Effect of hyper- and hypo-salt stresses on survival of a halotolerant *Brevibacterium so.* JCM 6894 during the heat treatment

Haruo Mini and Shinichi Nagata
Institute for Marine Cargo Transportation, Mobs University of Mercantile Marine, Mobs, Japan

Survival of the halotolerant *Brevibacterium so.* JCM 6894 was examined after heating in the hyper- and hypo-salt stress conditions. Heating for 30 min at 47 ^{0}C in 50 mM HEPES-TMAH buffer, pH 7.5, reduced the viable cell numbers by about a 5 log cycle, when cells grown in the absence of salts were used. By the addition of 2 M NaCl or KCl externally to give a hyper-salt stress to the cells, the viable cell numbers remained within a 0.1 log cycleof the former value after 30 min at 47 ^{0}C. For the hypo-salt stress experiment, cells grown in the presence of 2 M NaCl or KCl were subjected to heating in a 50 mM HEPS-TMAH buffer, pH 7.5, with dilution. As a result, the number of viable cells was reduced by 0.4-log cycle after 30 min at 47 ^{0}C when the cells grown in the presence of 2 M NaCl were examined. In the presence of 2 M KCl the reduction was a 1.6-log cycle. These results indicate that the hyper-salt stress rather than the hypo-salt stress is more effective, and that the Na^+ ions rather than K^+ ions play an important role at lower concentrations to increase the heat resistance of this strain.

1. INTRODUCTION

Microorganisms are surviving adapting to the changes of their surroundings. The osmotic change, oxidation, pH change, UV radiation and starvation are thought to be environmental stresses, which enhance the microbial tolerance to other stresses [1-5].

The objective of this study in the influence of Na^+ ions on the survival mechanism of a halotolerant *Brevibacterium* sp. JCM 6894 when subjected to sublethal environmental stresses. This strain grew in a wide range of NaCl or KCl concentrations from 0 to 2 M [6] and showed the resistance to the transient hyper-salt stress when the cells grown without salts addition were exposed to a buffer solution containing 2 M NaCl [7]. Therefore, we examined the effect of hyper-salt stress on the heat resistance of the resting *Brevibacterium* so. JCM 6894 cells without a pre-adaptation process by subjecting them to given concentrations of NaCl or KCl. We also examined the effect of hypo-salt stress on the heat resistance of this strain to compare with that of hyper-salt stress.

2. MATERIALS AND METHODS

2.1. Organisms and cultivation

For the heat resistant experiment with the hyper-salt stress, *Brevibacterium* so. JCM 6894 was grown aerobically at 30^{0}C for 24 h in a complex medium containing 5.0 g Bactopeptone (Difco, MI, USA), 1.0 g yeast extract (Dates), 0.1 g $CaC1_2.2H_2O$, 0.7 g $MgSO_4.7H_20$, 1.0 g NH_4Cl, 35 mg K_2HPO_4 and 15 mg KH_2PO_4 per liter. In case of the hyposalt stress, the strain was grown at 30 ^{0}C for 72 h in the complex medium containing 2 M NaCl or KCl. The pH was adjusted to 7.5 by tetramethylammonium hydroxide (TMAH).

2.2. Heating experiments

The cells grown at the stationary phase of growth in the absence of salts were harvested by centrifugation at 25 ^{0}C and washed twice with 50 mM N-(2-hydroxyethyl)piperazine-M-2-ethanesulfonic acid (HEPES)-TMAH buffer, pH 7.5. To keep the osmotic balance, the cells grown in the presence of 2 M NaCl or KCl were washed with 50 mM HEPES-TMAH buffer, pH 7.5, containing 2 M NaCl or KCl, respectively. Then the cells were suspended in the same buffer used for the washing. Fifty µL of cell suspension was added to 5 ml of the 50 mM HEPES-TMAH buffer used for heating, pH 7.5, containing the various concentrations of NaCl or KCl. At given times, 100 µl of aliquot was pipetted out and immediately diluted with 900 µl of 50 mM potassium phosphate (K-Pi) buffer, pH 7.5, containing the same concentration of NaCl or KCl.

2.3. Enumeration

The numbers of the viable cells are shown as the logarithmic value of colony-forming unit (CFU) per ml. After the serial dilution, 100 µl of sample was spread out onto the agar plate made of the complex

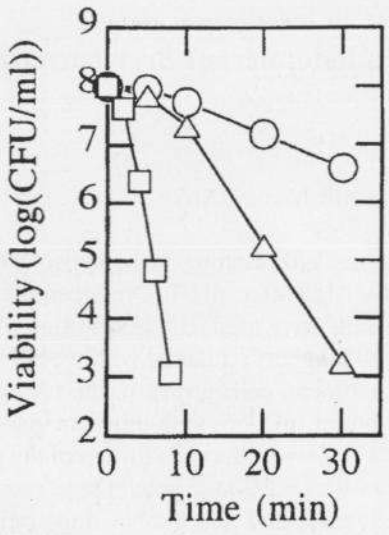

Figure 1. Survival curve of *Brevibacterium* so. JCM 6894 cells after heating.

Cells were heated in 50 mM HEPES-TMAH buffer, pH 7.5, at 43 ^{0}C (○), 47 ^{0}C (Δ) and 52 ^{0}C (□).

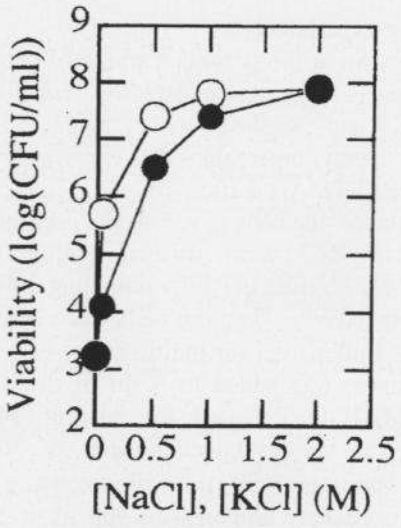

Figure 2. Effect of NaCl or KCl externally added on the heat resistance of *Brevibacterium* so. JCM 6894 cells.
Cells were heated in 50 mM HEPES-TMAH buffer, pH 7.5, at 47 ^{0}C in the presence of NaCl (○) or KCl (●).

medium containing 1.5% agar. The viable cells were counted after 3 days of incubation at 30 ^{0}C.

3. RESULTS AND DISCUSSION

3.1 Effect of hyper-salt stress on survival of the heat stressed *Brevibacierium so.* JCM 6894 cells

The resting *Brevibacterium* so. JCM 6894 cells grown without salts externally added were heated in 50 mM HEPES-TMAH buffer, pH 7.5, at 43 ^{0}C, 47 ^{0}C and 52 ^{0}C (Figure 1). For every survival curve, a shoulder was observed at the beginning of heating. About 1.4 and 4.8 - log cycles in total 8 log cycle of the freshly prepared viable cell numbers were reduced after heating for 30 min at 43 ^{0}C and 47 ^{0}C, respectively. At 52 ^{0}C, 8 min was sufficient to obtain a 5-log cycle reduction. Figure 2 shows the survival curve of the heat-stressed *Brevibacterium* so. JCM 6894 cells at 47 ^{0}C for 30 min as a function of the NaCl or KCl concentration from 0 to 2M. When the concentration was less than 1 M, NaCl was more effective than KCl in increasing the heat resistance. Especially, by the addition of 50 mM NaCl, the viability increased by more than a two-log cycle. The difference of the viability became smaller with increasing external concentrations of NaCl and KCl. In the presence of 0.05, 0.5 and 1 M NaCl or KCl, the difference of the viability at each concentration was 1.6-log, 0.9 - log and 0.4 - log cycles, respectively. In the presence of 2 M NaCl or KCl, the difference was not observed, and the viability for both was 7.9-log cycle. This value was almost the same as that without heating. These results indicate that the hyper-salt stress contributes to an increase in the heat resistance of this strain.

3.2 Effect of hypo-salt stress on.survival of the heat stressed *Brevibacterium so.* JCM 6894 cells

Figure 3 shows the survival curve of the heat stressed *Brevibacterium* so. JCM 6894 cells at 47 ^{0}C, 50 ^{0}C and 53 ^{0}C with the hypo-salt stress. The resting cells used were prepared from the cells grown in the presence of 2 M NaCl or KCl at 30 ^{0}C for 72 h. By an one-hundredth dilution of the cell suspension with the heating buffer, the simultaneous hypo-salt and heat stresses were given.

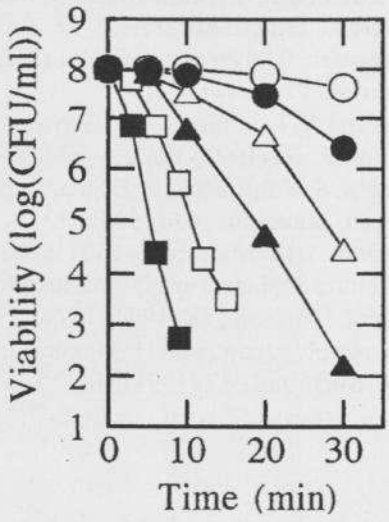

Figure 3. Effect of hypo-salt stress on the heat resistance of *Brevibacterium sp. JCM* 6894 cells.

Cells grown in the presence of 2 M NaCI (open symbol) or KCI (closed symbol) were heated in 50 mM HEPES-TMAH buffer, pH 7.5, at 47 ^{0}C (○, ●), 50 ^{0}C (Δ, ▲) and 53 ^{0}C (□,■).

The linear reduction of viable cell numbers, observed in previous experiments, did not occur through the heating at any temperatures examined for the cells grown in the presence of 2 M NaCl. Only 0.4 log cycle was reduced by heating for 30 min at 47 ^{0}C. In experiments with the resting cells grown in the presence of 2 M KCl, the reduced cell numbers after heating for 30 min at 47 ^{0}C were larger by 1.2-log cycle than in comparable experiments with 2 M NaCl. This phenomenon became more obvious with an increase in the heating temperature. The difference of survival between the cells grown in the presence of 2 M NaCl and KCl increased by 2.3-log and 3.1-log cycles at 50 ^{0}C for 30 min and at 53 ^{0}C for 9 min. respectively.

In the hypo-NaCI stress condition, where the final concentration of NaCl in the heating buffer was 20 mM, the viability at 47 ^{0}C for 30 min remained 7.6-log cycle. Even in the hypo-KCl stress, the viability was more than a 6-log cycle. Although the viability at 47 ^{0}C for 30 min. in the presence of 50 mM NaCl in the hyper-NaCl stress experiment was

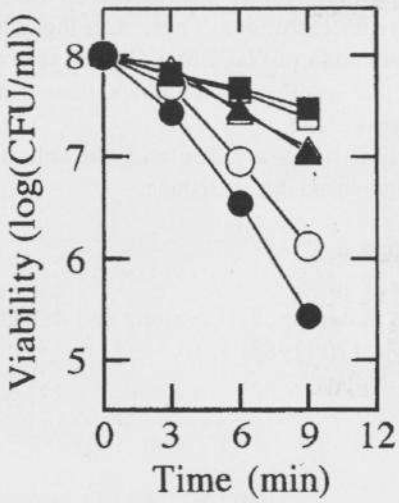

Figure 4. Effect of NaCl or KCl externally added on the heat resistance of hypo-salt stressed *Brevibacterium* sp. JCM 6894 cells.

Cells grown in the presence of 2 M KCl were heated in 50 mM HEPES-TMAH buffer, pH 7.5, containing 0.5 M (○,●), 1 M (Δ, ▲) and 2 M (□, ■) of NaCl (open symbol) or KCI (closed symbol) at 53 ^{0}C.

increased by more than a 2-log cycle in comparison with that in the absence of NaCl, the viable cell numbers were still less than 6-log cycle (Figure 2). These results indicate that the growth in the presence of 2 M NaCl or KCl as well as the hypo-salt stress is more effective to increase the heat resistance than 20 - 50 mM NaCl or KCl.

Nagata et al. [8] have measured the cell associated Na^+ and K^+ ion concentrations of this strain grown in the absence and presence of up to 2 M NaCl or KCl. When the cells were grown in the presence of 2 M NaCl, the concentration of the cell associated Na^+ ions was 1.3 M. On the other hand, the value for the cells grown in the presence of 2 M KCl was less than 10 mM. These data also support the importance of Na^+ ions to enhance the heat resistance of this strain.

The cells grown in the presence of 2 M KCl were used for heating at 53 ^{0}C for 9 min in the presence of various concentrations of NaCl or KCl to examine the importance of Na^+ ions closely related to the cell survival (Figure 4). As a result, the viability in the presence of 0.5 M NaCl was 0.7 log cycle higher than that in 0.5 M KCl. The difference of the viable cell numbers between NaCl and KCl in the same external concentrations became negligible at more

than 1 M, and the viability increased with increasing external salt concentrations. These data indicate that the excess amounts of NaCl or KCl lead to a larger increase in the viable cell numbers than those in hypo-salt stress alone.

Further study is necessary to clarify the role of Na^+ ions in the increased heat resistance.

REFERENCES

1. D. E. Jerkins, J. E. Schultz and A. Matin, J. Bacteriol., 170 (1988) 3910.
2. D. E. Jenkins, S. A. Chaisson and A. Matin, J. Bacteriol., 172 (1990) 2779.
3. F. Jorgensen, 0. Nybroe and S. Knochel, J. Appl. Bacteriol., 77 (1994) 340.
4. Y Lou and A. E. Yousef, Appl. Environ. Microbiol., 63 (1997) 1252.
5. R. Pag~n, S. Conffin and F. J. Sala, Appl. Environ. Microbiol., 63 (1997) 3225.
6. S. Nagata, Arch. Microbiol., 150 (1988) 302.
7. H. Mimura, S. Nagata and T. Matsumoto, Biosci. Biotech. Biochem., 58 (1994) 1873.
8. S. Nagata, Y Ogawa and H. Mimura, J. Gen. Appl. Microbiol., 37 (1991) 403