.9	59.2	6.0
Gly	69.9	5.0	62.8	8.7	16.3	1.6
Ala	69.0	4.9	25.9	3.6	28.7	2.9
Citrulline	31.4	2.2	7.7	1.1	23.7	2.4
Val	23.2	1.6	4.3	0.6	9.2	0.9
Leu	9.1	0.6	5.3	0.7	8.2	0.8
Ethanolamine	16.2	1.1	2.9	0.4	3.4	0.3
Hydroxylysine	13.6	1.0	11.9	1.6	0.9	0.1
Lys	28.2	2.0	10.6	1.5	9.1	0.9
Hydroxyproline	13.9	1.0	3.9	0.5	0.0	0.0
Proline	12.7	0.9	2.3	0.3	2.1	0.2
Total ^b	1314.1	93.1	664.0	91.5	929.0	93.9
	1409.4		726.5		988.4	
					401.5	90.4
					444.1	

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

^b The concentration of free amino acids (nmol/mg protein) 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, (Concentration of each free amino acid / Total concentration of free amino acids) $\times$ 100.

Effect of osmoprotectants and related compounds externally existing on the survivability of resting cells at hyper salt stress (grown at 0.5 M NaCl at 30°C)

The reduction of a 2-log cycle from the initial number of resting cells was observed after exposure to 1.8 M NaCl for 6 h at 30°C (Table 2.2). The value was maintained within the reduction of a 1-log cycle by the addition of 50 mM betaine as a final concentration, glycine derivatives such as dimethylglycine and sarcosine, choline chloride, and proline. The reduction of the 2-log cycle was observed in the presence of sodium glutamate, citrulline, and ectoine, and it was a 3-log cycle in the presence of trehalose.

When the temperature in the buffer was increased to 37°C, the number of surviving cells at 1.8 M NaCl was reduced by a 3-log cycle from the initial number of resting cells. The value remained within the reduction of a 2-log cycle when sodium glutamate, proline, or betaine existed externally. On the other hand, the values were reduced by a 4-log cycle with the addition of glycine derivatives sarcosine and dimethylglycine. In the presence of citrulline, choline chloride, ectoine, or trehalose, no mitigation effect was observed.

Survivability was drastically reduced when the temperature in the buffer was raised to 37°C. However, glutamate, proline, and betaine maintained the number of surviving cells at a 1-log cycle higher than that in the presence of 1.8 M NaCl alone, indicating that those osmoprotectants also mitigate thermal injury of the resting cells at hyper NaCl stress. Dimethylglycine shows the heat stress-relieving property (Bashir et al., 2014). However, the compound externally added did not enhance survivability at 37°C, indicating that the isolate's transport system(s) itself is temperature sensitive.

The number of surviving cells was reduced by a 2-log cycle from the initial number of resting cells with exposure to 1.2 M KCl and 50 mM NaCl for 3 h at 30°C. The values in the presence of each osmoprotectant, except of ectoine, reduced obviously more than that of the control. Especially, in the presence of sodium glutamate, the value reduced a 3-log cycle more than that of the control. The isolate accumulated glutamate and citrulline as major osmoprotectants when grown in the presence of 0.8 M KCl and 50 mM NaCl at 37°C. However, those osmoprotectants as well as others examined did not mitigate hyper KCl stress, indicating that externally existing osmoprotectants, except for ectoine, disrupt the osmoadaptation system(s) of the resting cells under hyper KCl stress. The concentration of Na^+ in the cytoplasm increases during the accumulation of osmoprotectants when Na^+ is co-transported into the cytoplasm coupled with betaine (Perez et al., 2014), or proline (Pirch et al., 2002), via the Na^+ /osmoprotectant symporters. The Na^+/H^+ antiporters regulate intracellular Na^+ concentration as well as pH (Padan et al., 2001). Reduction of the efficient extrusion of Na^+ via the Na^+/H^+ antiporters might occur at hyper KCl stress exposed externally to the resting cells.

The number of surviving cells was almost zero regardless of the presence and absence of each osmoprotectant at 1.2 M KCl and 50 mM NaCl when exposed at 37°C. Those results indicate that the osmoadaptation system(s) constitutively expressed is obviously temperature sensitive in the presence of hyper KCl stress.

TABLE 2.2 Effect of an osmoprotectant and related compounds externally existing on the survivability of resting cells at hyper salt stress (grown at 0.5 M NaCl at 30°C).

Osmoprotectant (50 mM)	Surviving cells (CFU · ml ⁻¹) ^a after exposure to			
	1.8 M NaCl for 6 h at 30°C	1.8 M NaCl for 6 h at 37°C	1.2 M KCl and 50 mM NaCl for 3 h at 30°C	1.2 M KCl and 50 mM NaCl for 3 h at 37°C
None	10 ^{5.1 ± 0.4}	10 ^{3.2 ± 0.5}	10 ^{5.1 ± 0.5}	< 10 ^{1.0b}
Sodium L-Glutamate	10 ^{5.6 ± 0.1}	10 ^{4.1 ± 0.3}	10 ^{2.2 ± 0.3}	< 10 ^{1.0b}
L-Citrulline	10 ^{5.9 ± 0.2}	10 ^{3.3 ± 0.1}	10 ^{3.0 ± 0.5}	< 10 ^{1.0b}
L-Proline	10 ^{6.2 ± 0.1}	10 ^{4.3 ± 0.5}	10 ^{2.9 ± 0.1}	10 ^{1.8 ± 1.6}
Choline chloride	10 ^{6.2 ± 0.1}	10 ^{3.5 ± 0.1}	10 ^{4.1 ± 0.1}	< 10 ^{1.0b}
Sarcosine	10 ^{6.2 ± 0.2}	10 ^{2.9 ± 0.2}	10 ^{3.2 ± 0.5}	< 10 ^{1.0b}
Dimethylglycine	10 ^{6.2 ± 0.1}	10 ^{2.8 ± 0.1}	10 ^{4.5 ± 0.1}	< 10 ^{1.0b}
Betaine	10 ^{6.6 ± 0.1}	10 ^{4.4 ± 0.5}	10 ^{4.1 ± 0.4}	10 ^{1.5 ± 1.3}
Ectoine	10 ^{5.8 ± 0.3}	10 ^{3.1 ± 0.5}	10 ^{5.3 ± 0.1}	< 10 ^{1.0b}
Trehalose	10 ^{4.8 ± 0.3}	10 ^{3.3 ± 0.3}	10 ^{4.2 ± 0.4}	< 10 ^{1.0b}

^a The initial number of resting cells was 10^{7.1 ± 0.2} (CFU · ml⁻¹). The numbers of the surviving cells after 6 h at 30 and 37 °C in the mixture containing 0.5 M NaCl were 10^{7.1 ± 0.2} and 10^{6.9 ± 0.1} (CFU · ml⁻¹), respectively. Surviving experiment was carried out at least three times independently, and the data are shown as the averaged value ± SD.

^b Averaged value was less than 10^{1.0} (CFU · ml⁻¹) (n=3).

Effect of osmoprotectants and related compounds externally existing on the survivability of resting cells at hyper KCl stress (grown at 0.8 M KCl and 50 mM NaCl at 37°C)

The numbers of surviving cells maintained at a 3-log order of magnitude after exposure to 1.2 M KCl and 50 mM NaCl for 3 h at 37°C (Table 2.3). The value maintained a 1-log order of magnitude higher in the presence of 50 mM betaine, or 50 mM proline, than that of the control. The survivability of resting cells prepared from cells grown at 0.5 M NaCl at 30°C was quite low regardless of the presence or absence of those osmoprotectants (see Table 2.2). When resting cells, that were prepared from preadapted cells in the growth to 0.8 M KCl and 50 mM NaCl at 37°C, were used, increased survivability was confirmed by exposure to 1.2 M KCl stress. In the growth condition, the occupation ratio of proline pooled as a free amino acid was 0.3% (data not shown). Those results indicate that the role of proline as an osmoprotectant is not primarily important to adaptation to the isolate's hyper KCl stress. Ectoine did not mitigate hyper KCl stress as well as it did hyper NaCl stress to resting cells regardless of the growth conditions so far examined, indicating that the transport system(s) of ectoine might be lacking in the isolate while *V. parahaemolyticus* possess the sole ectoine transporter, VP1456, that is induced at hyper NaCl stress (Ongagna-Yhombi et al., 2015) and the compound is critical for survivability at the given hyper NaCl stress (Ongagna-Yhombi and Boyd, 2013).

The number of surviving cells was reduced to $10^{4.6 \pm 0.5}$ (CFU·mL⁻¹) from $10^{6.5 \pm 0.1}$ (CFU·mL⁻¹) after 3 h of exposure to 0.8 M KCl and 50 mM NaCl at 37°C when resting cells examined was prepared from cells grown at 0.8 M KCl and 50 mM NaCl at 37°C (see footnotes in Table 2.3). The number of surviving cells was maintained when

exposed at 30°C in the buffer containing the same concentrations of KCl and NaCl as those examined at 37°C, indicating that osmoadaptation system(s) is temperature sensitive even for the resting cells prepared from pre-adapted cells in the growth to relatively high concentrations of KCl in the presence of 50 mM NaCl at 37°C.

TABLE 2.3 Effect of an osmoprotectant and related compounds externally existing on the survivability of resting cells at hyper KCl stress (grown at 0.8 M KCl and 50 mM NaCl at 37°C).

Osmoprotectant (50 mM)	Surviving cells (CFU · ml ⁻¹) ^a after exposure to 1.2 M KCl and 50 mM NaCl for 3 h at 37°C
None	10 ^{3.6 ± 0.4}
Sodium L-Glutamate	10 ^{2.2 ± 0.1}
L-Citrulline	10 ^{2.0 ± 0.5}
L-Proline	10 ^{4.4 ± 0.1}
Choline chloride	10 ^{2.5 ± 0.1}
Sarcosine	10 ^{3.2 ± 0.2}
Dimethylglycine	10 ^{2.6 ± 0.2}
Betaine	10 ^{4.9 ± 0.3}
Ectoine	10 ^{3.3 ± 0.1}
Trehalose	10 ^{2.4 ± 0.3}

^a The initial number of resting cells was 10^{6.5 ± 0.1} (CFU · ml⁻¹). The numbers of surviving cells after 3 h of exposure at 30°C and 37°C in the buffer containing 0.8 M KCl and 50 mM NaCl were 10^{6.5 ± 0.1} and 10^{4.6 ± 0.5} (CFU · ml⁻¹), respectively. Surviving experiment was carried out at least three times independently, and the data are shown as the averaged value ± SD.

Negative effect of relatively higher concentrations of betaine or proline externally added on the survivability of resting cells under hyper KCl stress (grown at 0.8 M KCl and 50 mM NaCl at 37°C)

A maximum concentration in each compound resulting in the highest number of surviving cells was 50 mM in the range of up to 200 mM (Fig. 2.4). The difference in the number of surviving cells at 50 mM as compared with 200 mM was a 3-log cycle. Relatively higher concentrations of such compounds seem to disrupt the osmoadaptation system(s) even for the resting cells described above when rapid adjustment of the osmotic balance across the cell membrane becomes necessary to survive under hyper KCl stress.

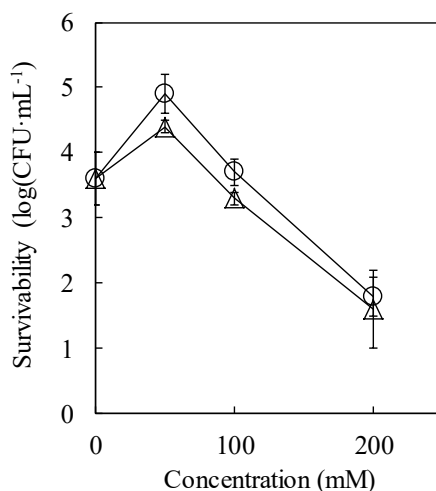


FIG. 2.4 Changes in the survivability of resting cells with an increase in the concentrations of betaine, or proline, under hyper KCl stress.

Resting cells prepared from cells grown for 10 h at 37°C in the presence of 0.8 M KCl and 50 mM NaCl were used in the experiments. Resting cells were added to give an initial number of $10^{6.5 \pm 0.1}$ (CFU·mL⁻¹) in 50 mM HEPES-TMAH buffer, pH 7.5, containing various concentrations of betaine (open circles), or proline (open triangle), as well as 1.2 M KCl and 50 mM NaCl. After 3 h of exposure at 37°C, the number of surviving cells was counted by the colony-counting method. Each experiment was carried out three times independently, and the average value $\pm$ SD is shown here.

Osmoadaptation of marine *Vibrio* sp. at hyper KCl stress

It took 24 h for getting the reduction of a 2-log cycle from the initial number of resting cells, $10^{7.1 \pm 0.2}$ (CFU·mL⁻¹), that were prepared from cells grown for 8 h at 0.5 M NaCl at 30°C, when exposed to 1.2 M NaCl at 30°C (Fig. 2.3). The period became shortened to approximate 3 h in the presence of 1.2 M KCl and 50 mM NaCl at 30°C, indicating that hyper KCl stress is more severe stress for the isolate than that of NaCl. The number of surviving cells drastically reduced to less than $10^{1.0}$ (CFU·mL⁻¹) at 37°C after 3 h of exposure to 1.2 M KCl and 50 mM NaCl (Table 2.2). Temperature sensitivity was also observed even when resting cells, $10^{6.5 \pm 0.1}$ (CFU·mL⁻¹), that were prepared from cells grown for 10 h in the presence of 0.8 M KCl and 50 mM NaCl at 37°C, were exposed to the same concentrations of KCl and NaCl for 3 h at 37°C (see footnote in Table 2.3). The results indicate that the osmoadaptation system(s) of the isolate to cope with high concentrations of KCl externally existing is vulnerable at 37°C.

We examined the effect of each osmoprotectant as well as its related compounds externally existing on the survivability of resting cells at hyper salt stress (Table 2.2 and Table 2.3). As a result, the number of surviving cells in the presence of 50 mM sodium glutamate, 50 mM proline, or 50 mM betaine was maintained a 1-log order of magnitude higher than that at 1.8 M NaCl alone at 37°C (Table 2.2). As for the adaptation to hyper KCl stress, the number of surviving cells was maintained a 1-log order of magnitude higher than that of the control, $10^{3.6 \pm 0.4}$ (CFU·mL⁻¹) when resting cells, that were prepared from preadapted cells grown in the presence of 0.8 M KCl and 50 mM NaCl at 37°C, were exposed to 1.2 M KCl and 50 mM NaCl at 37°C in the presence of 50 mM betaine, or 50 mM proline (Table 2.3). The results indicate that a

betaine and proline transporter(s) of the resting cells functions to mitigate hyper KCl stress at 37°C.

Summary

Survivability at hyper KCl stress was examined at 30°C and 37°C in the presence and absence of an osmoprotectant by using resting cells prepared from marine *Vibrio* sp. grown at early stationary phase. Survivability was decided by counting colonies. The number of initial cells, $10^{7.1 \pm 0.2}$ (CFU·mL⁻¹), was reduced to $10^{5.1 \pm 0.5}$ and $< 10^{1.0}$ (CFU·mL⁻¹) at 30°C and 37°C, respectively, by the exposure of resting cells, that were prepared from cells grown for 8 h at 0.5 M NaCl at 30°C, to 1.2 M KCl and 50 mM NaCl for 3 h. Betaine externally existed as a final concentration of 50 mM mitigated hyper KCl stress to the resting cells at 37°C. The number of surviving cells was maintained $10^{4.9 \pm 0.3}$ (CFU·mL⁻¹) when resting cells, $10^{6.5 \pm 0.1}$ (CFU·mL⁻¹), that were prepared from pre-adapted cells to relatively high concentration of KCl in the growth for 10 h at 0.8 M KCl and 50 mM NaCl at 37°C, were exposed to 1.2 M KCl, 50 mM NaCl, and 50 mM betaine at 37°C for 3 h. The results indicate that osmoadaptation system(s) in resting cells is temperature sensitive and betaine functions to mitigate hyper KCl stress to the resting cells at 37°C.

References

- Bashir, A., Hoffmann, T., Smits, S. H., and Bremer, E. (2014) Dimethylglycine provides salt and temperature stress protection to *Bacillus subtilis*. *Appl. Environ. Microbiol.*, 80, 2773–2785.
- Csonka, L.N., and Epstein, W. (1996) Osmoregulation. In *Escherichia coli and Salmonella: Cellular and Molecular Biology* (Neidhardt, F. C., ed.), pp. 1210–1223, ASM Press, Washington DC.
- Gundlach, J., Herzberg, C., Hertel, D., Thürmer, A., Daniel, R., Link, H., and Stülke, J. (2017) Adaptation of *Bacillus subtilis* to life at extreme potassium limitation. *mBio*, 8:e00861-17.
- Herz, K., Vimont, S., Padan, E., and Berche, P. (2003) Roles of NhaA, NhaB, and NhaD Na⁺/H⁺ antiporters in survival of *Vibrio cholerae* in a saline environment. *J. Bacteriol.*, 185, 1236–1244.
- Kapfhammer, D., Karatan, E., Pflughoeft, K. J., and Watnick, P. I. (2005) Role for glycine betaine transport in *Vibrio cholerae* osmoadaptation and biofilm formation within microbial communities. *Appl. Environ. Microbiol.*, 71, 3840–3847.
- Koga, T., Nakajyo, Y., and Komoto, A. (1996) Detection of Hsp60 (Gro-EL)-like proteins in *Vibrio parahaemolyticus* and *Vibrio* species by western immunoblotting analysis. *Lett. Appl. Microbiol.*, 23, 295–298.
- Larsen, H. (1986) Halophilic and halotolerant microorganisms—an overview and historical perspective. *FEMS Microbiol. Rev.*, 39, 3–7.
- Lee, J. H., Park, N. Y., Lee, M. H., and Choi, S. H. (2003) Characterization of the *Vibrio vulnificus* putAP operon, encoding proline dehydrogenase and proline

- permease, and its differential expression in response to osmotic stress. *J. Bacteriol.*, 185, 3842–3852.
- Makemson, J. C., and Hastings, J. W. (1979) Glutamate functions in osmoregulation in a marine bacterium. *Appl. Environ. Microbiol.*, 38, 178–180.
- Mimura, H., Nagata, S., and Matsumoto, T. (1994) Concentrations and compositions of internal free amino acids in a Halotolerant *Brevibacterium* sp. in response to salt stress. *Biosci. Biotechnol. Biochem.*, 58, 1873–1874.
- Mimura, H., Katakura, R., and Ishida, H. (2005) Changes of microbial populations in a ship's ballast water and sediments on a voyage from Japan to Qatar. *Mar. Pollut. Bull.*, 50, 751–757.
- Nakamura, T., Yuda, R., Unemoto, T., and Bakker, E. P. (1998) KtrAB, a new type of bacterial K⁺-Uptake System from *Vibrio alginolyticus*. *J. Bacteriol.*, 180, 3491–3494.
- Nagata, S., Ogawa, Y., and Mimura, H. (1991) Internal cation concentrations of the halotolerant bacterium *Brevibacterium* sp. in response to the concentrations and species of external salts. *J. Gen. Appl. Microbiol.*, 37, 403–414.
- Naughton, L. M., Blumberman, S. L., Carlberg, M., and Boyd, E. F. (2009) Osmoadaptation among *Vibrio* species and unique genomic features and physiological responses of *Vibrio parahaemolyticus*. *Appl. Environ. Microbiol.*, 75, 2802–2810.
- Ongagna-Yhombi, S. Y., and Boyd, E. F. (2013) Biosynthesis of the osmoprotectant ectoine, but not glycine betaine, is critical for survival of osmotically stressed *Vibrio parahaemolyticus* cells. *Appl. Environ. Microbiol.*, 79, 5038–5049.

- Ongagna-Yhombi, S. Y., McDonald, N. D., and Boyd, E. F. (2015) Deciphering the role of multiple betaine-carnitine-choline transporters in the Halophile *Vibrio parahaemolyticus*. *Appl. Environ. Microbiol.*, 81, 351–363.
- Padan, E., Venturi, M., Gerchman, Y., and Dover, N. (2001) Na^+/H^+ antiporters. *Biochim. Biophys Acta*, 1505, 144–157.
- Perez, C., Faust, B., Mehdipour, A. R., Francesconi, K. A., Forrest, L. R., and Ziegler, C. (2014) Substrate-bound outward-open state of the betaine transporter BetP provides insights into Na^+ coupling. *Nat. Commun.*, 5:4231. doi: 10.1038/ncomms5231.
- Pflughoeft, K. J., Kierek, K., and Watnick, P. I. (2003) Role of ectoine in *Vibrio cholerae* osmoadaptation. *Appl. Environ. Microbiol.*, 69, 5919–5927.
- Pirch, T., Quick, M., Nietschke, M., Langkamp, M., and Jung, H. (2002) Sites important for Na^+ and substrate binding in the Na^+ /proline transporter of *Escherichia coli*, a member of the Na^+ /solute symporter family. *J. Biol. Chem.*, 277, 8790–8796.
- Quinn, M. J., Resch, C. T., Sun, J., Lind, E. J., Dibrov, P., and Häse, C. C. (2012) NhaP1 is a $\text{K}^+(\text{Na}^+)/\text{H}^+$ antiporter required for growth and internal pH homeostasis of *Vibrio cholerae* at low extracellular pH. *Microbiology*, 158, 1094–1105.
- Radchenko, M. V., Waditee, R., Oshimi, S., Fukuhara, M., Takabe, T., and Nakamura, T. (2006) Cloning, functional expression and primary characterization of *Vibrio parahaemolyticus* K^+/H^+ antiporter genes in *Escherichia coli*. *Mol. Microbiol.*, 59, 651–663.
- Ressl, S., Terwisscha van Scheltinga, A. C., Vonnrhein, C., Ott, V., and Ziegler, C. (2009) Molecular basis of transport and regulation in the Na^+ /betaine symporter BetP. *Nature*, 458, 47–52.

Wilson, T. H., and Ding, P. Z. (2001) Sodium-substrate cotransporter in bacteria.

Biochim. Biophys. Acta, 1505, 121–130.

Chapter 3

Mitigation of Hyper KCl Stress at 42°C with Externally Existing Sodium

Glutamate to a Halotolerant *Brevibacterium* sp. JCM 6894

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* (Pflughoeft 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; Ressler 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).

Membrane-binding transporters from halotolerant bacteria are shown in Fig. 3.1 (Krämer and Morbach, 2004; Kronemeyer et al., 1995; Ochrombel et al., 2011; Peter et al., 1998; Schiller et al., 2004; Steger et al., 2004; Trötschel et al., 2003; Wang et al., 2018).

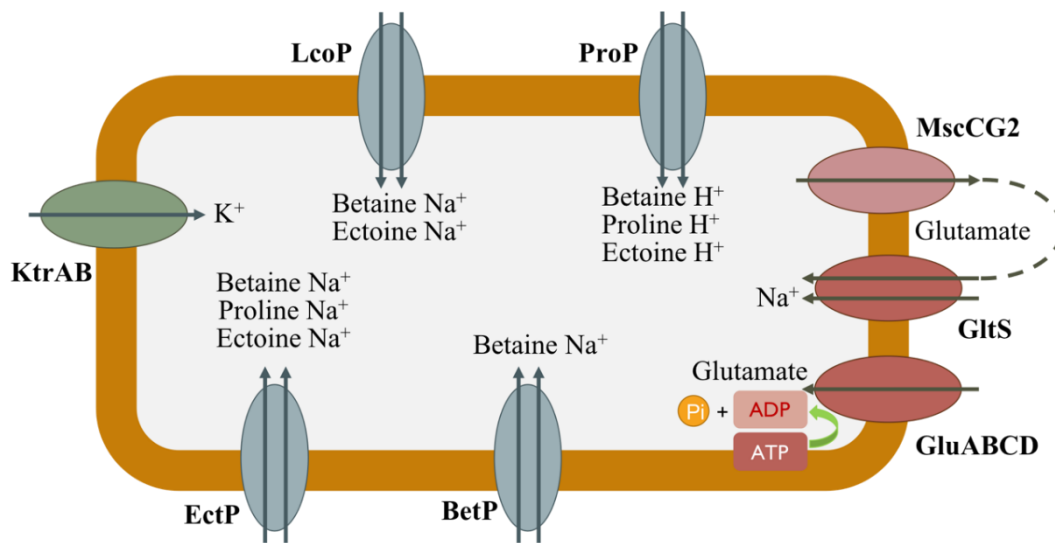


FIG. 3.1 Membrane-binding transporters from halotolerant bacteria.

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, in press). 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 μ L) 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 ($10,000 \times g$, 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 μ L) 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. 3.2A) and 37°C (Fig. 3.2B). 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. 3.2A). 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. 3.2B). 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.

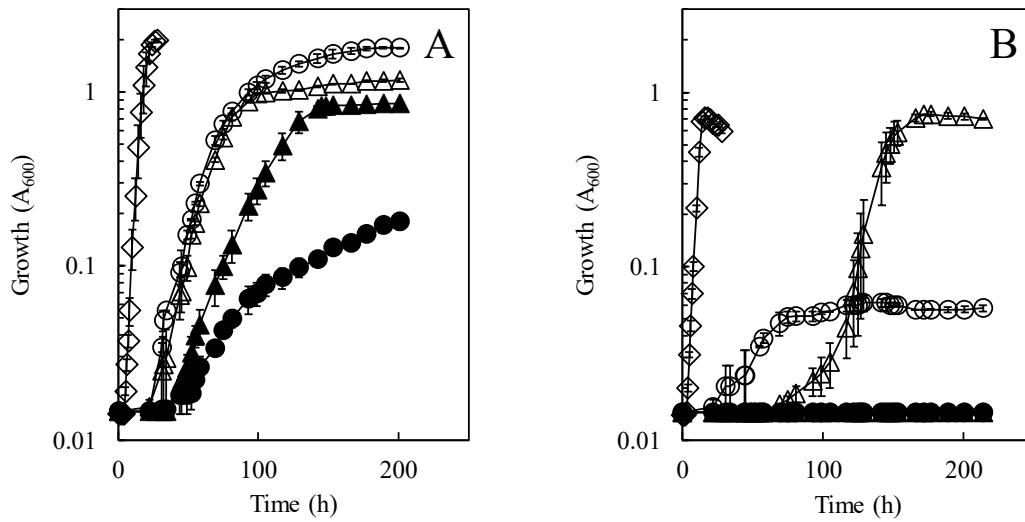


FIG. 3.2 Growth curves of *Brevibacterium* sp. under hyper salt stress in response to incubation temperatures of 30°C, 37°C, and 42°C.

Growth experiments were carried out at 30°C in the absence (open diamonds) and presence of 2.3 M NaCl (open circles), 2.3 M KCl (open triangles), 2.6 M NaCl (closed circles), and 2.6 M KCl (closed triangles) (Fig. 3.2A). The experiments were also carried out at 37°C in the absence (open diamonds) and presence of 2.3 M NaCl (open circles), 2.3 M KCl (open triangles), 2.6 M NaCl (closed circles), and 2.6 M KCl (closed triangles) (Fig. 3.2B).

Each experiment was carried out three times independently, and the averaged value $\pm$ SD is shown here.

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, $10^{7.4 \pm 0.1}$ (CFU·mL⁻¹), was maintained within a 7-log order of magnitude after 120 h at 30°C in the absence of salts (Fig. 3.3A). The number of surviving cells was reduced to $10^{3.1 \pm 0.2}$ (CFU·mL⁻¹) after 72 h at 37°C. At 42°C, it was reduced exponentially to less than $10^{3.0}$ (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. 3.3B). The values for 120 h at 37°C and 120 h at 42°C were $10^{4.2 \pm 0.3}$ (CFU·mL⁻¹) and $10^{2.9 \pm 0.7}$ (CFU·mL⁻¹), respectively, in the presence of 3.3.M KCl (Fig. 3.3C).

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. 3.2B and Fig. 3.3C).

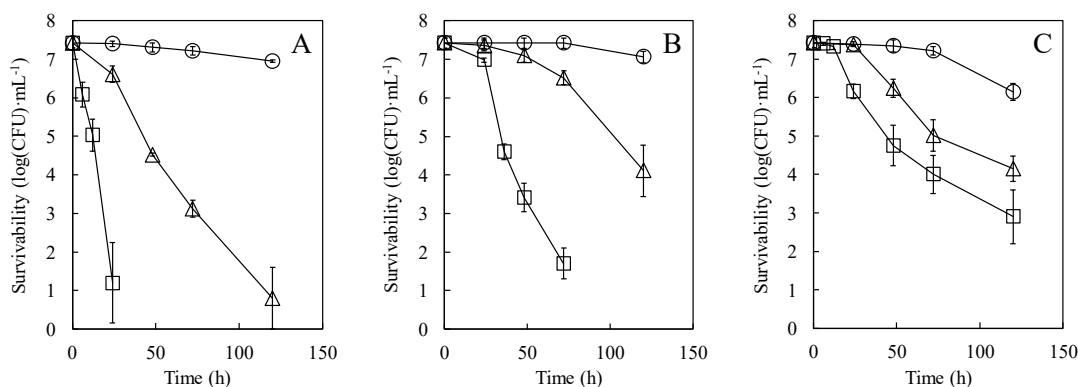


FIG. 3.3 Changes in the survivability of resting cells at hyper salt stress with exposure to temperature stress.

Resting cells, which were prepared from cells grown for 28 h at 30°C in the absence of externally added salts, were used in the experiments. Changes in survivability were examined in the absence of salts at 30°C (open circles), 37°C (open triangles), and 42°C (open squares) (Fig. 3.3A), in the presence of 3.3 M NaCl at 30°C (open circles), 37°C (open triangles), and 42°C (open squares) (Fig. 3.3B), and in the presence of 3.3 M KCl at 30°C (open circles), 37°C (open triangles), and 42°C (open squares) (Fig. 3.3C).

Each experiment was carried out three times independently, and the average value $\pm$ SD is shown here.

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, $10^{6.8 \pm 0.1}$ (CFU·mL⁻¹), was reduced gradually to $10^{5.1 \pm 0.1}$ (CFU·mL⁻¹) for 240 h at 37°C in the presence of 2.3 M KCl (Fig. 3.4), 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 $10^{4.4 \pm 0.1}$ (CFU·mL⁻¹) when exposed to 3.3 M KCl for 240 h at 37°C. It was $10^{3.4 \pm 0.1}$ (CFU·mL⁻¹) after 120 h at 42°C.

The time of exposure required to obtain a 3-log cycle of reduction at 37°C was 120 h longer for resting cells prepared from pre-adapted cells grown in the presence of 2.3 M KCl at 37°C than for resting cells prepared from cells grown in the absence of salts at 30°C. Similar results were observed at 42°C (see Fig. 3.3C). 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).

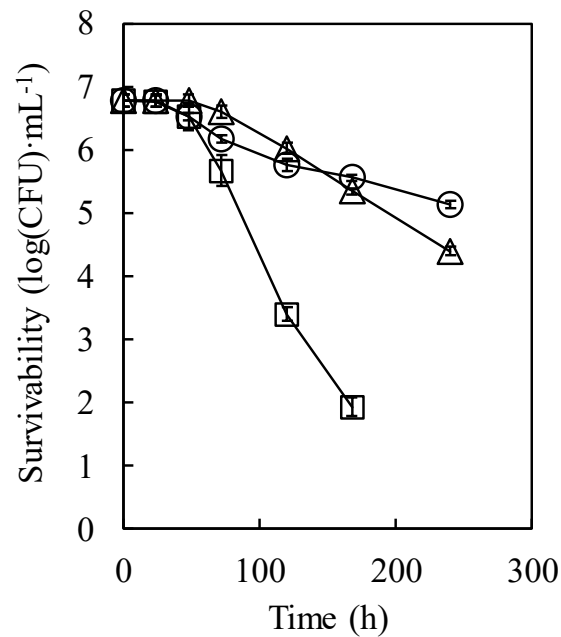


FIG. 3.4 Changes in the survivability of resting cells at hyper KCl with exposure to temperature stress.

Resting cells, which were prepared from cells grown for 189 h at 37°C in the presence of 2.3 M KCl, were used in the experiments. Changes in survivability were examined in the presence of 2.3 M KCl at 37°C (open circles), 3.3 M KCl at 37°C (open triangles), and 3.3 M KCl at 42°C (open squares).

Each experiment was carried out three times independently, and the average value $\pm$ SD is shown here.

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

Concentrations and compositions of free amino acids accumulated in cytoplasm were measured when grown in the absence (Table 3.1) and presence of hyper NaCl or KCl stress at 30 and 37°C (Table 3.2).

The value of the total content, 517.4 (nmol mg·protein⁻¹), was less than 50% for the strain grown at 37°C in comparison with that at 30°C (Table 3.1). Glutamic acid, hydroxyproline, and proline were major free amino acids when grown at 30°C without salts added externally. The occupation ratios of glutamic acid were more than 70% of the total content at 30 and 37°C. At 37°C, the total content was reduced to less than half that at 30°C.

The strain might utilize free amino acids preferentially to synthesize stress-induced proteins when grown at 37°C (Hantke et al., 2019; Lindquist, 1992). The occupation ratios of β -alanine and β -aminoisobutyric acid were less than 0.3%. Amino acids seemed to increase in the cytoplasm when inorganic salts were added to the nutrient medium to enhance the growth of the strain at 30°C (Mimura et al., 1994).

The total content—569.9 (nmol mg·protein⁻¹)—of free amino acids accumulated in the cytoplasm in the presence of 2.3 M KCl at 30°C was less than 60% of that obtained in the presence of 2.3 M NaCl at 30°C, although the major compositions of free amino acids were not changed, except for γ -aminobutyric acid (Table 3.2). The amino acid was not detected in cells grown in the presence of 2.3 M KCl at 30 and 37°C. The content of hydroxyproline grown at 37°C in the presence of 2.3 M KCl was 5 times higher than that at 30°C. Hydroxyproline accumulated in the cytoplasm seems to contribute not only to mitigating hyper KCl stress but also to mitigating temperature stress to the strain. The

total content of free amino acids accumulated in cells grown in the presence of 2.6 M KCl at 30°C was 1.2 times higher than that in the presence of 2.3 M KCl at 30°C.

TABLE 3.1 Free amino acids pooled in the cytoplasm of cells grown without salts externally added.

Amino acid ^a	Cells were grown without salts added externally at					
	30°C for 28 h			37°C for 18 h		
	nmol/mg protein	Occupation ratio (%) ^c	mM ^d	nmol/mg protein	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
Hypro	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 concentration of free amino acids (nmol/mg protein) 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: (Concentration of each free amino acid / Total concentration of free amino acids) × 100.

^d Internal water space of 5.5 (μL/mg protein) was used to estimate each amino acid content (mM) in the cytoplasm (Nagata et al., 1991).

TABLE 3.2 Free amino acids pooled in the cytoplasm of cells grown with hyper salt stress.

Amino acid ^a	Cells were grown with											
	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	Occupation ratio (%) ^c	mM ^d	nmol/mg protein	Occupation ratio (%) ^c	mM ^d	nmol/mg protein	Occupation ratio (%) ^c	mM ^d	nmol/mg protein	Occupation ratio (%) ^c	mM ^d
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 ^b	923.0	96.80	230.90	542.8	95.2	135.8	584.6	94.2	146.2	654.2	95.2	163.7
	953.0			569.9			620.2			687.1		

^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 concentration of free amino acids (nmol/mg protein) in this table is shown above, and the concentration of total free amino acids detected is shown

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

^d Internal water space of 4.0 (μL/mg protein), 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.

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 3.3). 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 sodium glutamate, but not of 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.

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; Ressler 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).

TABLE 3.3 Effect of each osmoprotectant externally existing on the survivability of *Brevibacterium* sp. after exposure to hyper salt stress at 42°C.

Osmoprotectant (mM)	(50)	Resting cells ^a were exposed to		
		3.3 M NaCl	3.3 M KCl	
		for 36 h at 42°C	for 72 h at 42°C	for 120 h at 42°C
None		$10^{4.6 \pm 0.2}$	$10^{4.0 \pm 0.5}$	$10^{2.9 \pm 0.7}$
Sodium L-Glutamate		$10^{5.2 \pm 0.2}$	$10^{7.2 \pm 0.1}$	$10^{5.6 \pm 0.1}$
Potassium L-Glutamate		$10^{5.6 \pm 0.3}$	$10^{5.1 \pm 0.2}$	$10^{3.9 \pm 0.5}$
L-Proline		$10^{7.1 \pm 0.2}$	$10^{6.5 \pm 0.1}$	$10^{4.2 \pm 0.6}$
L-Hydroxyproline		$10^{6.1 \pm 0.3}$	$10^{4.0 \pm 0.3}$	$10^{2.5 \pm 0.4}$
Betaine		$10^{6.8 \pm 0.1}$	$10^{3.8 \pm 0.7}$	$10^{2.7 \pm 0.7}$
Ectoine		$10^{6.8 \pm 0.2}$	$10^{4.7 \pm 0.7}$	$10^{2.4 \pm 0.4}$

^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 $10^{7.4 \pm 0.1}$ (CFU·mL⁻¹).

^b Surviving experiment was carried out three times independently, and the data are shown as the averaged value $\pm$ SD.

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⁺ at 42°C. 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. 3.5). The number of surviving cells at 3.3 M KCl alone was reduced to $10^{5.7 \pm 0.2}$ (CFU·mL⁻¹) from $10^{6.8 \pm 0.1}$ (CFU·mL⁻¹). The value was increased with an increase in the concentrations of sodium glutamate and showed the maximum value— $10^{6.8 \pm 0.1}$ (CFU·mL⁻¹)—at 50 mM. The value barely changed up to 200 mM. In the presence of proline, the maximum value of $10^{6.5 \pm 0.1}$ (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. The value was reduced gradually with an increase in the concentrations of hydroxyproline, reaching $10^{5.0 \pm 0.2}$ (CFU·mL⁻¹) at 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 3.3 and Fig. 3.5). On the other hand, externally existing proline, betaine, and ectoine (50 mM) functioned better than 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 (Kronmeyer 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 at 37°C, were exposed

to 3.3 M KCl at 42°C. Güler et al. (2016) reported that K^+ binding reduces the interaction of BetP with negatively charged phospholipids in the cell membrane of *C. glutamicum* by competing with positively charged residues of BetP. The phospholipid dependency of stress-sensing and adaptation mechanisms is essential in *E. coli* (Rowlett et al., 2017). The reduction of survivability in the presence of 1 mM betaine might be related to changes in the composition ratios of phospholipids in the cell membrane, which are interacting with betaine and its transporters in the presence of 3.3 M KCl.

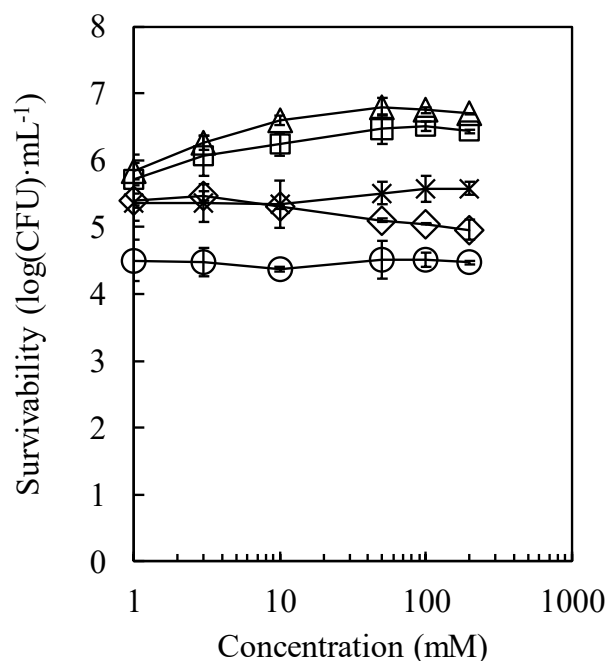


FIG. 3.5 Changes in the survivability of resting cells after exposure to 3.3 M KCl at 42°C in the presence of different concentrations of each osmoprotectant.

Resting cells, which were prepared from cells grown for 189 h at 37°C in the presence of 2.3 M KCl, were used in the experiments. Changes in survivability 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 $\pm$ SD is shown here.

Summary

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.

References

- Bracher, S., Schmidt, C. C., Dittmer, S. I., and Jung, H. (2016) Core transmembrane domain 6 plays a pivotal role in the transport cycle of the sodium/proline symporter PutP. *J. Biol. Chem.*, 291, 26208–26215.
- Csonka, L.N., and Epstein, W. (1996) Osmoregulation. In *Escherichia coli and Salmonella: Cellular and Molecular Biology* (Neidhardt, F. C., ed.), pp. 1210–1223, ASM Press, Washington DC.
- Grammann, K., Volke, A., and Kunte, H. J. (2002) New type of osmoregulated solute transporter identified in halophilic members of the *bacteria* domain: TRAP transporter TeaABC mediates uptake of ectoine and hydroxyectoine in *Halomonas elongata* DSM 2581^T. *J. Bacteriol.*, 184, 3078–3085.
- Gundlach, J., Herzberg, C., Hertel, D., Thürmer, A., Daniel, R., Link, H., and Stülke, J. (2017) Adaptation of *Bacillus subtilis* to life at extreme potassium limitation. *mBio*, 8, e00861-17.
- Güler, G., Gärtner, R. M., Ziegler, C., and Mäntele, W. (2016) Lipid-protein interactions in the regulated betaine symporter BetP probed by infrared spectroscopy. *J. Biol. Chem.*, 291, 4295–4307.
- Hantke, I., Schäfer, H., Janczikowski, A., and Turgay, K. (2019) YocM a small heat shock protein can protect *Bacillus subtilis* cells during salt stress. *Mol. Microbiol.*, 111, 423–440.
- Hunte, C., Screpanti, E., Venturi, M., Rimon, A., Padan, E., Michel, H. (2005) Structure of a Na⁺/H⁺ antiporter and insights into mechanism of action and regulation by pH. *Nature*, 435, 1197–1202.

- Koshy, C., Schweikhard, E. S., Gärtner, R. M., Perez, C., Yildiz, O., and Ziegler, C. (2013) Structural evidence for functional lipid interactions in the betaine transporter BetP. *EMBO J.*, 32, 3096–3105.
- Krämer, R., and Morbach, S. (2004) BetP of *Corynebacterium glutamicum*, a transporter with three different functions: betaine transport, osmosensing, and osmoregulation. *Biochim. Biophys. Acta.*, 1658, 31–36.
- Kronemeyer, W., Peekhaus, N., Krämer, R., Sahm, H., and Eggeling, L. (1995) Structure of the *gluABCD* cluster encoding the glutamate uptake system of *Corynebacterium glutamicum*. *J. Bacteriol.*, 177, 1152–1158.
- Larsen, H. (1986) Halophilic and halotolerant microorganisms—an overview and historical perspective. *FEMS Microbiol. Rev.*, 39, 3–7.
- Lindquist, S. (1992) Heat-shock proteins and stress tolerance in microorganisms. *Curr. Opin. Genet. Dev.*, 2, 748–755.
- Marinelli, F., Kuhlmann, S. I., Grell, E., Kunte, H.-J., Ziegler, C., Faraldo-Gómez, J. D. (2011) Evidence for an allosteric mechanism of substrate release from membrane-transporter accessory binding proteins. *Proc. Natl. Acad. Sci. USA*, 108, E1285 — 1292.
- Mimura, H., Nagata, S., and Matsumoto, T. (1994) Concentrations and compositions of internal free amino acids in a halotolerant *Brevibacterium* sp. in response to salt stress. *Biosci. Biotech. Biochem.*, 58, 1873–1874.
- Mimura, H., and Nagata, S. (1998) Effect of hyper-salt stress on the heat resistance of a halotolerant *Brevibacterium* sp. JCM6894. *J. Ferment. Bioeng.*, 85, 185–189.
- Nagata, S. (1988) Influence of salts and pH on the growth as well as NADH oxidase of the halotolerant bacterium A505. *Arch. Microbiol.*, 150, 302–308.

- Nagata, S., Ogawa, Y., and Mimura, H. (1991) Internal cation concentrations of the halotolerant bacterium *Brevibacterium* sp. in response to the concentrations and species of external salts. *J. Gen. Appl. Microbiol.*, 37, 403–414.
- Nagata, S., Adachi, K., and Sano, H. (1996) NMR analyses of compatible solutes in a halotolerant *Brevibacterium* sp. *Microbiology*, 142, 3355–3362.
- Naughton, L. M., Blumerman, S. L., Carlberg, M., and Boyd, E. F. (2009) Osmoadaptation among *Vibrio* species and unique genomic features and physiological responses of *Vibrio parahaemolyticus*. *Appl. Environ. Microbiol.*, 75, 2802–2810.
- Ochrombel, I., Ott, L., Krämer, R., Burkovski, A., and Marin, K. (2011) Impact of improved potassium accumulation on pH homeostasis, membrane potential adjustment and survival of *Corynebacterium glutamicum*. *Biochim. Biophys. Acta*, 1807, 444–450.
- Olkhova, E., Raba, M., Bracher, S., Hilger, D., and Jung, H. (2011) Homology model of the Na⁺/proline transporter PutP of *Escherichia coli* and its functional implications. *J. Mol. Biol.*, 406, 59–74.
- Peter, H., Weil, B., Burkovski, A., Krämer, R., and Morbach, S. (1998) *Corynebacterium glutamicum* is equipped with four secondary carriers for compatible solutes: identification, sequencing, and characterization of the proline/ectoine uptake system, ProP, and the ectoine/proline/glycine betaine carrier, EctP. *J. Bacteriol.*, 180, 6005–6012.
- Perez, C., Faust, B., Mehdipour, A. R., Francesconi, K. A., Forrest, L. R., and Ziegler, C. (2014) Substrate-bound outward-open state of the betaine transporter BetP provides insights into Na⁺ coupling. *Nat. Commun.*, doi.org/10.1038/ncomms5231.

- Pflughoeft, K. J., Kierek, K., and Watnick, P. I. (2003) Role of ectoine in *Vibrio cholerae* osmoadaptation. *Appl. Environ. Microbiol.*, 69, 5919–5927.
- Ressl, S., Terwisscha van Scheltinga, A. C., Vonnheim, C., Ott, V., and Ziegler, C. (2009) Molecular basis of transport and regulation in the Na⁺/betaine symporter BetP. *Nature*, 458, 47–52.
- Rowlett, V. W., Mallampalli, V. K. P. S., Karlstaedt, A., Dowhan, W., Taegtmeier, H., Margolin, W., and Vitrac, H. (2017) Impact of membrane phospholipid alterations in *Escherichia coli* on cellular function and bacterial stress adaptation. *J. Bacteriol.* 199:e00849-16.
- Schiller, D., Krämer, R., and Morbach, S. (2004) Cation specificity of osmosensing by the betaine carrier BetP of *Corynebacterium glutamicum*. *FEBS Lett.*, 563 108–112.
- Steger, R., Weinand, M., Krämer, R., and Morbach, S. (2004) LcoP, an osmoregulated betaine/ectoine uptake system from *Corynebacterium glutamicum*. *FEBS Lett.*, 573, 155–160.
- Teichmann, L., Kümmel, H., Warmbold, B., and Bremer, E. (2018) OpuF, a new *Bacillus* compatible solute ABC transporter with a substrate-binding protein fused to the transmembrane domain. *Appl. Environ. Microbiol.*, 84, e01728-18.
- Tolner, B., Poolman, B., and Konings, W. N. (1992) Characterization and functional expression in *Escherichia coli* of the sodium/proton/glutamate symport proteins of *Bacillus stearothermophilus* and *Bacillus caldovenax*. *Mol. Microbiol.*, 6, 2845–2856.
- Trötschel, C., Kandirali, S., Diaz-Achirica, P., Meinhardt, A., Morbach, S., Krämer, R., and Burkovski, A. (2003) GltS, the sodium-coupled L-glutamate uptake system of *Corynebacterium glutamicum*: identification of the corresponding gene and impact on L-glutamate production. *Appl. Microbiol. Biotechnol.*, 60, 738–742.

- Wang, Y., Cao, G., Xu, D., Fan, L., Wu, X., Ni, X., Zhao, S., Zheng, P., Sun, J., and Ma, Y. (2018) A novel L-glutamate exporter of *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.*, 84, e02691-17.
- Yin, Y., and Mimura, H. (2020) Changes in the survivability of marine *Vibrio* sp. under hyper KCl stress in the presence of betaine as well as with exposure to 37°C. *Biocontrol Sci.*, 25, 17–24.

Chapter 4

General Conclusion

As for a halophilic marine *Vibrio* sp. September 1, Na^+ externally existing was essential for the growth under hyper KCl stress at 37°C. The result indicates that osmoprotectants are transported coupled with Na^+ via membrane-binding transporter(s). Sodium cation that was co-transported with osmoprotectants into the cytoplasm needs to be excluded from the cytoplasm by membrane-binding Na^+/H^+ antiporter. Those transporters need not to be inactivated by the exposure to higher concentrations of KCl. Resting cells, that were prepared from cells grown in the presence of 0.5 M NaCl at 30°C, showed temperature sensitivity when exposed to 1.2 M KCl and 50 mM NaCl at 37°C, regardless of the absence and presence of osmoprotectants. The adaptation system(s) of the strain to hyper KCl stress is temperature sensitive.

In general, bacterial osmoadaptation study has been focused on the osmoadaptation system(s) that functions to cope with higher concentrations of NaCl existing externally. However, a halotolerant marine *Brevibacterium* sp. JCM 6894 could grow in the presence of 2.3 M KCl at 37°C, while the growth was strongly repressed in the presence of 2.3 M NaCl at 37°C. The result indicates that the osmoadaptation system(s) of the strain to hyper NaCl stress is temperature sensitive. Osmoadaptation of *Brevibacterium* sp. JCM 6894 to hyper KCl stress with exposure to temperature stress was different with that of *Vibrio* sp. September 1. Osmoadaptation system(s) of *Brevibacterium* sp. JCM 6894 that functions under hyper KCl stress coexisting with temperature stress might be unique. Further study is necessary to make clear how the strain can adapt to hyper KCl stress with temperature stress simultaneously existing.

Betaine and proline (50 mM) externally existing mitigated hyper NaCl stress to *Vibrio* sp. September 1 at 30 and 37°C. Glutamate (50 mM) was the most functional osmoprotectant to mitigate hyper KCl stress to *Brevebacterium* sp. JCM 6894 at 42°C. Those osmoprotectants need to be accumulated in the cytoplasm after transported via transporters. Therefore, salt tolerance of transporters is primarily essential to survive when cells are exposed to hyper salt stress in the presence of osmoprotectants. Throughout the experiments, it was suggested that bacterial halotolerance depended on the function of constitutively expressed membrane-binding transporters that were not inactivated by the exposure to higher concentrations of salts.

List of papers published

Yue Yin and Haruo Mimura, Changes in the survivability of marine *Vibrio* sp. under hyper KCl stress in the presence of betaine as well as with exposure to 37°C, *Biocontrol Science*, 25, pp. 17–24 (2020).

Yue Yin and Haruo Mimura, Mitigation of hyper KCl stress at 42°C with externally existing sodium glutamate to a halotolerant *Brevibacterium* sp. JCM 6894, *Biocontrol Science*, 25, 139–147 (2020).

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