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

YUE YIN, AND HARUO MIMURA *

Graduate School of Maritime Sciences, Kobe University, 5-1-1 Fukae, Higashinada, Kobe 658-0022, Japan

Received 14 May, 2019/Accepted 29 October, 2019

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.

Key words : Marine *Vibrio* sp. / Betaine / Proline / Survivability / Hyper KCl Stress.

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; Ressl et al., 2009) and the proline transporter of *Escherichia coli* (Pirch et al., 2002) need Na$^+$.

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.

*Corresponding author. Tel: +81-78-431-6344, Fax: +81-78 431-6365, E-mail : hmimura(a)maritime.kobe-u.ac.jp
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 stational 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. 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.

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 μL) 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 × 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. 1A). 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. 1B). 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; Ressl et al., 2009; Wilson and Ding, 2001).

**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 $10^{2.3 ± 0.2}$ and $10^{3.2 ± 0.3}$ (CFU·mL$^{-1}$), respectively, from the initial number of $10^{7.1 ± 0.2}$ (CFU·mL$^{-1}$) (Fig. 2A). The values were reduced almost exponentially with exposure to 1.8 M NaCl, and it showed $10^{2.7 ± 0.4}$ (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. 2B). The value was reduced slightly to $10^{6.7 ± 0.2}$ (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 $10^{3.9 ± 0.8}$ (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 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⁺.

**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 1). The occupation ratios were
FIG. 2. 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. 2A). 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.
Each experiment was carried out three times independently, and the average value ± SD is shown here.

TABLE 1. Free amino acids pooled in the cytoplasm of cells grown with hyper salt stress.

| Amino acid | Cells were grown with | 0.5 M NaCl at 30°C | Occupation ratio (%) | 0.5 M NaCl at 37°C | Occupation ratio (%) | 1.2 M NaCl at 37°C | Occupation ratio (%) | 0.8 M KCl and 0.05 M NaCl at 37°C | Occupation ratio (%) |
|------------|-----------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|--------------------------|---------------------|
|            |                       | nmol mg⁻¹ protein   |                     | nmol mg⁻¹ protein   |                     | nmol mg⁻¹ protein   |                     | nmol mg⁻¹ protein       |                     |
| Asp        |                       | 11.8                | 0.8                 | 16.5                | 2.3                 | 21.6                | 2.2                 | 26.3                    | 5.9                 |
| Asn        |                       | 5.4                 | 0.4                 | 21.5                | 3.0                 | 2.5                 | 0.3                 | 1.4                     | 0.3                 |
| Glu        |                       | 996.5               | 70.7                | 481.9               | 66.3                | 744.1               | 75.3                | 131.8                   | 29.7                |
| α-Aminoadipic acid |           | 13.2                | 0.9                 | 6.6                 | 0.9                 | 59.2                | 6.0                 | 95.9                    | 21.6                |
| Gly        |                       | 69.9                | 5.0                 | 62.8                | 8.7                 | 16.3                | 1.6                 | 17.0                    | 3.8                 |
| Ala        |                       | 69.0                | 4.9                 | 25.9                | 3.6                 | 28.7                | 2.9                 | 25.7                    | 5.8                 |
| Citrulline |                       | 31.4                | 2.2                 | 7.7                 | 1.1                 | 23.7                | 2.4                 | 80.8                    | 18.2                |
| Val        |                       | 23.2                | 1.6                 | 4.3                 | 0.6                 | 9.2                 | 0.9                 | 4.2                     | 0.9                 |
| Leu        |                       | 9.1                 | 0.6                 | 5.3                 | 0.7                 | 8.2                 | 0.8                 | 5.3                     | 1.2                 |
| Ethanolamine|                      | 16.2                | 1.1                 | 2.9                 | 0.4                 | 3.4                 | 0.3                 | 0.9                     | 0.2                 |
| Hydroxylsine|                     | 13.6                | 1.0                 | 11.9                | 1.6                 | 0.9                 | 0.1                 | 2.2                     | 0.5                 |
| Lys        |                       | 28.2                | 2.0                 | 10.6                | 1.5                 | 9.1                 | 0.9                 | 5.4                     | 1.2                 |
| Hydroxyproline|                   | 13.9                | 1.0                 | 3.9                 | 0.5                 | 0.0                 | 0.0                 | 4.7                     | 1.1                 |
| Totalb     |                       | 1301.4              | 92.2                | 661.7               | 91.2                | 926.9               | 93.7                | 401.5                   | 90.4                |

b The concentration of free amino acids (nmol mg⁻¹ 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) × 100.
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, Beneckea 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 glutamate and glutamine 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 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 glutamate and glutamine 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·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.

**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). 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 for 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.

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 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). When resting cells, that were prepared from pre-adapted 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 $10^{5.0} \pm 0.1$ (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 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.

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. 3). 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.

### Table 2. 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).

| 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                   | $10^{6.1} \pm 0.4$          | $10^{3.2} \pm 0.5$          | $10^{5.1} \pm 0.6$          | $< 10^{1.0b}$          |
| Sodium L-Glutamate     | $10^{5.6} \pm 0.1$          | $10^{4.1} \pm 0.3$          | $10^{5.2} \pm 0.3$          | $< 10^{1.0b}$          |
| L-Citrulline           | $10^{6.9} \pm 0.2$          | $10^{3.3} \pm 0.1$          | $10^{5.3} \pm 0.5$          | $< 10^{1.0b}$          |
| L-Proline              | $10^{6.2} \pm 0.1$          | $10^{4.3} \pm 0.5$          | $10^{5.2} \pm 0.1$          | $10^{5.8} \pm 1.6$     |
| Choline chloride       | $10^{5.2} \pm 0.1$          | $10^{3.5} \pm 0.1$          | $10^{4.1} \pm 0.1$          | $< 10^{1.0b}$          |
| Sarcosine              | $10^{6.2} \pm 0.2$          | $10^{2.9} \pm 0.2$          | $10^{5.3} \pm 0.5$          | $< 10^{1.0b}$          |
| Dimethylglycine        | $10^{6.6} \pm 0.1$          | $10^{2.8} \pm 0.1$          | $10^{4.5} \pm 0.1$          | $< 10^{1.0b}$          |
| Betaine                | $10^{5.8} \pm 0.3$          | $10^{4.4} \pm 0.5$          | $10^{4.1} \pm 0.4$          | $< 10^{1.0b}$          |
| Ectoine                | $10^{7.1} \pm 0.2$          | $10^{3.1} \pm 0.5$          | $10^{5.3} \pm 0.1$          | $< 10^{1.0b}$          |
| Trehalose              | $10^{6.8} \pm 0.3$          | $10^{3.3} \pm 0.3$          | $10^{4.2} \pm 0.4$          | $< 10^{1.0b}$          |

$^a$The initial number of resting cells was $10^{6.1} \pm 0.2$ (CFU·mL$^{-1}$). The numbers of surviving cells after 6 h of exposure at 30°C and 37°C in the buffer containing 0.5 M NaCl were $10^{5.1} \pm 0.2$ and $10^{6.9} \pm 0.1$ (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 $10^{6.0}$ (CFU·mL$^{-1}$) (n=3).
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, $10^{11.1 \pm 0.2}$ (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). 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 $10^{3.4}$ (CFU·mL$^{-1}$) at 37°C after 3 h of exposure to 1.2 M KCl and 50 mM NaCl (Table 2). Temperature sensitivity was also observed even when resting cells, $10^{5.5 \pm 0.1}$ (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 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 and Table 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). 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, $10^{3.0 \pm 0.4}$ (CFU·mL$^{-1}$) when resting cells, that were prepared from pre-adapted 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 3). The results indicate that a betaine and proline transporter(s) of the resting cells functions to mitigate hyper KCl stress 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.

Koga, T., Nakajo, 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.

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.

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., 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.

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.

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.

Pfugheoff, K. J., Kierk, K., and Watenick, 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.

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., Tenvischa van Scheltinga, A. C., Vonrhein, 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.