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Full Length Research Paper

The synthesis and role of hydroxyectoine in halophilic bacterium *Halomonas ventosae* DL7

Daochen Zhu[1,2]*, Chenxiang Wang[3], Shoko Hosoi-Tanabe[4], Weimin Zhang[2] and Shinichi Nagata[4]

[1]School of Environmental Engineering, Jiangsu University, 301 Xuefu Road Zhenjiang 212013, China.
[2]Guangdong Provincial Open Laboratory of Applied Microbiology, Guangdong Provincial, Key Laboratory of Microbial Culture Collection and Application, Guangdong Institute of Microbiology, Guangzhou 510070, China.
[3]Environmental Science and Engineering College, Dalian Maritime University, 1 Linghai Road, Dalian 116026, China. [4]Environmental Biochemistry Division, Research Center for Inland Seas, Kobe University, 5-1-1 Fukae, Higashinada-ku, Kobe 658-0022, Japan.

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Hydroxyectoine and ectoine were accumulated in the moderately halophilic bacterium *Halomonas ventosae* DL7 as dominant compatible solutes in response to the external thermo- and osmo-stresses (30-42°C, 1-3 M NaCl). As the byproduct of ectoine, hydroxyectoine was directly converted from ectoine mediated by the ectoine hydroxylase in DL7, and the hydroxylase gene sequence was obtained by PCR. Further, the activity of ectoine hydroxylase of strain DL7 cells *in vitro* was determined at 30 to 42°C, and the thermo-stress conduce the inhibition of this enzyme activity. In addition, DL7 cells showed similar growth level when grown in presence of same level ectoine or hydroxyectoine (1 mM) in medium with high salinity (2-2.7 M NaCl). However, the presence of hydroxyectoine showed higher growth level than ectoine when DL7 cells were incubated at 42°C. The results showed that the alteration of temperature instead of salinity stress obviously stimulated the expression of ectoine hydroxylase, but inhibited its activity, and hydroxyectoine with ectoine together played an important role as thermo- and osmo-protectants in strain DL7 to cope with high temperature and salinity environment.

Key words: *Halomonas ventosae* DL7, compatible solutes, hydroxyectoine, ectoine hydroxylase, thermo-stress, osmo-stress.

INTRODUCTION

To cope with a variety of high osmotic environments, halophilic and halotolerant bacteria developed a strategy to accumulate a particular class of highly soluble, small molecular organic compounds which named compatible solutes (Kunte, 2006; Roberts, 2005). Among compatible solutes, ectoine and its hydroxylated derivative, hydroxyectoine are one of the most widely produced solutes. And ectoines are increasingly concerned for their superior function on the enzyme, nucleic acid and cell wall protective ability against a variety of stress factors such as heating, freeze-thawing and freeze-drying (Lippert and Galinski, 1992; Louis et al., 1994). The OH group of hydroxyectoine can partly replace the loss of water molecules from hydrate shell to further stabilize the native structure of the biopolymers compare to ectoine (Graf et al., 2008). Hydroxyectoine is proposed as suitable healthy cells protectant during chemotherapy and molecular chaperon for Alzheimer's disease treat-ment, and it can protect skin cells against the aging and function as the PCR enhancer to improve the yield of target production (Furusho et al., 2005; Kanapathipillai et al., 2005). It would have a significantly applicable value in the field of bio-industry, medical therapy, and cosmetic ingredients, etc. Therefore, the synthesis pathway of hydroxyectoine and its physiological role in micro-organisms were interesting to researchers. Studies have suggested that the biosynthesis of hydroxyectoine as

*Corresponding author. E-mail: dczhucn@hotmail.com. Tel/fax: +86-511-88796122.

thermo-protectant has been proved to parallel with high environmental temperature (García-Estepa et al., 2006; Prabhu et al., 2004). The biosynthetic pathway of hydroxyectoine was directly converted via ectoine hydroxylase from ectoine in halophilic or halotolerant bacteria and induced by osmotic stress of high salinity, and ectoine hydroxylase has been found in strains *Streptomyces chrysomallus, Streptomyces coelicolor* and *Chromohalobacter salexigens* (Bursy et al., 2008; García-Estepa et al., 2006; Prabhu et al., 2004) . Further study revealed that the ectoine hydroxylase is a member of the non-heme- containing iron (II) and 2- oxoglutarate-dependent dioxygenase superfamily, and its *in vitro* activity depends on iron (II), molecular oxygen, and 2-oxoglutarate (Bursy et al., 2008; Reuter et al., 2010). The chemical synthesis of hydroxyectoine, however, is still difficult at present. Therefore, the production of hydroxyectoine by microorganisms is currently a simple and meaningful method. To produce hydroxyectoine using halophilic bacteria, the fed-batch and milking method has been developed and the effective factors for the synthesis of hydroxyectoine in the production process were studied. For example, the carbon source xylose can significantly improve the conversion of ectoine to hydroxyectoine in recombinant *E. coli* BL21, which carries ectoine hydroxylase gene (*thpD*) (Nagata et al., 2008b).

Moderately halophilic bacterium, *Halomonas ventosae* DL7, which has been isolated from Dalian saltern, China, displays the ability to synthesize a large amount of ectoine as well as minor hydroxyectoine under hyper-osmolality (Zhu et al., 2007). And the synthesized amount of hydroxyectoine significantly increased in strain DL7 when subjected to heat stimulation (Zhu et al., 2008b). Strain DL7 as an excellent ectoine or hydroxyectoine producer has a potential application value for producing ectoines in factory on a larger scale. But there is still unclear for the effect factor of hydroxyectoine synthesize and activity of ectoine hydroxylase in DL7. Therefore, to elucidate the role and synthesis characteristics of hydroxyectoine in DL7 is an interesting work.

The goal of this study was to investigate the effects of hydroxyectoine and ectoine on the growth of strain DL7 when subjected to high salinity and temperature environments. And the *in vitro* activity of ectoine hydroxylase with the fluctuant salinity and temperature were determined. We also evaluated hydroxyectoine as a vital protectant for strain DL7 cells to defend off the environmental thermo-stress.

MATERIALS AND METHODS

Bacteria and growth media

H. ventosae DL7 was aerobically grown in a nutrient medium (g/l): Bacto peptone (Difco Laboratories, Detroit, MI, USA), 5.0 and Bacto yeast extract (Difco), 3.0. Glucose mineral medium (MM63) was also used in this study: 100 mM KH_2PO_4, 75 mM KOH, 15 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 3.9 µM $FeSO_4$, and 20 mM glucose as carbon source. NaCl was added as described in the text and pH was adjusted to 7.2 with NaOH.

PCR amplification and gene analysis

To isolate the gene encoding ectoine hydroxylase of *H. ventosae* DL7, we designed degeneration of oligonucleotides for PCR. Based on the *ectD* conservation regions from *Virgibacillus salexigens* (AY935522) and *Chromohalobacter salexigens* (AM231629), the universal degenerate primers (5'-GAMAGCMAKGASATCCGKT-3' and 5'- GARCCATGCAKYGTRTT-3') were employed. Genomic DNA of *H. ventosae* DL7 and *C. salexigens* DSM 3043T were isolated using Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. PCR was performed under the following condition: 5 min at 94°C, 30 cycles of 30 s at 94°C plus 1 min at 55°C plus 30 s at 72°C, and finally 5 min at 72°C. Electrophoresis was performed on 1% (w/v) agarose gel in TAE buffer at 100 V for 30 min. The PCR product on the gel was extracted by using a PCR Purification Kit (QIAGEN, Hilden, Germany), cloned into the pGEM-T Vector System (Promega), and sequenced using an ABI PRISMA 377 sequencer. The nucleotide sequence of the PCR product has been deposited in the GSDB, DDBJ, EMBL, and NCBI databases (Accession No. EU732521).

Enzyme assay and the conversion of ectoine to hydroxyectoine

Ectoine hydroxylase activity in the extracts of DL7 cells were assayed as described before (Bursy et al., 2007), 70 µl protein fractions were suspended in 100 µl reaction mixture that contained 10 mM TES buffer, pH 7.5, 10 mM 2-oxoglutarate, 5 mM ectoine, 1 mM $FeSO_4 \cdot 7H_2O$, 1.3 kilo units beef liver catalase (Roche, Germany). The reaction mixture was incubated at 30 or 42°C for 20 min and stopped by adding 100 µl acetonitrile. Supernatant was analyzed by HPLC. One unit of ectoine hydroxylase activity is defined as the conversion of 1 µM of ectoine to 1 µM of hydroxyectoine per min.

Release of hydroxyectoine and ectoine by downshock

H. ventosae DL7 was aerobically grown in the nutrient medium containing 3 M NaCl until stationary phase of growth and harvested. The pellets were washed once by KPi buffer with the same NaCl concentration as that of corresponding nutrient medium, and then resuspended in deionized water or 2% NaCl solution. The cells were incubated for 10-120 min at 30°C. After centrifugation the supernatant fractions were analyzed by HPLC. Release rate of hydroxyectoine and ectoine from the cells were calculated as described previously (Nagata et al., 2008a).

Extraction and determination of compatible solutes

Cells were aerobically grown in the nutrient medium containing 1-3 M NaCl at 30 or 42°C. Cells were harvested by centrifuging (10,000×*g*, 4°C, 10 min), and then the pellets were washed once with 50 mM potassium phosphate (KPi) buffer (pH 7.2) containing the same concentration of NaCl corresponding to growth medium. Extraction of intracellular compatible solutes was carried out as described previously (Zhu et al., 2008a). Fraction of 80% (v/v) ethanol extraction as well as supernatant after osmotic downshock analyzed and quantified by HPLC system (L-5020, Hitachi, Tokyo, Japan) with a Bio-Sil C_8 90-5S reversed phase column (Bio-Rad

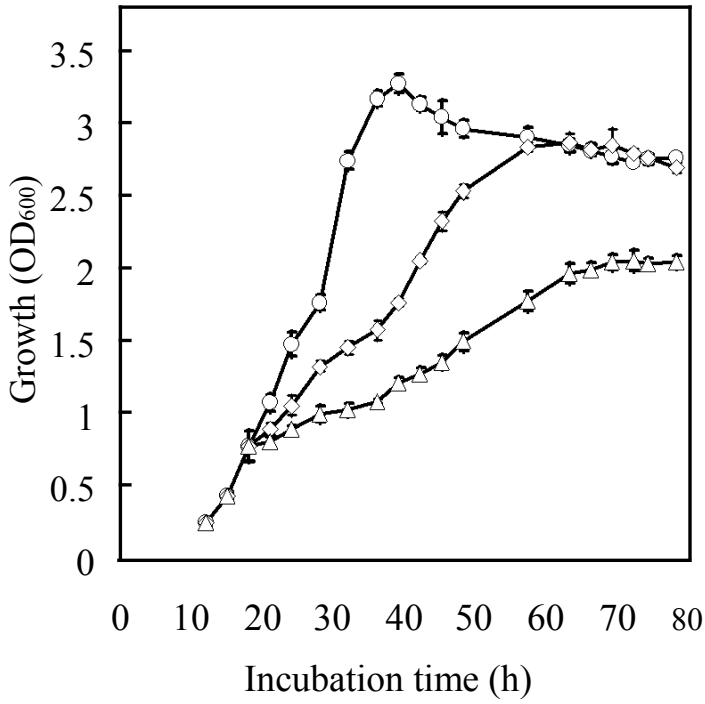

Figure 1. The growth of strain DL7 cells at different incubation temperatures. Strain DL7 was pre-incubated in a nutrient medium containing 3 M NaCl for 18 h at 30°C and then separately incubated at 30°C (cycles), 37°C (diamonds), or 42°C (triangles). Values are the averages ± SD from three independent experiments.

Laboratories, Hercules, CA, USA) as described previously (Nagata et al., 2006; Zhu et al., 2010). Five microliters of samples were eluted with 50 mM KPi buffer at pH 6.0 with a flow rate of 1 ml/min at 35°C and detected by UV absorbance at 210 nm. Concentrations of ectoine and hydroxyectoine were expressed as milligram per gram CDW.

RESULTS AND DISCUSSION

Effect of temperature on the growth of *H. ventosae* DL7

The influence of temperature on the cells growth of strain DL7 was examined using the nutrient medium containing 3 M NaCl at 30-42°C. Results showed that the growth of cells was severely inhibited with the increasing of incubation temperature, and reached stationary phase at 36 h of incubation at 30°C, which prolonged to 57 and 66 h with the incubation temperature of 37 and 42°C, respectively. Optical density (OD_{600}) was significantly reduced from 3.3 at 30°C to 2.8 at 37°C and 2.1 at 42°C, respectively (Figure 1). In our previous report, the amount of hydroxyectoine synthesized was very low comparing with ectoine when cells grown at 30°C and the concentration of hydroxyectoine in the cells remarkably increased with the increase of temperature and reached the maximum of 36.6 mg/g CDW at 42°C, (Zhu et al., 2008a) . It suggests that the synthesis of hydroxyectoine was a strategy of strain DL7 cells to defend off the environmental thermo-stress.

Ectoine hydroxylase in *H. ventosae* DL7

Ectoine hydroxylase catalyzing the conversion of ectoine into hydroxyectoine had been proved in several halophilic bacteria (Bursy et al., 2007; García-Estepa et al., 2006; Nagata et al., 2008a). The PCR strategy was carrying out to clarify whether this hydroxylase was present in strain DL7, and we assumed that a homologous hydroxylase gene to those of *C. salexigens* and *V. salexigens* was existed in this strain to design a pair of degenerated primers for PCR amplification, and a 480-bp gene fragment was obtained and subsequently sequenced (EU732521), the amino acid sequence deduced from the DNA sequence of this fragment showed 54.8% identity with the ectoine hydroxylases from *C. salexigens* and *V. salexigens* (Figure 2). These data confirmed the presence of hydroxylase in *H. ventosae* DL7, by which the conversion of ectoine should be the important pathway for the synthesis of hydroxyectoine. To prove this opinion, the activity of ectoine hydroxylase of strain DL7 was determined.

To investigate the activity of ectoine hydroxylase under

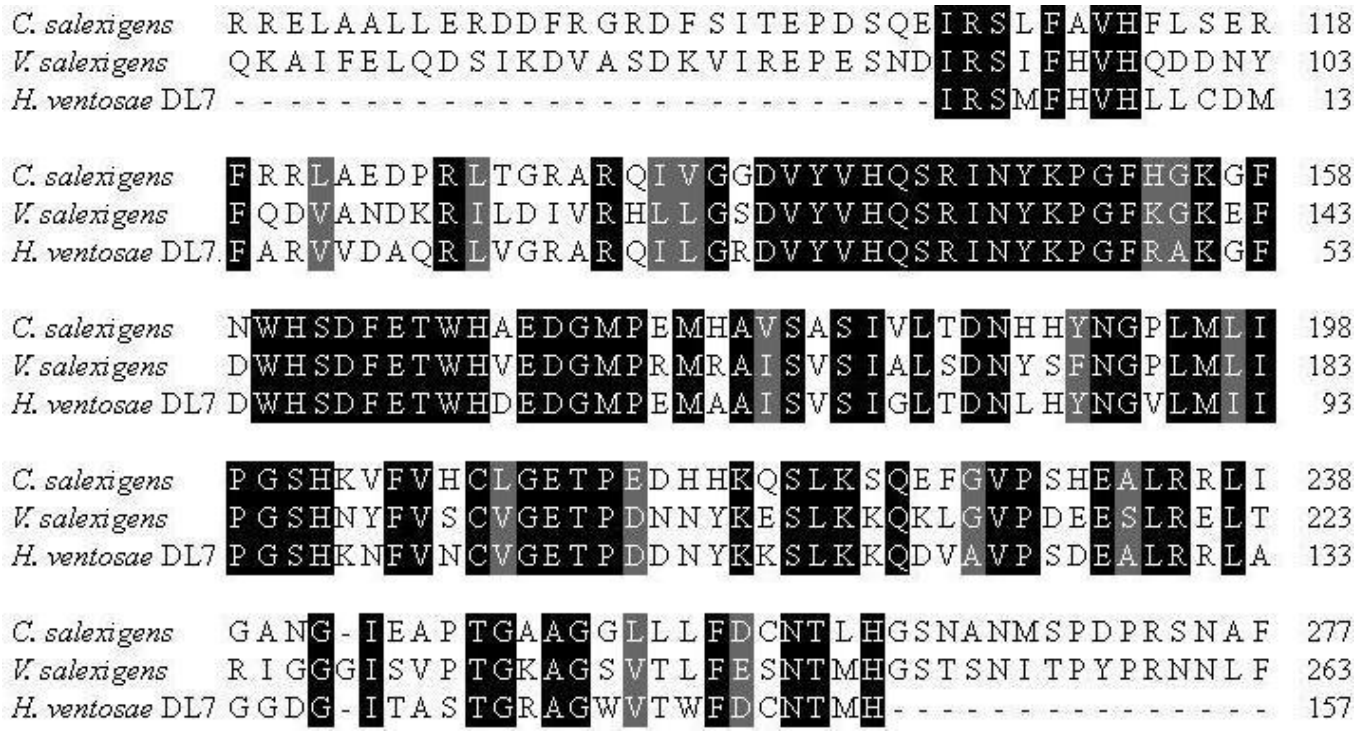

Figure 2. Alignment of the sequences of reading frame *ectD* (EU732521) of *H. ventosae* DL7 with the sequences of the ectoine hydroxylases of *V. salexigens* (AY935522) and *C. salexigens* (AM231629). Identical positions and conservative fragments are shown against a dark and gray background, respectively.

Table 1. Ectoine hydroxylase activity (cell free extract) from DL7*

Reaction temperature (°C)	NaCl (M)	Activity (Unit)	Protein (mg)**
30	0	43.7	916.5
42	0	29.5	920.3
30	1	42.1	918.4

*Cell free extract was prepared from 20 g cell pellets grown in 3 M NaCl at 30°C.
** Protein (mg): the protein content of 70 µl protein fractions extract from DL7 cells.

the heat or salt stress, we assayed the cell-free extracts from strain DL7 cells *in vitro* at 30 or 42°C, without or with 1 M NaCl. Results showed that the activity of hydroxylase was reduced from 43.7 to 29.5 U, when the temperature was increased from 30 to 42°C (Table 1). However, the activity was stable with the fluctuation of NaCl concentration (0-1 M) at 30°C. It revealed that the conversion of ectoine to hydroxyectoine was inhibited by the higher incubation temperature. On the other hand, the activity of hydroxylase had no obviously fluctuation with or without salt. In addition, similar result has also been found in our previous research, i.e., the *in vivo* activity of hydroxylase was inhibited by high temperature, and hydroxyectoine showed similar conversion rate in the presence of different NaCl concentrations (0.5-1 M) (Zhu et al., 2008a). Therefore, these *in vivo* and *in vitro* data supported our suggestion that the hydroxylase activity was sensitive to temperature but was not disturbed by salinity.

Based on these data, we could deduce that the increased amount of hydroxyectoine synthesized following the enhancing of thermo-stress might be that the heat stimulated the cells to produce more amount of ectoine hydroxylase instead of induction of the increasing of the enzyme activity. Further, to detect whether there is a high temperature intermediated operon to trigger the synthesis of hydroxylase in DL7 is an interesting work in future.

Release of hydroxyectoine and ectoine by osmotic downshock

To investigate the role of hydroxyectoine and ectoine for

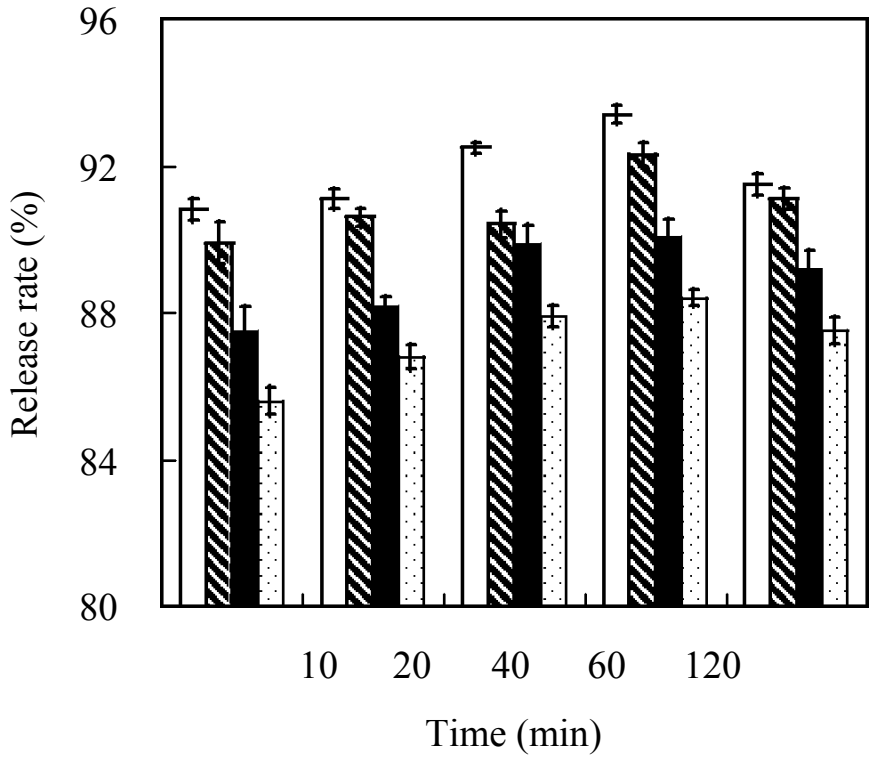

Figure 3. Releases of hydroxyectoine and ectoine by *H*. *ventosae* DL7 subjected to osmotic downshock. Downshock procedures were handled in deionized water to release ectoine (white bars) and hydroxyectoine (hatched bars), or in 2% NaCl to release ectoine (black bars) and hydroxyectoine (macula bars) during 10-120 min incubation. Values are the averages ± SD from three independent experiments.

strain DL7 in response to the salt or heat stress, it is necessary to use the "clearing cells", which have no intracellularly compatible solutes. Therefore, the release of compatible solutes from cells and at the same time to keep alive of cells was concerned for preparation of the compatible solutes free cells. When halophilic bacteria are confronted with a rapid osmotic downshock, most of the small components in the cytoplasm were released into the environments (Frings et al., 1995; Nagata et al., 1996; 1998; Sauer et al., 1998). In this consideration, we tried to examine the release rates of hydroxyectoine and ectoine following a shift in medium osmolarity, that is, cells were suspended in 2% (0.34 M) NaCl or deionized water from 3 M NaCl medium. When the strain was downshocked in deionized water, both intracellular ectoine and hydroxyectoine were rapidly released to environment. During less than 10 min of downshock treatment, 90.8 and 89.9% of release rates were achieved for ectoine and hydroxyectoine, respectively (Figure 3). However, since strain DL7 is a moderately halophilic bacterium and a minimum concentration of Na^+ is essential for growth, the cells would burst by osmotic downshock from high salinity environment to deionized water and could not be recovered to use for further study. To remain the growth of cells after downshock, we tried to examine 2% NaCl solution instead of deionized water. As a consequence, the cells were kept intact after re-incubation in 2% NaCl solution for 1 h (data not shown). Release rates of ectoine and hydroxyectoine reached maximum at 60 min of treatment, and resulted in 90.1 and 88.4%, respectively. The survival rates of cells treated by downshock (3 M NaCl to 2% NaCl and 2 M NaCl to 2% NaCl) were determined as 95.3 and 97.6%, respectively. It was suggested that the numbers of damaged cells were negligible for the present condition, and 2% NaCl solution was chosen to prepare the solutes free cells for the following study.

Thermo- and osmo-protection of compatible solutes

To gain insight into the role of hydroxyectoine and ectoine in strain DL7 when cells were subjected to thermo-stress, we tried to examine the behavior of strain DL7 under high temperature and salinity in the presence or absence of compatible solutes. Cells were osmotic downshock treated by 2% NaCl solution to avoid the interference of intracellular solutes, and pellets were then resuspended in MM63 medium containing 1-2.7 M NaCl without or with 1 mM each of ectoine or hydroxyectoine at 30 or 42°C. We obviously observed that the presences of hydroxyectoine and ectoine significantly promoted the

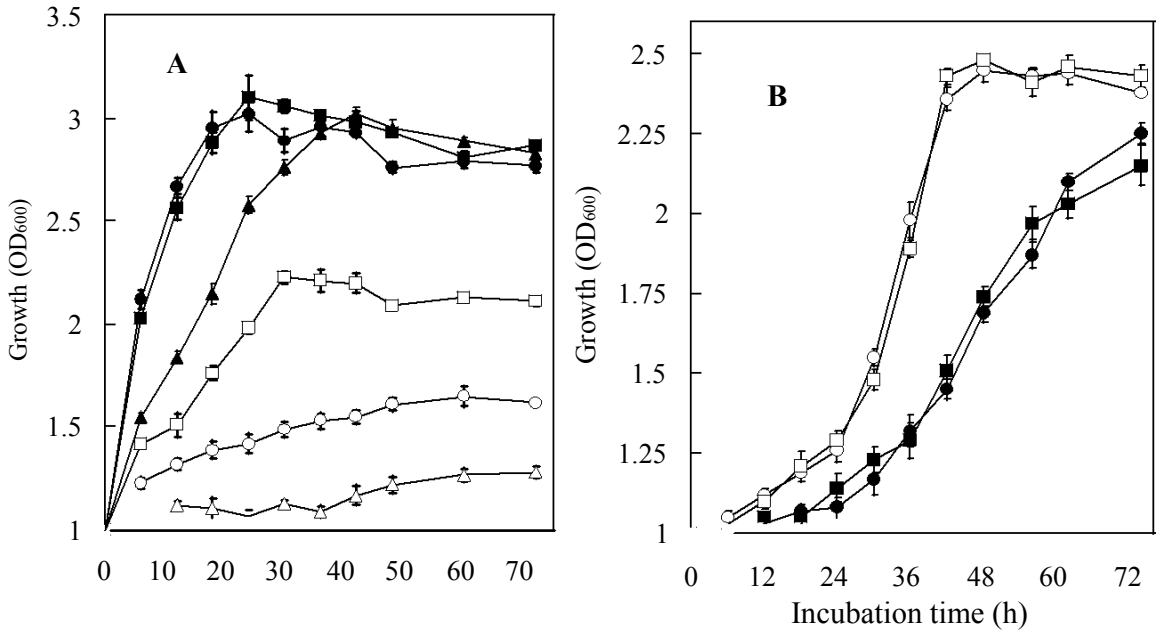

Figure 4. Supplement effects of hydroxyectoine and ectoine on the growth of *H. ventosae* DL7 under high temperatures or high salinities. A. Cells were grown in MM63 medium containing 1 M NaCl at 42°C (open symbols) or 30°C (closed symbols) without (triangles), with 1 mM ectoine (circles) or 1 mM hydroxyectoine (squares). B. Cells were grown in MM63 medium containing 2 M (open symbols) or 2.7 M NaCl (closed symbols) at 30°C with 1 mM ectoine (circles) and 1 mM hydroxyectoine (squares). Values are the averages ± SD from three independent experiments.

Table 2. The accumulation of ectoine and hydroxyectoine by downshocked cells of *H. ventosae* DL7*

Compatible solutes	NaCl (M)	Concentrations (μM /g CDW)			
		Incubation time (h)			
		1	3	5	10
Ectoine	1	241±14.8	245±10.6	253±3.5	258±7.8
	2	327±10.6	368±12.7	395±5.6	411±4.2
Hydroxyectoine	1	234±12.7	244±2.8	241±7.1	244±10.6
	2	304±9.1	355±4.2	369±5.7	390±4.9

*Downshock treatment was performed according to the method described in Materials and Methods. The downshocked cells were incubated in MM63 medium at 42°C with 1 mM each ectoine or hydroxyectoine. The values are the means ± SD from three independent experiments.

growth of cells at 42°C from the growth curve, and the optical density of hydroxyectoine showed 1.5 times higher than ectoine after 24 h of incubation (Figure 4A). However, there was no obviously difference between the growth of DL7 in presence of hydroxyectoine and ectoine at 30°C. In addition, the downshocked compatible solutes free DL7 cells to accumulate hydroxyectoine and ectoine reached almost same level with 1 M or 2 M NaCl concentrations (Table 2). It suggests that the presence of hydroxyectoine result in the growth of cells higher than ectoine were not induced by different concentrations of hydroxyectoine and ectoine accumulated in cells. The above data elucidated that hydroxyectoine protected DL7 cells more effectively than ectoine at 42°C.

To investigate the protection functions of hydroxyectoine and ectoine on *H. ventosae* DL7 to defend off high salinity, the compatible solutes free cells were incubated in MM63 medium containing 2 or 2.7 M NaCl in presence of 1 mM each of hydroxyectoine or ectoine and the growth was detected. Results verified that the supplementation of ectoine or hydroxyectoine conferred a similar cell growth both in presence of 2 and 2.7 M NaCl (Figure 4B), that is, ectoine and hydroxyectoine have similar protection effect for cells grown under high salinity environment.

It was understood that the accumulation of compatible

solutes was osmo-adapted by environmental stresses for halophilic bacteria. In this study, we described the synthesis of hydroxyectoine and activity of hydroxylase in strain DL7, showed that thermo-stress stimulated the yield of hydroxyectoine but inhibited the activity of hydroxylase. We also noticed the hydroxylase express system might be induced by thermo-stress instead of osmotic-stress. The study for charactering the hydroxylase in structure and exploring its molecular mechanism are interesting work in the near future.

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