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# Isolation and characterisation of moderately halophilic bacterium *Halomonas ventosae* DL7 synthesizing ectoine as compatible solute

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**Abstract -** A moderately halophilic Gram-negative bacterium, strain DL7, was isolated from saltern sediment of Dalian, China. Phylogenetic analysis based on 16S rRNA gene sequences indicated that this isolate belongs to the genus *Halomonas*. Based on the taxonomic and DNA sequence in addition to high DNA-DNA homologies, we concluded that this strain was similar with the type strain of *Halomonas ventosae*. Ectoine, one of the representative compatible solutes, was mainly detected in the cells as a result of $^{1}$H- and $^{13}$C-NMR measurements when grown in the presence of wide concentration ranges of NaCl. The results showed that higher amount of ectoine was synthesized in a shorter incubation time compared with those of other strains reported earlier.

**Key words:** compatible solute, ectoine, *Halomonas ventosae*, 16S rRNA sequence.

## INTRODUCTION

Halophilic or halotolerant bacteria inhabiting saline environments can adapt their metabolic systems to wide variations of high osmotic pressure. A common strategy for osmoadaptation in these microorganisms involves the accumulation of organic molecules by *de novo* synthesis or through transport into the cell cytoplasm from the surrounding environment. Such osmolytes, termed as compatible solutes, do not interfere with the central metabolism even at high concentrations in the cytoplasm. In recent years, compatible solutes such as amino acids and their derivatives, quaternary amines, sugars, and small peptides are well known to protect proteins from thermal stress, proteolysis, freezing, desiccation or attack of oxygen radicals (Poolman and Glaasker, 1998).

Among compatible solutes studied, ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) showed a superior ability in stabilizing enzymes, nucleic acids, membranes and whole cells; utilisation in curing some diseases such as cell death by the polyglutamine-induced toxicity or as potential inhibitors associated with neurodegenerative diseases have been well studied (Furusho *et al*., 2005; Kanapathipillai *et al*., 2005). Thus, ectoine has a foreseeable wide range of applications in the biochemical, medical and cosmetic sectors (Lippert and Galinski, 1992), but at present, the important constrain is the difficulty in synthesizing it by chemical methods (Margesin and Schinner, 2001).

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In the present work, we report the isolation and characterisation of novel strain *Halomonas ventosae* DL7, which was obtained from saltern sediments of Dalian China. Ectoine is the main compatible solute produced by this isolate when grown in the presence of 1-4 M NaCl. Further we aimed to investigate the characteristics of ectoine synthesis in this strain in comparison with those of some earlier reported strains. To our knowledge this is the first report which described the characteristics of ectoine synthesis in the species *Halomonas ventosae* in more detail.

## MATERIALS AND METHODS

**Bacteria and culture conditions.** For isolation of microorganisms, sediment samples were incubated at 30 °C in a nutrient medium that consists (g/l) of 5.0 Bactopeptone (Difco Laboratories, MI, USA); 3.0 Bacto yeast extract (Difco); 0-4 M NaCl. pH of the medium was adjusted to 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 with NaOH. Then the incubated sediments were inoculated on agar medium with same composition as the nutrient medium at 30 °C for 72 h, from which we picked up single colonies and subcultured repeatedly to ensure the purity. The reference strains used in this study were the type strains belonging to the genus *Halomonas*: *H. ventosae* DSM 15911$^{T}$, *H. elongata* ATCC 33173$^{T}$, *H. halodenitrificans* DSM 735$^{T}$, *H. marina* DSM 4741$^{T}$, *H. pacifica* DSM 4742$^{T}$, *H. salina* DSM 5928$^{T}$ and *H. alimentaria* DSM 15356$^{T}$.

**Morphological and physiological characterisation.** Gram staining was determined for the cells grown for 24 h

in the liquid nutrient medium. Motility was determined using soft agar nutrient medium (0.5 %, w/v). Poly-β-hydroxybutyrate (PHB) stain techniques were performed as described by Jenkins (1992).

Oxidase reaction was performed with 1% tetramethyl-*p*-phenylenediamine. Catalase activity was determined by bubble formation in 3% hydrogen peroxide solution.

Growth at different salt concentrations were determined by spreading 20 µl of each inoculum onto the surface of solid nutrient medium with NaCl concentration of 0, 0.5, 1, 2, 3, 20, 23, and 25% (w/v).

The growth at different pH values was determined in solid nutrient medium, in which the pH was adjusted to 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 with HCl or NaOH.

Nutritional tests were made using a modified medium (%, w/v): 7.5 NaCl, 0.2 KCl, 0.02 $MgSO_4 \cdot 7H_2O$, 0.1 $KNO_3$, 0.1 $(NH_4)_2HPO_4$, 0.05 $KH_2PO_4$. Each substrate (D-trehalose, D-fructose, lactose, starch, L-serine, glycerol) sterilised by filtration was added (0.1%, w/v) to the modified medium. When amino acids were used as substrates, the medium did not contain $KNO_3$ nor $(NH_4)_2HPO_4$.

**DNA base composition and DNA-DNA hybridization.** DNA was extracted from colonies and purified using the method of Marmur (1961). The purity was assessed from the $A_{260}/A_{280}$ and $A_{230}/A_{260}$ ratios. The G + C content (% mol) of DNA was determined from the midpoint of thermal denaturation profile $T_m$ (Marmur and Doty, 1962) obtained with a model UV-VIS DU600 spectrophotometer (Beckman, USA) at 260 nm. The $T_m$ of reference DNA from *Escherichia coli* NCTC 9001 was 74.6 °C in 0.1 x SSC (Owen and Picher, 1985).

DNA-DNA hybridization experiment was performed with *Halomonas ventosae* DSM 15911$^T$. The hybridization was carried out with the renaturation rate method described by De Ley *et al.* (1970), with a modification described by Escara and Hutton (1980).

**Sequencing of 16S rRNA genes and phylogenetic analysis.** 16S rRNA was amplified by PCR using the two universal primers 27F (5'-AGAGTTTGATCC/ATGGCTCAG-3') and 1541R (5'-AAGGAGGTGAT- CCAGCC-3') (Woese *et al.,* 1983). PCR reactions were carried out under conditions described previously (Springer *et al.*, 1993). The PCR product was purified with a PCR purification kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's protocol. The 16S rRNA was sequenced using the ABI PRISMA 377 sequencer.

The alignment of sequences was done with Clustal W software (Thompson *et al.*, 1994). Sequences are all of the family *Halomonadaceae* retrieved from the NCBI database, and thus only type strains of species were taken into account. The sequence analyses were performed with software MEGA3 (Kumar *et al.*, 2004). Phylogenetic trees were constructed by the neighbour-joining method (Saitou and Nei, 1987) and maximum-likelihood method (Felsenstein, 1981).

Nucleotide sequence had been deposited in the GenBank international nucleotide sequence database. The accession numbers of the 16S rRNA nucleotide sequence of strain DL7 is DQ316071.

**Extraction and determination of intracellular solutes by NMR spectroscopy.** Extraction of intracellular compatible solutes was carried out as described previously with a slight modification (Nagata *et al.*, 1996; Wang *et al.*, 2006). Strain DL7 was grown in 50 ml of the medium containing 3 M NaCl at 30 °C until late exponential phase of growth. Cells were harvested by centrifugation (10000 *x g*, 4 °C, 10 min). The pellets were washed with 50 mM potassium phosphate ($KP_i$) buffer (pH 7.2) containing isotonic NaCl concentrations with the growth medium. Pellets were resuspended in the same volume of 80% (v/v) ethanol and allowed to stand for 12 h to extract the intracellular solutes and centrifuged. Supernatant fraction was dried and resuspended in 0.5 ml $D_2O$. NMR spectra were recorded on a JEOL JNM-AL300 (JEOL Ltd., Tokyo, Japan) with a probe temperature of 20-22 °C as described elsewhere (Nagata *et al.*, 1998).

**HPLC analysis of ectoine synthesized by strain DL7 and reference strains.** Samples of 5 µl of fraction extracted by 80% (v/v) ethanol containing compatible solutes were analysed by HPLC (Hitachi L-5020, Japan) system on an Octyl-80Ts column (Tosoh, Japan), with 50 mM $KP_i$ buffer (pH 6.0) as eluent, at a flow rate of 1 ml/min at 25 °C, ectoine was monitored at 210 nm and quantified by comparison with an standard ectoine sample. Intracellular ectoine concentration was calculated as gram per litre culture.

## RESULTS AND DISCUSSION

Totally, seven different strains which possess the capability to grow in high salinity and accumulate compatible solutes were isolated. Among them one strain exhibited to accumulate higher concentration of ectoine as compatible solutes in comparison with those of other strains when they were grown under high salinity conditions (data not shown). This strain, designated as DL7, was selected for further study.

### Morphological and physiological characterisation

The strain DL7 was an aerobic and Gram-negative with short rod shape. In addition it showed positive in motility test using soft agar medium. Colony formed was cream-yellow in colour, smooth and circular/slightly irregular on nutrient agar medium after 2 days of incubation at 30 °C. This strain was found to be tolerant and can grow in nutrient medium containing wide range of NaCl concentration, 1-20% (w/v). The strain did not grow in the absence of NaCl and in the presence of > 25% (w/v) NaCl. The growth was permitted for pH between 6.5 and 10.0. The strain DL7 was positive for oxidase and catalase activities. Among different substrates examined as single carbon source growth was not observed when starch or lactose was used. Physiological properties of strain DL7 and other related species of genus *Halomonas* are shown in Table 1. From its morphological and physiological properties, strain DL7 appears to be the most similar to *H. ventosae* and *H. alimentaria* (Table 1).

### Phylogenetic analysis

In the 16S rRNA sequencing experiment, we obtained almost complete gene sequence of strain DL7. Comparative analysis was subjected to the similarity searches with the database (Genbank) to infer a possible phylogenetic classi-

TABLE 1 - Differential phenotypic characteristics of strain DL7 and other related type strains of *Halomona*s species

| Characteristics | Strain DL7 | *H. ventosae* DSM 15911$^T$ | *H. alimentaria* DSM 15356$^T$ | *H. pacifica* DSM 4742$^T$ | *H. salina* DSM 5928$^T$ | *H. halodenitrificans* DSM 735$^T$ |
|---|---|---|---|---|---|---|
| Colony pigmentation | Cream yellow | Cream yellow | Cream yellow | Cream | Cream yellow | Cream |
| Cell morphology | Short rod | Short rod | Short rod | Rod | Short rod | Short rod |
| Motility | + | + | –[b] | +[c] | –[d] | –[b] |
| PHB stain | + | + | ND[f] | +[c] | +[d] | +[b] |
| NaCl range (%, w/v) | 1-20 | 0.5-20 | 0-20 | 0.5-20 | 2-20 | 3-20 |
| pH range | 6.5-10.0 | 6.0-10.5 | 6.5-10.0 | 6.0-9.5 | 5.5-10.0 | 6.0-10.5 |
| Catalase | + | + | +[e] | +[c] | +[d] | +[b] |
| Oxidase | + | + | +[e] | +[c] | +[d] | +[b] |
| Growth on[a] | | | | | | |
| D-Trehalose | + | + | + | + | + | + |
| D-Fructose | + | + | + | + | + | + |
| Lactose | – | – | – | – | – | – |
| Starch | – | – | – | – | – | – |
| L-Serine | + | + | + | + | + | + |
| Glycerol | + | + | + | + | – | + |
| DNA G+C content | 67.8 | 72.6[e] | 63[b] | 67-68[c] | 60.7-64.2[d] | 64-66[c] |

[a] See nutritional tests in Materials and Methods section; [b] (Yoon *et al*., 2002); [c] (Mata *et al*., 2002); [d] (Lim *et al*., 2004); [e] (Martínez-Cánovas *et al*., 2004); [f] ND, not determined.

fication, which revealed that the strain DL7 belonged to the genus *Halomonas*. Phylogenetic tree was constructed based on the neighbour-joining method, which showed the position of strain DL7 was placed within the genus *Halomonas* and formed a cluster with *H. ventosae* (Fig. 1). The level of 16S rRNA gene sequence similarity of the strain DL7 with *H. ventosae* DSM 15911$^T$, *H. alimentaria* JCM 10888$^T$, *H. pacifica* DSM 4742$^T$, *H. halodenitrificans* ATCC 13511$^T$, *H. salina* DSM 5928$^T$, and *H. halophilia* DSM4770$^T$ were 99.6, 98.5, 97.4, 96.9, 96.5 and 93.0%, respectively. The G + C content of DNA from the strain DL7 was 67.8%. DNA-DNA hybridization was done for further discrimination of the strain DL7 from *H. ventosae* DSM 15911$^T$. The highest similarity was found between the strain DL7 and *H. ventosae* DSM 15911$^T$ with a re-association value of 89.7%.

We concluded that the strain DL7 belongs to the species *H. ventosa*e on the bases of the closest phylogenetic affinity to *H. ventosae* and the similarity between the strain DL7 and *H. ventosae* higher than the consensus value of 70% for delineating a species (Stackebrandt and Goebel, 1994).

**Identification of compatible solutes in strain DL7**

Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique for the determination of molecular structure of biomolecules. Until now $^1$H- and $^{13}$C-NMR spectroscopies have been successfully applied for the analyses of compatible solutes (Nagata *et al*., 1996; Calderon *et al*., 2004). We evaluated the spectrum of compatible solutes synthesized *de novo* in the strain DL7 cells when grown in the presence of 1-4 M NaCl. Representative $^1$H-NMR spectrum of cell extracts from this bacterium is shown in Fig. 2A. The signals corresponding to the hydrogen atoms of solutes were found to be the same as those of standard ectoine, which was also verified by the measurement of $^{13}$C-NMR (Fig. 2B). HPLC analyses showed that the extract of strain DL7 had the same retention time with standard ectoine (data not shown). Based on NMR spectra and HPLC analyses, ectoine accumulated in cells were highly pure even in the form of crude extract of cells. Thus, it is proved that ectoine is the main solute accumulated in strain DL7 when grown in the present conditions.

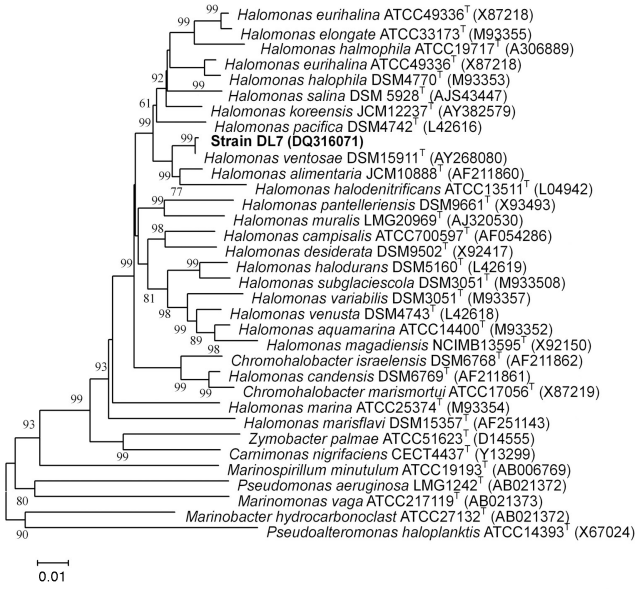


FIG. 1 - Phylogenetic tree based on 16S rRNA gene sequence similarity data for the isolate DL7. The tree was constructed using the neighbour joining method. Present analysis showed the position of strain DL7 among the genus *Halomonas* and some species of other genera of *Halomonadaceae* family. Numbers at branching nodes are bootstrap values (percentages of 1000 replications); only values greater than 50% are indicated. Scale bar represents 0.01 substitutions per nucleotide position.

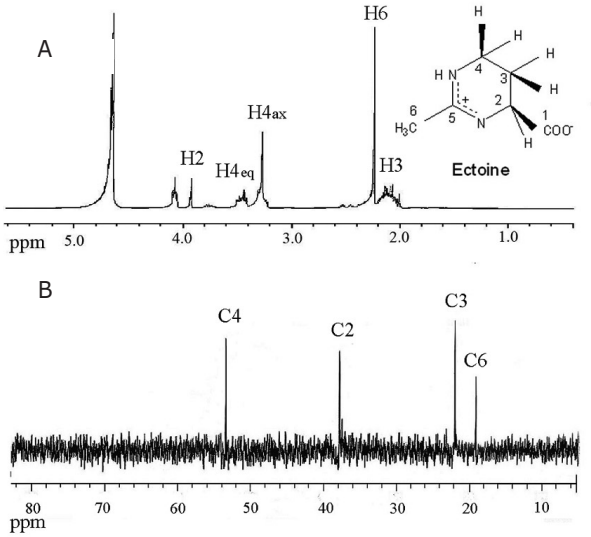


FIG. 2 - $^{1}$H-NMR (A) and $^{13}$C-NMR (B) spectra of ethanol extracts of strain DL7. Ethanol extracts were prepared and analysed as described in Materials and Methods. Strain DL7 was grown in a nutrient medium containing 3 M NaCl at 30 °C. Peaks derived from ectoine are labeled with H or C plus the number of the carbon atom to which the hydrogen atoms are attached. ax: axial; eq: equatorial.

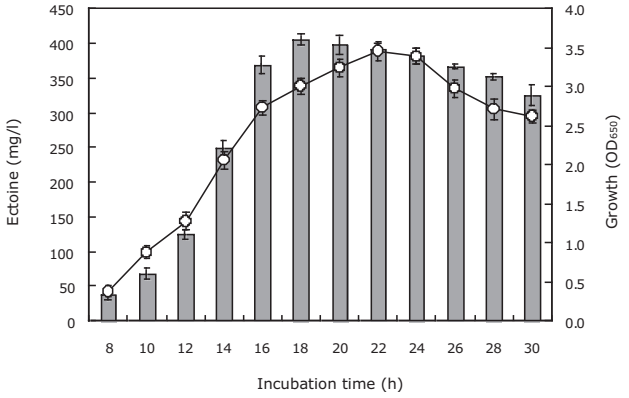


FIG. 3 - Correlation between growth and synthesis of ectoine by strain DL7. Cells were grown aerobically in a nutrient medium containing 2 M NaCl (open circles) at 30 °C. Ectoine concentration (shaded bars) was determined by HPLC at different growth period. Details are given in Materials and Methods. Cell growth was determined by measuring the $OD_{650}$. The values are the means ± SD from three independent experiments.

**Changes of ectoine synthesis during growth of strain DL7**

To determine the concentration of ectoine synthesized at different growth periods, cells were incubated in the nutrient medium containing 2 M NaCl at 30 °C (Fig. 3). Lower level of ectoine synthesis was observed during 12 h of incubation. But it increased abruptly from 14 to 18 h of incubations, following the dramatic increase of cell growth and reaching a maximum after 18 h, where ectoine concentration resulted in 404.8 mg/l. Then, ectoine concentration gradually decreased from 18 to 22 h of incubations, although the cell growth reached the maximum ($OD_{650}$ = 3.5). These results show that the synthesis of ectoine in strain DL7 attained the maximum during the stationary phase of growth, which promoted us to perform all measurements in cultures grown in this period.

**Effect of salt on the growth of and accumulation of compatible solutes in strain DL7**

The influence of NaCl concentration on cell growth as well as ectoine synthesis was examined using the nutrient medium containing 1-4 M NaCl concentrations. When cells were grown in the presence of 1-2 M NaCl, we observed the concentration of ectoine significantly increased with the increase of salinity (Fig. 4). However, the incubation time to reach the maximal concentration was shortened; from 22 to 18 h. Concentration of ectoine continued to increase with an increase of salinity, 2.5-3 M, but the incubation time was prolonged. The highest concentration of ectoine in the cells were observed for the cells incubated in the presence of 3 M NaCl for 44 h, where the incubation time was 2.4 fold longer than that of 2 M NaCl. The synthetic rate of ectoine was reduced when the NaCl concentration increased to more than 3.5 M. When the cells were grown in the presence of 4 M NaCl, in which the ectoine concentration only resulted in 146.5 mg/l after 144 h of incubation. These results show that the growth of strain DL7 is optimum in the presence of 2 M NaCl and the ectoine concentration reached maximal in the shortest incubation time compared with other NaCl concentrations. Taking these data into account, we conclude that the amount of ectoine synthesized by strain DL7 is up-regulated by NaCl in the concentration 1-3 M.

To determine whether the ectoine synthesis was dependent on the adaptation reaction against sodium salt-stress or osmotic adaptation reaction, the supplementation effect of KCl and saccharide to induce the osmotic pressure were examined by checking the cell growth. In the presence of 1 M KCl or 1 M glucose and 1 M sucrose, however, the cells did not show the growth. In addition, the cells showed no growth in the medium with $NaCO_3$ or $NaNO_3$ instead of NaCl (data not shown). This indicated that both $Na^+$ and $Cl^-$ are essential for the growth of strain DL7. In the current study, minimal amount of $Na^+$ was essential for bacterial growth. There are four different functions of $Na^+$ for bacterial cells such as sodium ion-solutes co-transport systems, sodium-coupled energy conservation and energy transduction, pH homeostasis and activation of specific enzymes (Peter, 1987). However, the role of $Cl^-$ ion in the growth of strain DL7 is still not clear.

**Comparison of ectoine synthesis with some species of genus *Halomonas***

To gain insights into characteristics of ectoine synthesis in the strain DL7, *H. ventosae* and *H. elongata*, we tried to detect the amounts of ectoine synthesized following the growth curve in the presence of 1-3 M NaCl. The maximum of ectoine concentrations in strain DL7 and *H. ventosae* were higher than that of *H. elongata* in the presence of 1 M NaCl (Fig. 5). Interestingly, ectoine concentration in strain DL7 suddenly increased from 14 to 18 h of incubations in the presence of 2 M NaCl, but *H. ventosae* and *H. elongata* reached the maximum until 22-24 h. Although three

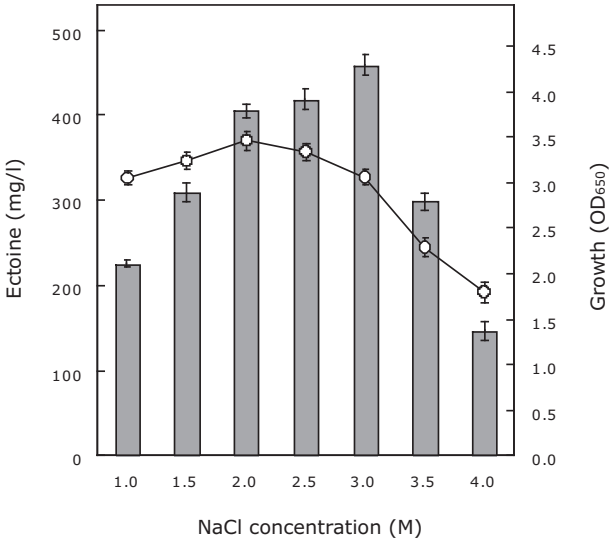


FIG. 4 - Effect of NaCl concentrations on ectoine synthesis and growth of strain DL7. Analysis of ectoine concentration (bars) was carried out according to the method described in the legend of Fig. 3. The strain DL7 was grown in nutrient medium containing 1-4 M NaCl at 30 °C. Cell growth was determined by measuring the $OD_{650}$ (open circles). The values are the means ± SD from three independent experiments.

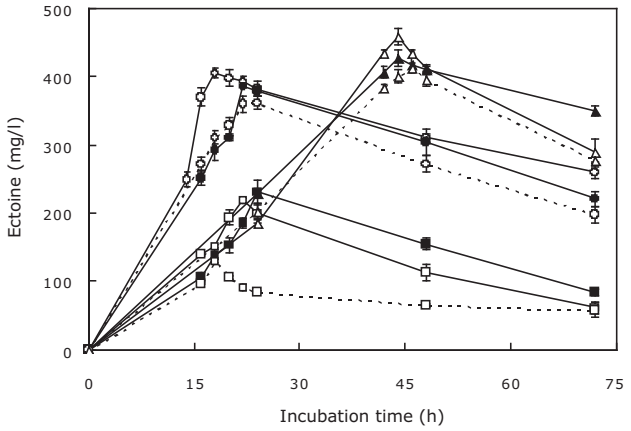


FIG. 5 - Comparison of ectoine synthesis by strain DL7, *Halomonas ventosae* and *Halomonas elongata*. Cells of strain DL7 (open symbols), *H. ventosae* (closed symbols) and *H. elongata* (dotted lines) were grown aerobically in nutrient medium containing 1 M (squares), 2 M (circles), and 3 M NaCl (triangles) at 30 °C. Ectoine concentrations in cells were determined by HPLC after extraction with 80% ethanol. Details are given in Materials and Methods. The values are the averages ± SD from three independent experiments.

kinds of strains showed the similar characteristics of ectoine synthesis in the presence of 3 M NaCl, the maximal ectoine concentration in strain DL7 was higher than the other two. Correspondingly, ectoine synthesis in some of the species belonging to the genus *Halomonas* such as *H. salina*, *H. pacifica*, *H. marina*, *H. alimentaria*, and *H. halodenitrificans* was also been examined (Table 2). In comparison with those strains, strain DL7 showed significantly higher level of ectoine synthesis in the presence of 1-3 M NaCl. We found that the strain DL7 could grow in a broad salinity range 1-20% NaCl. Thus, the cytoplasmic ectoine concentration was rapidly and finely adjusted in response to variable concentrations of NaCl. In fact, the cytoplasmic amount of ectoine significantly increased in response to the increase in the medium salinity, up to maximum in 3 M NaCl. Moreover, when the strain DL7 cells were suspended in deionised water, > 90% ectoine in the cells was rapidly released from the cell cytoplasm (data not shown). These data suggests that ectoine is one of the main compatible solutes in the genus *Halomonas*, and especially the strain DL7 synthesized the highest concentration of ectoine among seven strains examined. We concluded that the strain DL7 is a good source for production of ectoine. There were some significant properties of the strain DL7 which fulfil the requirements of ectoine production such as the large amount of ectoine synthesis in a shorter incubation time, rapid release of the accumulated ectoine and ability to grow well in both low and high-salt media. Thus, our data suggested that the strain DL7 has an excellent potential as the ectoine producer. In addition, we found that the response to elevated temperatures of strain DL7 involves the synthesis of hydroxyectoine (data not shown). Hydroxyectoine has gained more attention compared to other compatible solutes due to its superior effect of enzyme protection. In the near future, the synthesis of ectoine and hydroxyectoine, metabolic pathway, transport and leaking channel will be an interesting work to be conducted in the strain *H. ventosae* DL7.

TABLE 2 - Maximal amount of ectoine synthesis (mg/l) in strain DL7 and six *Halomonas* species growing in medium containing 1-3 M NaCl

| NaCl (M) | Strain DL7 | *H. ventosae* DSM 15911$^T$ | *H. pacifica* DSM 4742$^T$ | *H. salina* DSM 5928$^T$ | *H. halodenitrificans* DSM 735$^T$ | *H. alimentaria* DSM 15356$^T$ | *H. marina* DSM 4741$^T$ |
|---|---|---|---|---|---|---|---|
| 1 | 225.3 ± 3.7 (22)* | 230.3 ± 5.1 (24) | 76.2 ± 6.6 (42) | 84.7 ± 2.0 (24) | 129.8 ± 4.7 (24) | 93.4 ± 4.6 (42) | 59.5 ± 3.2 (45) |
| 2 | 404.8 ± 8.0 (18) | 385.2 ± 1.9 (22) | 204.7 ± 3.0 (48) | 252.6 ± 7.2 (42) | 253.8 ± 6.4 (42) | 213.8 ± 3.2 (42) | 156.3 ± 11.2 (45) |
| 3 | 458.9 ± 11.5 (44) | 426.1 ± 12.0 (44) | 266.9 ± 11.9 (48) | 292.8 ± 3.0 (45) | 326.3 ± 15.3 (45) | 185.8 ± 4.5 (45) | 66.8 ± 4.8 (45) |

* Values are means ± SD from three independent experiments; the values in parentheses are the optimal incubation time (h) to synthesize ectoine.

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