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Growth and Physiological Properties of Wild Type and Mutants of *Halomonas subglaciescola* DH-1 in Saline Environment

Hye Jeong Ryu, Yoo Jung Jeong, and Doo Hyun Park*

Department of Biological Engineering, Seokyeong University, Seoul 136-704, Korea

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A halophilic bacterium was isolated from fermented seafood. The 16S rDNA sequence identity between the isolate and *Halomonas subglaciescola* AJ306801 was above 95%. The isolate that did not grow in the condition without NaCl or in the condition with other sodium (Na^+) or chloride ions (Cl^-) instead of NaCl was named *H. subglaciescola* DH-1. Two mutants capable of growing without NaCl were obtained by random mutagenesis, of which their total soluble protein profiles were compared with those of the wild type by two-dimensional electrophoresis. The external compatible solutes (betaine and choline) and cell extract of the wild type did not function as osmoprotectants, and these parameters within the mutants did not enhance their growth in the saline environment. In the proton translocation test, rapid acidification of the reactant was not detected for the wild type, but it was detected for the mutant in the condition without NaCl. From these results, we derived the hypothesis that NaCl may be absolutely required for the energy metabolism of *H. subglaciescola* DH-1 but not for its osmoregulation, and the mutants may have another modified proton translocation system that is independent of NaCl, except for those mutants with an NaCl-dependent system.

Key words: *Halomonas subglaciescola*, osmoprotectant, osmoregulation, compatible solute, proton translocation, saline environment

There are various species belonging to the genus *Halomonas* capable of growth in condition with NaCl above 3.0 M (Ventosa *et al.*, 1998). Halophilic bacteria have been reported to accumulate the compatible solutes that confer protection against the deleterious effect of low water activity (Galinski, 1995). Various studies about *Halomonas* sp. have concentrated on compatible solutes and the osmoprotection mechanism (Cánovas *et al.*, 1996; Cánovas *et al.*, 1998; Ono *et al.*, 1999; Gadda and McAllister-Wilkins, 2003; Prabhu *et al.*, 2004). Cánovas *et al.* (1998) reported that when the external compatible solute choline is added to a culture of *H. elongata*, this extends the saline range from 2.5 M to at least 3.5 M NaCl, and the optimal saline from 1.75 M to 2.5 M NaCl. Cánovas *et al.* (1996) reported that the externally provided betaine, choline, or choline-*O*-sulfate (but not proline, ectoine or proline betaine) enhances the growth of *H. elongata* on 3.0 M NaCl, and betaine and choline stimulate the growth of *H. elongata* DSM 3043 over a wide range of saline. Grammann *et al.* (2002) reported that a mutant of *H. elongata* DSM 3043, which is defective in ectoin synthesis, is able to tolerate elevated salt concentrations only in the presence of external compatible solutes. The compatible solute functions as an osmoregulatory agent and it is produced from a gene expressed by an osmotically induced response (Mljica *et al.*, 1997).

We isolated a halophilic bacterium from a fermented salt shellfish (Korean Jeotgal). The isolate did not grow in a condition without NaCl, but it grew maximally in a condition with 2.0 M NaCl. We obtained two mutants of the isolate capable of growing in a condition without NaCl by the random mutagenesis, and compared the physiological properties between the wild type and mutants of the isolate. In this paper we investigated the relationship between NaCl and the external compatible solute, and their influence on the growth of halophilic bacteria. We also studied the possibility that NaCl may function as an inducer for the energy metabolism or maintenance of the membrane potential dependent on the cation (Na^+).

Materials and Methods

Materials

Yeast extract and peptone, the ingredients used for the medium, were purchased from Difco (USA). Other chemicals used for the experiment were purchased from Sigma (USA). All chemicals used for two-dimensional electrophoresis were purchased from Invitrogen (USA).

* To whom correspondence should be addressed.
(Tel) 82-2-940-7190; (Fax) 82-2-919-0345
(E-mail) baakdoo@skuniv.ac.kr

Microorganism and culture condition

To test if an organism was isolated from a fermented salt shellfish, it was cultivated in a modified Luria-Bertani (LB) medium, containing (g/L) 2.5 g of yeast extract and 5.0g of peptone supplemented with 3.0 M NaCl. The culture was incubated at 30°C with vigorous shaking at 200 rpm.

Identification

The isolate was identified with a 16S rDNA sequence. 16S ribosomal DNA of the isolate was amplified by direct PCR using the universal primers 5'-GAGTTGGATCCTGGCTCAG-3' and 5'-AAGGAGGGGATCCAGCC-3'. The reaction mixture consisted of 300 mM Tris-HCl (pH 8.8), 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 20 pM of each primer, 20 mM of each dNTP, 2U *Taq* polymerase (Genenmed, USA), and a 20 ng template. Amplification reactions were performed using a PCR machine (T Gradient model, Biometera, German). The PCR products were directly sequenced with an ABI Prism 3700 Genetic analyzer upon request to a professional company (Macrogen, Korea) with a DNA analysis system. The 16S rDNA sequences were analyzed using the Genbank database and a phylogenetic tree was composed on the basis of 16S rDNA sequence homology.

Selection of mutant

The mutants capable of growing in the medium without NaCl were obtained by random mutagenesis. Methylmethane sulfonate and nitrosoguanidine were each used as a mutagen. The bacterial cells that had been cultivated for 24 h were harvested by centrifugation (5000×g and 4°C for 30 min) and washed twice with saline. A 0.1 volume of 1.0 mM mutagen was added to the bacterial suspension and incubated at 4°C for 3 h. The bacterial cells treated with mutagen were spread on an agar medium plate without NaCl. The colonies that had been grown on the agar plate medium were transferred to a broth medium without NaCl and the minimum inhibitory concentration (MIC) of NaCl for the selected mutant was determined by a series culture containing a different concentration of NaCl.

Determination of optimal concentration of NaCl

The optimal concentration of NaCl for the growth of the wild type and mutants was determined by series cultures, each containing a different concentration of NaCl from 0.0 M to 3.5 M.

Effect of external compatible solutes on the growth

Two mM betaine and choline chloride were added to the medium containing a series concentration of NaCl from 0.0 M to 3.5 M before inoculation, respectively. The MIC of NaCl for the test organism in the culture with and without the compatible solutes was compared with each other.

Preparation of cell free extract as compatible solute

The bacterial cells harvested from a 48 h old culture were washed with a 50 mM phosphate buffer (pH 7.0) twice and disrupted by ultrasonication. The cell debris was discarded by centrifugation (5,000×g at 4°C for 30 min). The supernatant was used as the cell free extract and filtrated by a 0.22 μm pore-membrane filter before being used. The protein concentration of the filtrate was determined by using the Bradford reagent (Bio-Rad, Sweden). To examine the function of the cell free extract as a compatible solute, the filtrate (final 10 mg/ml protein) was added to the mutant culture growing under a saline environment from 0.0 to 3.4 M NaCl.

Protein gel electrophoresis

Two-dimensional gel electrophoresis was basically performed according to O'Farrells procedure (Ores, 1990). Iso-electric focusing (IEF) and SDS-PAGE were carried out by using the Novex (Invitrogen, USA) apparatus, and the ready-made reagents, buffers, IEF-strips, molecular weight marker and gels of the Novex kit were used according to the user's instruction manual.

Measurement of proton translocation test

Proton translocation was measured under an oxygenic atmosphere. The proton translocation by cell suspensions was measured as described by Fitz and Cypionka (Fitz and Cypionka, 1989). The cells were harvested by centrifugation (5,000×g at 20°C for 30 min), washed twice with 100 mM KCl, and resuspended in a KKG solution (pH 7.1) containing 100 mM KSCN, 150 mM KCl, and 1.5 mM glycylglycin. The cell suspension was preincubated in KKG without NaCl for 30 min at room temperature. The pH electrode (Toyo glass electrode, Japan) was placed in the reactant (cell suspension) of the reactor that was connected to a recorder for the conversion of pH variation to a recordable signal. Measurements were taken upon the addition of 0.1 volumes of substrate or of a substrate supplemented with 3.0 M NaCl to the reactant of KKG without NaCl. The 10 times-concentrated LB ingredients in KKG without NaCl were used as the substrate and the acidification of the reactant by the addition of the substrate was measured.

Results and Discussion

The bacterial isolate from the fermented seafood was identified on the basis of the 16S rDNA sequence (Keneko *et al.*, 1979). The genetic relationships of the 16S rDNA from the isolate with other species are shown in the dandrograms of Fig. 1. The 16S rDNA identity of the isolate with *Halomonas subglaciescola* AJ306801, which is a standard strain used for the identification of an isolate, was above 95% as shown in the phylogentic tree of Fig. 1. On the basis of this result, the isolate was named *Halomonas*

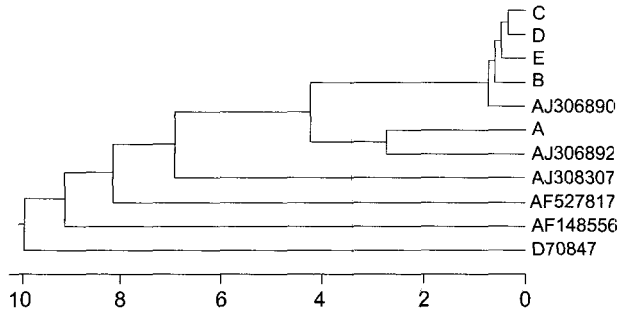

Fig. 1. Phylogeny of five *Halomonas* sp. isolated from salted seafoods. *Halomonas marina* (AJ306890), *Halomonas subglaciescola* (AJ306892), *Pseudomonas fluorescens* (AJ308307), *Escherichia coli* (AF527817), *Burkaholderia cepaciea* (AF148556), and *Phodobacter sphaeroides*(D70847) were used as the standard organisms for a comparison of homology and a measurement of phylogenic distance. A-*Halomonas subglaceiscola* DH-1 B-*Halomonas marina* IN, C-*Halomonas marina* SQ, D-*Halomonas marina* SH-1, E-*Halomonas marina* SH-2. The bar scale indicates the difference of % unit.

subglaciescola DH-1.

A high salt requirement is a specific attribute of halophilic bacteria (Boch *et al.*, 1994). Generally, halophilic bacteria are incapable of growing in a condition without NaCl and they require the NaCl concentration to be above 1.0 M for normal growth (Kushner, 1989). Halophilic bacteria are different from halotolerant microorganisms in terms of requirement for NaCl (Vreeland *et al.*, 1980). Halotolerant bacteria are capable of growing in a condition without NaCl and they have an ability to tolerate high salt concentrations. Both halophilic and halotolerant bacteria produce a compatible solute that provides an osmotic balance without interfering in the metabolic functions of the bacterial cells (Adams *et al.*, 1987; Choquet *et al.*, 1991; Del Mora *et al.*, 1994; Cummings and Gilmor, 1995; Frings *et al.*, 1995). In the current study, the wild type of *H. subglaciescola* DH-1 grew to a small degree in the condition without NaCl or with an NaCl concentration below 0.5 M. However, the mutants were capable of growing in the medium without NaCl and they grew to a small degree in the condition with an NaCl concentration above 0.5 M.

The total protein profile between the wild type and the mutants was compared by two-dimensional elelecrophoresis. As shown in the two-dimensional electrophoresis profiles of Fig. 2, about 50% and 30% of the proteins from mutant A and mutant C disappeared, respectively. This was used as a visible clue for solving the differences of physiological functions, including the osmoprotection activity and some of the metabolisms dependent on NaCl, between the wild type and mutants. As shown in Fig. 3, the wild type of *H. subglaciescola* DH-1 did not grow in the condition without NaCl, however, mutants A and C grew maximally in this condition. The optimal concentration of NaCl for the wild type and mutants of *H. subglaciescola* DH-1 was 1.7-2.5 M and 0.0-0.5 M, respectively. The compatible

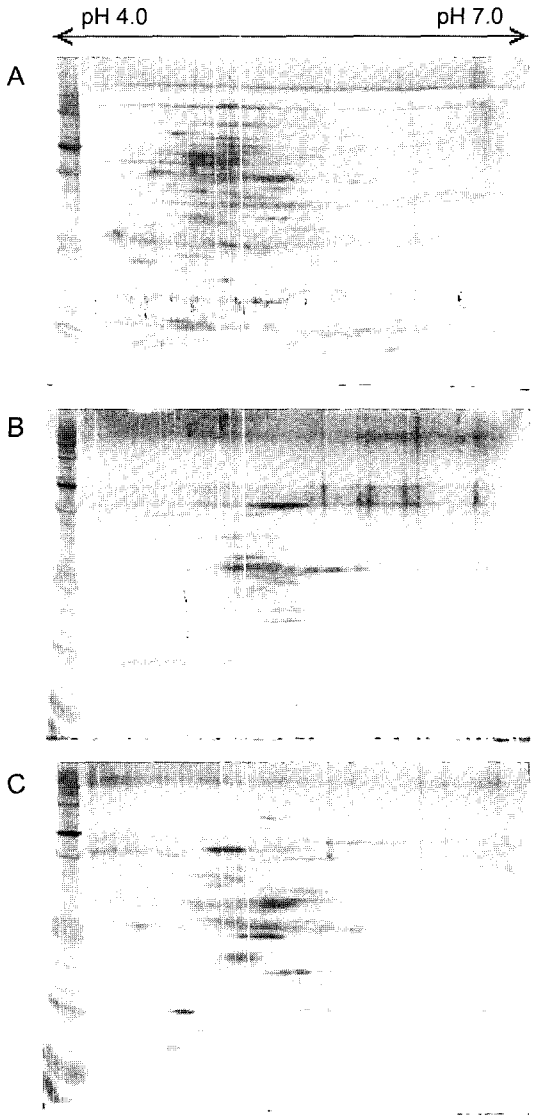

Fig. 2. Two-dimensional profiles of the soluble proteins extracted from the wild type (A), mutant A (B) and mutant C (C) of *Halomonas subglaciescola*. The test organism was cultivated in the modified Luria-Bertani (LB) medium, containing (g/L) 2.5 g of yeast extract and 5.0g of peptone supplemented with 3.0 M NaCl. The culture was incubated at 30°C with vigorous shaking at 200 rpm for 48 hr. The bacterial cells were harvested, washed twice with a 50 mM Tris-HCl buffer (pH 7.5), and disrupted by an ultrasonic treatment. Cell debris was discarded by centrifugation (5,000×g at 4°C for 30min). The supernatant was used as protein samples after filtration by a 0.22 μm pore-membrane filter before being used. The protein samples were resolved on a pH scale from 4 to 7 of the linear pH gradient in the first dimension and by SDS PAGE in the second dimension.

solutes were reported to be produced in proportion to the NaCl concentration. When grown in a complex medium with 3.4 M NaCl, all heterotrophic halophile bacteria were found to synthesize over 1.0 M of glycine betaine (Imhoff and Rodriguez-Valera, 1984; Imhoff, 1993), or found to modify the osmolytes (Deax and Tayler, 1996) inside the bacterial cytoplasm. As shown in the comparison of growth

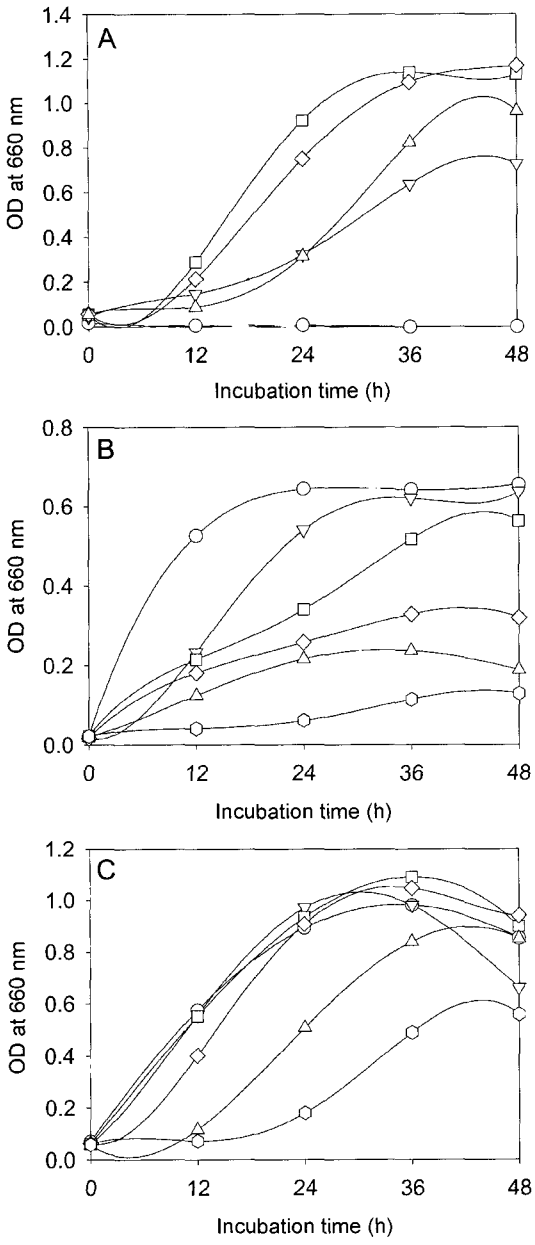

Fig. 3. Growth of *Halomonas subglaciescola* DH-1 (A) in the medium without NaCl (○), with 0.85 M NaCl (▽), 1.7 M NaCl (□), 15.7 M NaCl (◇), and 3.4 M NaCl (△), and mutant A (B) and mutant C (C) in the medium without NaCl (○), with 0.17 M NaCl (▽), 0.34 M NaCl (□), 0.51 M NaCl (◇), 0.68 M NaCl (△) and 0.85 M NaCl (⬡), respectively.

rates between the wild type and mutants, the mutants completely lost their osmoprotection ability, which is similar to the non-halophilic bacteria. We suppose that the loss of the osmoprotection of the mutants might be caused by a deficiency of the internal compatible solute.

The wild type growing in a high saline environment may produce an internal compatible solute, which may act as an external osmoprotectant for mutants or other non-halophilic bacteria (Cánovas *et al.*, 1998; Cánovas *et al.*

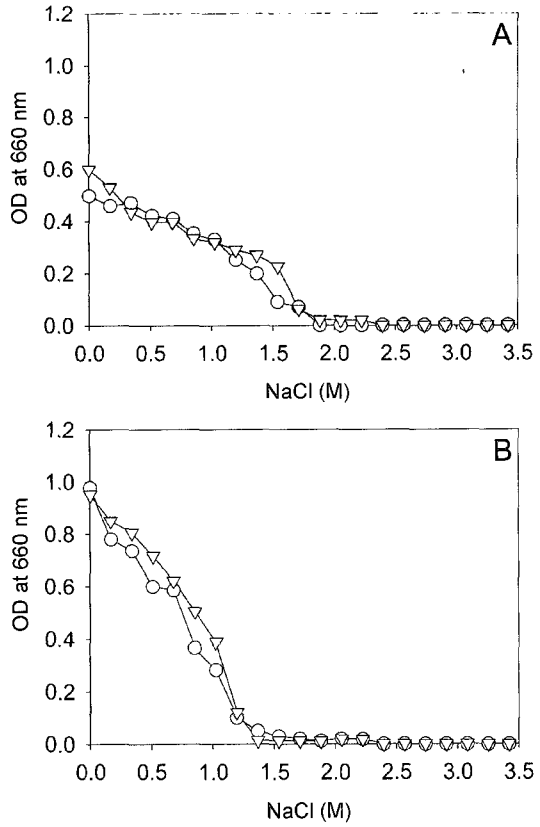

Fig. 4. Effects of the cell free extract of *Halomonas subglaciescola* DH-1 wild type on the growth of mutant A (A) and mutant C (B) without (○) and with (▽) cell free extract.

1998). To test the effect of the cell free extract as an external osmoprotectant, the cell free extract (the final concentration was 10 mg/ml based on the protein concentration) of the wild type was added to the culture of the mutants. As shown in Fig. 4, the growth of mutants was not enhanced by the addition of the cell free extract in both the low and high saline environment. This serves two possibilities: the cell free extract of the wild type was not working as the external compatible solute, or the internal compatible solutes produced by the wild type were not taken up by the mutants. In the test that used another compatible solute on the basis of the method reported by Cánovas *et al.* (Cánovas *et al.*, 1998), we also did not observe the effect of the external compatible solute for the osmoprotection of the wild type and mutants. Fig. 5 shows that the effect of the compatible solutes on the osmoprotection of the wild type and mutants was not distinct. As shown in Fig. 5A, choline was shown to act as an effective osmoprotectant against NaCl at around 2.0 M, but the overall growth of *H. subglaciescola* DH-1 in the conditions with an NaCl concentration from 0.5 M to 3.0 M was not enhanced. Furthermore, the growth of the mutants was not enhanced by the external compatible solutes choline and betaine as shown in Fig. 5B and 5C. It is possible that both the wild type and mutants of *H. sub-*

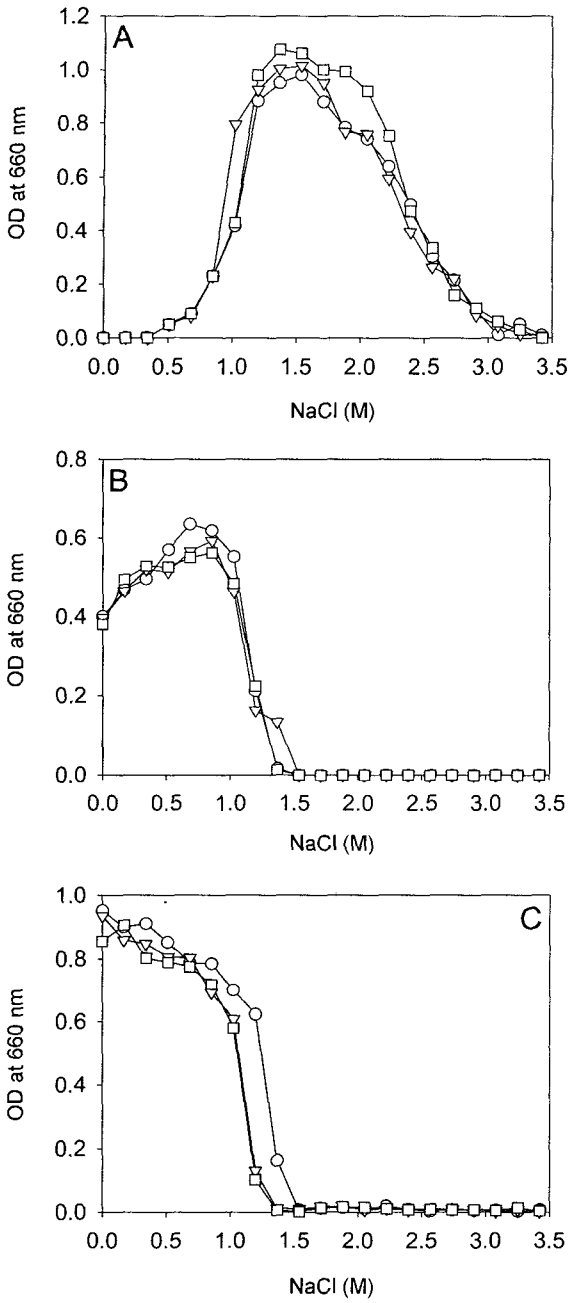

Fig. 5. Effects of compatible solutes on the growth of the wild type (A), mutant A (B) and mutant C (C) of *Halomonas subglaciescola* DH-1. The bacteria were cultivated on the LB medium without a compatible solute (○), with 2.0 mM betaine (▽) and 2.0 mM choline (□), respectively.

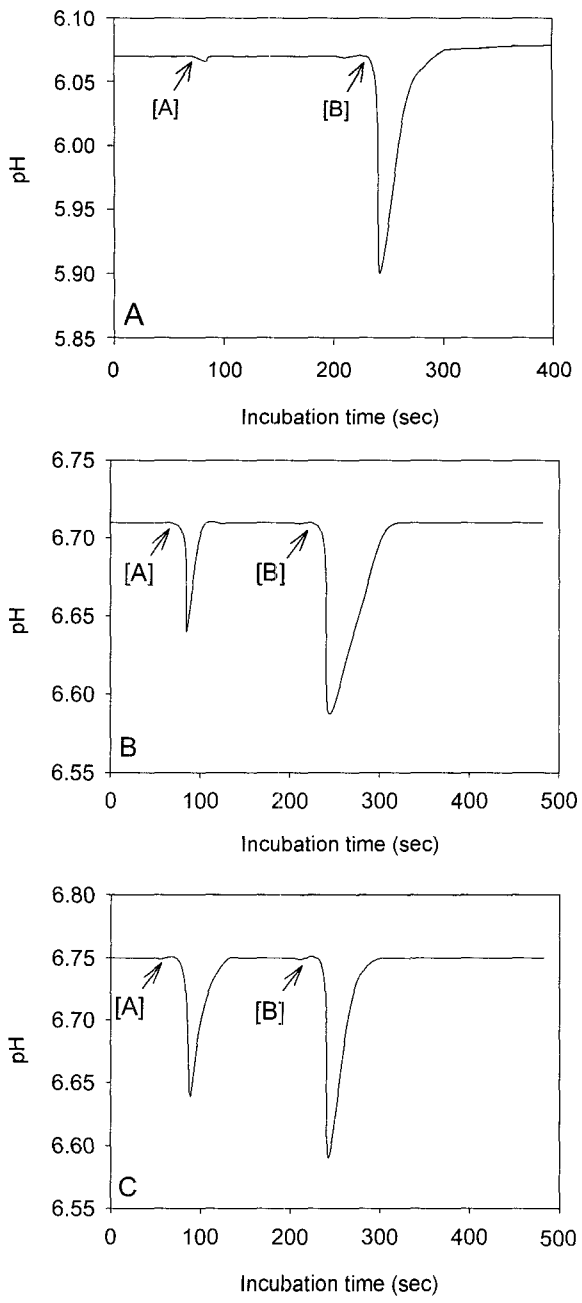

Fig. 6. Proton translocation after the addition of substrate only [A] or after the substrate was supplemented with 3.0 M NaCl [B] to the cell suspension of wild type (A), mutant A (B) and mutant C (C) of *Halomonas subglaciescola* DH-1. The cell suspension was preincubated in KKG (100 mM KSCN, 150 mM KCl and 1.5 mM glycylglycin) without NaCl for 30 min before the test. Cell density was adjusted to 5.0 as the optical density at 660 nm and the 10 times-concentrated LB ingredients in KKG without NaCl were used as a substrate.

glaciescola DH-1 may not take up the compatible solutes, or the wild type may so sufficiently produce other internal compatible solutes as not to be influenced by the external compatible solutes. However, the reason why the external compatible solute did not affect the osmoprotection of the mutants is not clear. As a solution to this question, we suggest that *H. subglaciescola* DH-1 may have a different osmoregulation system from other halophilic bacteria independent of the compatibility in a high saline environment.

Both the wild type and mutants of *H. subglaciescola* DH-1 were never grown in a medium with $NaHCO_3$, Na_2HPO_4, $NaNO_3$, Na_2SO_4 and Na_2CO_3 instead of Na^+,

nor with CsCl, RbCl, KCl and LiCl instead of Cl^-, respectively (data not shown). However, *Micrococcus varians* has been reported to be able to grow in 1.5 to 2.0 M LiCl, RbCl or CsCl in the presence of 60 mM Na^+ (Kamekura and Omishi, 1982), and *H. elongata* and *H. halophilus* have been reported to grow well on $NaNO_3$ (Vreeland and Martia, 1980) and Na_2SO_4 (Orea, 1990), respectively. This shows that *H. subglaciescola* DH-1 depends on both Na^+ and Cl^- for normal growth and energy metabolism. In most cases within the current study, the minimum concentration of Na^+ was essential for bacterial growth. This may be due, in part, to the requirement for Na^+ gradients to drive the transport processes in the bacterial cell membrane. Two possible mechanisms have been suggested: the activity of the Na^+/H^+ antiport and the presence of a primary respiration-driven Na^+ pump (Ken-Dor *et al.*, 1986). Ken-Dror *et al.* (Ken-Dor *et al.*, 1986; Ken-Dor *et al.*, 1986) reported that the movement of Na^+ is directly coupled to electron transport, and the uncoupler-insensitive primary Na^+ pump may play an important role in the regulation of intracellular salt concentration, which serves as an important clue for the examination of the electron transport system dependent on a high concentration of NaCl (Kim *et al.*, 2003; Kim, 2003).

In the bacterial respiration system, the proton motive force is generated coupled to substrate oxidation and O_2 reduction. Proton translocation was compared between the wild type of *H. subglaciescola* DH-1 grown in the medium with 2.5 M NaCl and the mutants grown in the medium without NaCl (Fig. 6). The cells were incubated in the absence of NaCl for at least 30 min prior to the addition of substrate or substrate supplemented with 2.5 M NaCl, and then proton translocation was measured by using the pH variation of the reactant. Rapid acidification of the reactant with the wild type was detected directly after the addition of the substrate supplemented with 2.5 M NaCl but not by the addition of substrate only as shown in Fig. 6A. However, the proton translocation by the mutants was detected in both of the reactants with and without NaCl, as shown in Fig. 6B and 6C. It is possible that the wild type of *H. subglaciescola* DH-1 may have a primary respiration-driven Na^+ pump coupling with proton translocation, but the mutants of *H. subglaciescola* DH-1 may have a respiration-driven proton translocation system working with NaCl and another modified respiration-driven proton translocation system working without NaCl (Ken-Dor *et al.*, 1986).

Compatible solutes work as an osmoprotectant for halophilic bacteria but they are not needed for bacterial growth in a condition without NaCl. A growth condition without NaCl is thought to be more profitable for halophilic bacteria because this condition allows them to save energy for the biosynthesis of compatible solutes. However, *Halomonas* sp. cannot grow in a medium without NaCl. We did not find a solution from published papers to answer the question on why halophilic bacteria cannot grow in a condition without NaCl.

Organisms living in the natural ecosystem have competitively progressed, and thermodynamically dominant organisms can survive and propagate their offspring. Based on this information, we propose the hypothesis that *H. subglaciescola* DH-1 evolved the NaCl-dependent energy metabolism during a period of prolonged exposure to a saline environment in which NaCl may have been absolutely required for the maintenance of the energy metabolism but not for the osmoregulation coupled to the production of compatible solutes. Compatible solutes have been reported to be produced for the osmoregulation in a high saline environment but they do not function. In a low saline environment (Kushner, 1989). The energy consumption required for competition with other microorganisms in the natural ecosystem may be greater than that required for the biosynthesis of compatible solutes. Accordingly, it is thought that halophilic bacteria growing in a saline environment do not need to compete, or compete little, with other microorganisms because non-halophiles cannot, or have difficulty, growing in a high saline environment that is above 0.6 M NaCl.

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