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Effect of NaCl on *Halomonas subglaciescola* DH-1 Incapable of Growing at Non-Salinity

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A halophilic bacterium, *H. subglaciescola* DH-1, grew at 2.0 M salinity, but did not grow at 0.8 M salinity when cultivated at higher temperature (40°C) than optimum (30°C). When the cell extract of strain DH-1 was heated at 50°C for 60 min in the absence of NaCl, isocitrate dehydrogenase and malate dehydrogenase lost their activities, but when it was heated in the presence of 2.0 M NaCl, the activity was maintained. Meanwhile, the cell extract of *E. coli* did not catalyze the reduction of NAD^+ to NADH coupled with the oxidation of isocitrate and malate at higher salinities than 1.0 M. The pH range for DH-1 was 7 to 10, and that for *E. coli* was 5 to 9. DH-1 was not grown in conditions with sodium salts other than NaCl.

Key words: *Halomonas subglaciescola*, halophile, halotolerant, compatible solutes, alkalophile

Introduction

Halophilic organisms living in saline conditions are challenged by high osmotic pressure (ion strength) and low water potential [19]. Non-halophilic organisms exposed to salinity must adjust their cytoplasmic water potentials to levels higher than the surrounding environment, which occurs through an ATP-dependent biochemical reaction [2]. Various species belonging to genus *Halomonas* produce and accumulate compatible solutes [3-5, 15, 25]. The compatible solutes added to cultures of *H. elongata* extended the range of salinity from 2.0 M to 3.5 M and the optimal salinity from 1.75 M to 2.5 M [6]. *H. variabilis* was dependent on the compatible solutes to protect themselves against high salinity [11, 12]. A mutant of *H. elongata*, which is incapable of synthesizing ectoin, tolerated elevated salt levels through the addition of external compatible solutes [16]. In most instances documented to date, halophiles grew at salinity from 0.05 M to 3.5 M or at non-salinity; one exception to this is *H. eurihalina* [22, 29]. However, Ryu *et al.* [30] reported that *Halomonas subglaciescola* DH-1 was not grown in non-saline conditions or at salinities lower than 0.8 M, and its salinity range and optimal salinity for growth were not enhanced by addition of external compatible solutes such as choline and betaine.

In this study, we tested the effect of NaCl on viability and intracellular enzymes in unfavorable conditions, and compared the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of the cell-free extract of DH-1 grown at different salinities to verify the influence of NaCl on the physiology of *H. subglaciescola* DH-1.

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Materials and Methods

Microorganism and cultivation

The DH-1 strain [30] was cultivated in a medium containing 100 mM glucose, 2 g yeast extract/L, 1 g peptone/L, 2 g K_2HPO_4/L, 3.0 M NaCl, and 1 ml trace mineral stock solution/L. The trace mineral stock solution contained 0.01 g $MnSO_4$/L, 0.01 g $MgSO_4$/L, 0.01 g $CaCl_2$/L, 0.002 g $NiCl_2$/L, 0.002 g $CoCl_2$/L, 0.002 g $SeSO_4$/L, 0.002 g WSO_4/L, 0.002 g $ZnSO_4$/L, 0.002 g $Al_2(SO_4)_3$/L, 0.0001 g $TiCl_3$/L, 0.002 g $MoSO_4$/L, and 10 mM EDTA. The bacterial culture was incubated at 30°C with vigorous shaking at 200 rpm. The pH of the medium was controlled with 50 mM carbonate buffer (pH 8-10), 50 mM citrate buffer (pH 3-6), and 50 mM phosphate buffer (pH 7).

Identification

The isolate was identified as *Halomonas subglaciescola* using the 16S rDNA sequence, as described in the previous

study [30].

Protein gel electrophoresis

Bacterial cells were harvested and washed three times with saline (0.85% NaCl) by centrifugation at 5,000 × g for 30 min. The washed cells were resuspended in the SDS-PAGE sample buffer and then cooked in 100°C water for 20 min. The sample buffer containing bacterial cell materials was centrifuged at 10,000 × g for 40 min, and the supernatant was then used as a sample. SDS-PAGE was basically performed according to Laemmli's procedure [20].

Preparation of crude enzyme

Bacterial cells harvested from a 48-hr-old cultures were washed three times with a 50 mM phosphate buffer (pH 7.0) and disrupted by 2 seconds of on-off-pulsed ultrasonic disrupter (400 Watt) at 4°C for 60 min. Cell debris was discarded by centrifugation (5,000 × g at 4°C for 30 min), and supernatant was filtrated with a 0.22 μm-pore membrane filter for sterilization. The protein concentration of the cell extract was determined using Bradford reagent (Bio-Rad, Sweden). The cell extract was used as a crude enzyme to assay isocitrate dehydrogenase (iCDH) and malate dehydrogenase (MDH). iCDH and MDH catalyze the reduction of NAD^+ to NADH coupled to the oxidation of isocitrate to α-ketoglutarate and malate to oxaloacetate, respectively. The NAD^+-dependent enzymes were chosen based on a direct assay by spectrophotometry at the same time during the reactions. NAD^+-independent enzymes have to be indirectly assayed by using analytical methods such high-performance liquid chromatography (HPLC) and gas chromatography (GC) after reaction.

Enzyme assay

The reaction mixture was composed of 5 mM NAD^+, 10 mM substrate (isocitrate or malate), crude enzyme (2 mg protein/ml as final concentration), 50 mM phosphate buffer (pH 7.0), and 2.0 M NaCl. The substrate was omitted from the reaction mixture for the control test. Dehydrogenase activity of the cell extract was spectrophotometrically assayed at 340 nm by measuring the NADH reduced coupled to the enzymatic oxidation of substrates. The specific activity was determined by the concentration (μM) of NADH produced coupled to the oxidation of substrates per minute and mg protein. The extinction coefficient of NADH (ε_{340}) is 6230 $M^{-1}cm^{-1}$.

Heat treatment of crude enzyme

Crude enzyme of DH-1 was incubated at 30°C, 40°C, and 50°C for 60 min in the absence of NaCl or in the presence of 2.0 M NaCl, and activity was assayed at 2.0 M salinity.

Results

Bacterial metabolism can be influenced by environmental variation, which can be estimated by differences in protein expression. Soluble protein patterns of DH-1 grown in the salinity range from 0.8 to 3.6 M for 24 and 48 hr were compared. As shown in Fig. 1, SDS-PAGE patterns of the soluble proteins were entirely the same, and were not related to the NaCl concentration. This shows that no specific protein expression was induced by NaCl, or that NaCl cannot be a factor that acts to regulate gene expression. Generally, NaCl added to bacterial culture is not transferred into the cytoplasm, but instead increases external osmotic

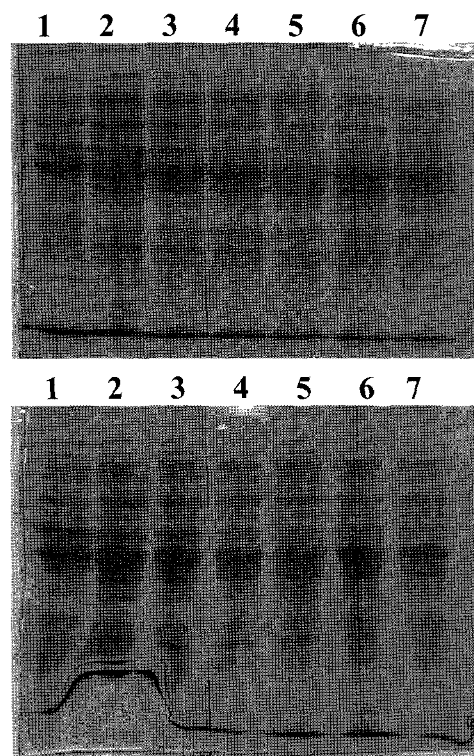

Fig. 1. SDS-PAGE pattern of the soluble protein of *H. subglaciescola* DH-1 grown in medium containing 0.8 M NaCl (lane 1), 1.3 M NaCl (lane 2), 1.8 M NaCl (lane 3), 2.3 M NaCl (lane 4), 2.8 M NaCl (lane 5), 3.3 M NaCl (lane 6), and 3.6 M NaCl (lane 7) for 24 hr (upper) and 48 hr (lower).

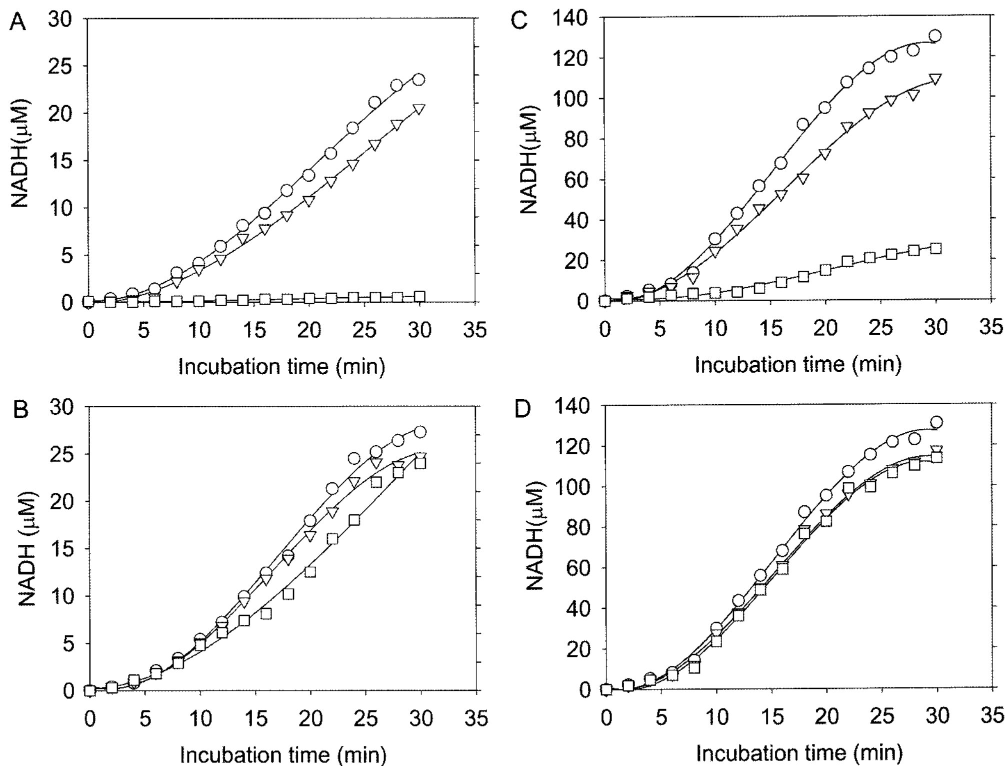

Fig. 2. Influence of heat treatment on the activity of isocitrate dehydrogenase (A,B) and malate dehydrogenase (C,D) of *H. subglaciescola* DH-1 crude enzyme. The enzyme was incubated at 30°C (○), 40°C (▽), and 50°C (□) for 60 min in the condition without (A,C) and with 2.0 M NaCl (B,D), respectively. The enzyme activity was assayed in the presence of 2.0 M NaCl, and the protein concentrations of enzymes were adjusted to a final 2 mg/ml.

pressure, which causes non-halophilic bacterium such as *E. coli* to consume more energy. Crude enzyme extracted from *E. coli* catalyzed the reduction of NAD^+ to NADH to a large extent, coupled to oxidation of isocitrate (14.6 μM NAD^+ min^{-1}mg $protein^{-1}$) or malate (22.7 mM NAD^+ min^{-1} mg $protein^{-1}$) at non-salinity, but did not have the same effect at 1.0M salinity. When the cell extract of DH-1 was incubated at 30°C, 40°C, and 50°C for 60 min in the presence of 2.0 M NaCl, iCDH and MDH did not lose their activities, as shown in Fig. 2. When the same experiments were performed in the presence of other alkaline metal ions (K^+, Li^+, Cs^+, and Rb^+), iCDH and MDH completely lost their activities. The iCDH and MDH of *E. coli* was not detected at 1.0 M salinity, even without heat treatment. This result presents the possibility that DH-1 may survive or grow at higher temperatures depending on the NaCl concentration. As shown in Fig. 3, DH-1 even grew at 40°C in the presence of 2.0M NaCl. However, the bacterial growth was greatly decreased at 30°C, and stopped at 40°C in the presence of 0.8M NaCl, which is the minimal salinity required for

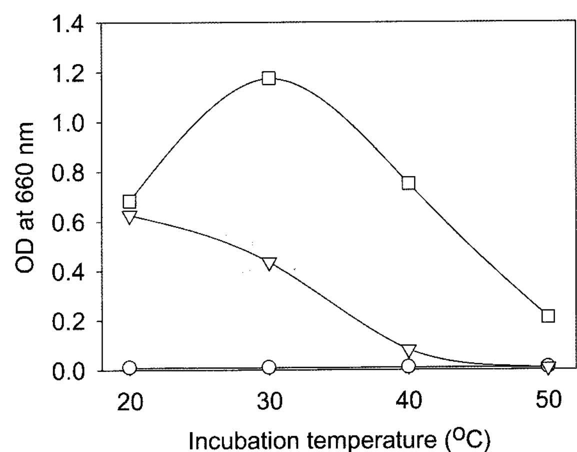

Fig. 3. Growth of *H. subglaciescola* DH-1 at different temperatures and NaCl concentrations (○, 0.0 M; ▽, 0.8 M; □, 2.0 M) for 48 hr.

DH-1. This result is a clue that at least optimal salinity (1.5~2.5 M) may be required for DH-1 to tolerate higher temperatures. Meanwhile, pH variation also may be an environmental factor for halophiles or non-halophiles. High sodium ion concentrations around bacterial cells may allow

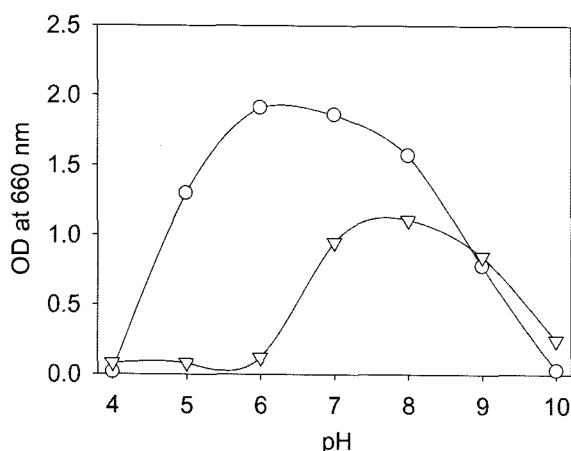

Fig. 4. Growth of *E. coli* (○) and *H. subglaciescola* DH-1 (▽) at different pH for 48 hr. The medium pH was adjusted by mixing with citrate buffer, phosphate buffer, or carbonate buffer after autoclaving. *E. coli* growth was tested to compare the optimum pH between a halophilic and a non-halophilic bacterium. The NaCl contained in medium was adjusted to 2.0 M for *H. subglaciescola* DH-1, but was adjusted to only 0.17 M for *E. coli*.

DH-1 to adjust to higher pH. As shown in Fig. 4, DH-1 grew in the pH range from 7 to 10, which is considerably higher than the pH range of 5 to 9 for *E. coli*, and the optimal pH for DH-1 and *E. coli* was around 8 and 6, respectively. It is possible that DH-1 may have a different metabolic system from other halophiles or halotolerant bacteria that are capable of growing in a wide salinity range from 0.05 to 3.0 M NaCl. The *E. coli* strain used for this research is halotolerant, and can grow in the salinity range from 0 to 0.9 M NaCl. Alkalophiles have been known to grow in alkaline environments and generate ATP in a manner dependent on the sodium motive force. This means that other alkaline metal ions such as potassium, lithium, cesium, and rubidium cannot substitute for sodium in generating ATP. The effects of different salts such as $NaNO_3$, Na_2SO_4, Na_2HPO_4, KCl, LiCl, RbCl, and CsCl on the growth of DH-1 was tested to verify which specific salt may substitute for sodium salt. As shown in Table 1, DH-1 never grew in media containing salts other than NaCl. This is clear evidence that DH-1 depends on the proper concentration of salts to produce energy, but not to maintain osmotic pressure. Theoretically, the osmotic pressure can be regulated with other sodium salts such as potassium ions, which function as a factor for the maintenance of the proton motive force [1].

Discussion

In previous research [30], it was confirmed that DH-1 did not grow at salinity lower than 0.8 M, the optimal salinity range for DH-1 was 1.5~2.5 M NaCl, and the growth of mutants of DH-1 incapable of growing at higher salinity than 1.0 M was not enhanced by the addition of external compatible solutes (choline and betaine) or the cell extract of DH-1. The cell extract of DH-1 is believed to contain compatible solutes. These results act as evidence that DH-1 is not dependent on the compatible solutes for its tolerance to high saline conditions. In general, the compatible solutes confer protection of non-halophiles, slight halophiles, and moderate halophiles against the deleterious effects of high salinity [1, 8, 9, 14, 22]. The reports on compatible solute synthesis show that specific enzymes may be differentially expressed in halophilic bacteria grown at the different salinities [10, 17, 21]. Mojica *et al.* [23] reported that soluble proteins synthesized in *H. elongata* grown at different salinities differed according to the NaCl concentration. However, no specific protein band was observed on the SDS-PAGE patterns of soluble proteins extracted from DH-1 grown at different salinities. DH-1 is capable of adapting to various salinities, from 0.8 to 3.6 M NaCl, without production of specific protein and physiological variation, which makes it different from other halophilic bacteria capable of synthesizing the compatible solutes [7, 13, 16]. DH-1 did not grow at non-salinity or at salinities lower than 0.8 M, and never grew in conditions with sodium and chloride salts other than NaCl. The iCDH and MDH lost their activities following heat treatment in the absence of NaCl. The iCDH and MDH may be not representative of all intracellular enzymes, but are essential enzymes for energy metabolism, which are only found in the TCA cycle. The most likely explanation of these results is that NaCl may be absolutely required for some intracellular enzymes of DH-1, and a proper concentration of

Table 1. Growth of *H. subglaciescola* DH-1 in the presence of different salts, of which the concentrations were adjusted to 2.0 M.

Salts	NaCl	LiCl	KCl	RbCl	CsCl	$NaNO_3$	Na_2SO_4	Na_2HPO_4
Growth (OD at 660 nm)	1.2	0.028	0.008	0.023	0.011	0.035	0.042	0.031

NaCl may still exist in the cytoplasm. Some data reported by other researchers show results that are very similar to ours. Peterson and Salin [26] reported that hydroperoxidase purified from *Halobacterium halobium* was greatly diminished at low salinity, and later [27] reported that the catalase that was purified from the halophilic archaeon, *Halobacterium halobium*, lost its activity when incubated in a buffer lacking NaCl. Kobayashi *et al.* [18] reported that the amylase purified from a *Haloalkaliphilic archeon* exhibited maximal activity and was stable at 2.5 M salinity. From these results, we cannot establish why NaCl is absolutely required for the growth and enzyme activity of DH-1 in both normal and unfavorable environments. If the growth and metabolism of DH-1 depend on Na^+, the optimal pH for growth should be higher than 7 because the external Na^+ level must be higher than the internal level. DH-1 grew maximally at pH 8, and was capable of growing at pH 10, which is similar to the growth pattern of alkalophiles for pH. Some alkalophiles were reported to depend on Na^+ to generate an ion gradient between the outside and the inside of bacterial cells and to maintain the membrane potential [24, 28]. In a previous study [30], the proton translocation measured with DH-1 was activated by the addition of NaCl. NaCl-dependent growth and proton translocation activation do not provide definitive evidence that DH-1 produces energy in its coupling with the Na^+-motive force, but may be a clue that NaCl is a nutritional factor for DH-1 rather than an environmental factor.

REFERENCES

1. Abee, T., K. J. Hellingwerf, and W. N. Konings. 1988. Effects of potassium ions on proton motive force in *Rhodobacter sphaeroides*. *J. Bacteriol.* **170**: 5647-5653.
1. Adams, R., J. Bygraves, M. Kogul, and N. J. Russell. 1987. The role of osmotic effects in haloadaptation of *Vibrio costicola*. *J. Gen. Microbiol.* **133**: 1861-1870.
2. Amezaga, M. R. and I. R. Booth. 1999. Osmoprotection of *Escherichia coli* by peptone is mediated by the uptake and accumulation of free proline but not of proline-containing peptides. *Appl. Envir. Microbiol.* **65**: 5272-5278.
3. Barth, S., M. Huhn, B. Matthey, A. Klimka, E. A. Galinski, and A. Engert. 2000. Compatible-solute-supported periplasmic expression of functional recombinant proteins under stress conditions. *Appl. Envir. Microbiol.* **66**: 1572-1579.
4. Boch, J., B. Kempt, and E. Bremer. 1994. Osmorgulation in *Bacillus subtilis*. Synthesis of the osmoprotectant glycine betaine from exogenously provided choline. *J. Bacteriol.* **176**: 5364-5371.
5. Boch, J., B. Kempf, R. Schmid, and E. Bremer. 1996. Synthesis of the osmoprotectant glycine-betaine in *Bacillus subtilis*: characterization of *gbs*AB gene, *J. Bacteriol.* **178**: 5121-5129.
6. Cánovas D., C. Vargas, L. N. Csonka, A. Ventosa, and J. J. Nieto. 1998. Synthesis of glycine betaine from exogenous choline in the moderately halophilic bacterium *Halomonas elongata*. *Appl. Environ. Microbiol.* **64**: 4095-4097.
7. Choquet, C. G., I. Ahoshai, M. Klein, and D. J. Kushner. 1991. Formation and role of glycine betaine in the moderate halophile *Vibrio costicola*: site for action of Cl^- ions. *J. Bacteriol.* **171**: 880-886.
8. Ciulla, R. A., M. R. Diza, B. F. Taylor, and M. F. Roberts. 1997. Organic osmolytes in aerobic bacteria from Mono Lake, an alkaline, moderately hypersaline environment. *Appl. Environ. Micrbiol.* **63**: 220-226.
9. Cummings, S. P. and D. J. Gilmour. 1995. The effect of NaCl on the growth of *Halomonas* species: accumulation and utilization of compatible solutes. *Microbiology* **141**: 1413-1418.
10. Del Moral, A., J. Severin, A. Ramos-Cormenzana, H. G. Truper, and E. A. Galinski. 1994. Compatible solutes in new moderately halophilic isolates. *FEMS Microbiol. Lett.* **122**: 165-172.
11. Dobson, S. J. and P. D. Frenzmann. 1996. Unification of the genera *Deleya*, *Halomonas*, and *Halovibrio* and the species *Parcoccus halodenitrificans* into a single genus, *Halomonas* and placement of the genus *Zymobacter* in the family *Halomondaceae*. *Int. J. Syst. Bacteriol.* **46**: 550-558.
12. Fendrich, C. 1986. *Halovibrio variabilis* gen. nov. sp. nov. *Pseudomonas halophila* sp. nov. and a new halophilic aerobic coccoid eubacterium from Great Salt lake, Utah. *Syst. Appl. Microbiol.* **11**: 36-43.
13. Frings, E., H. J. Junte, and E. A. Galinski. 1993. Compatible solutes in representatives of the genera *Brevibacterium* and *Corynebacterium*: occurrence of tetrahydropyrimidine and glutamine. *FEMS Microbiol. Lett.* **109**: 25-32.
14. Frings, E., T. Sauer, and E. A. Glinski. 1995. Production of hydroxyectoin: high cell-density cultivation and osmotic downshock of *Marinococcus* strain M52. *J. Biotechnol.* **43**: 53-61.
15. Galinski, E. A. 1995. Osmoadaptation in bacteria. *Adv. Microb. Physiol.* **19**: 273-328.
16. Grammann, K., A. Volke, and H. J. Künte. 2002. New type of osmoregulated solute transporter identified in Halophilic members of the Bacteria Domain: TRAP transporter TeaABC mediates uptake of ectoine and hydroxyectoine in *Halomonas elongate* DSM2581. *J. Bacteriol.* **184**: 3078-3085.
17. Hagemann, M., S. Richter, and S. Mikkat. 1996. The *ggtA* gene encodes a subunit of the transport system for the osmoprotective compound glucosylglycerol in *Synechocystis* sp. strain PCC 6803. *J. Bacteriol.* **179**:714-720.
18. Kobayashi, T., H. Kanai, T. Hayashi, T. Akiba, R. Akaboshi, and K. Horikoshi. 1992. Haloalkaliphilic maltotriose-forming alpha-amylase from the archaebacterium *Natronococcus*

sp. strain Ah-36. *J. Bacteriol.* **174**: 3439-3444.
19. Kraegeloh, A and H. J. Kunte. 2002. Novel insights into the role of potassium for osmoregulation in *Halomonas elongata*. *Extremophiles*. **6**: 453-462.
20. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.
21. Lai, M. C., D. R. Yang, and M. J. Chuang. 1999. Regulatory factors associated with synthesis of the oxmolyted glycine betaine in the halophilic methanoarchaeon *Methanolhalophilus protucafensis*. *Appl. Environ. Microbiol.* **65**: 828-833.
22. Mellado, E., M. T. Garcia, J. J. Nieto, K. N. Timmis, and A. Veatosa. 1995. Phylogenetic inferences and taxonomic consequences of 16S ribosomal DNA sequence comparison of *Chromohalobacter marismortui*, *Vocaniella eurihalina*, and *Deleya salina* and reclassification of *V. eurihalina* as *Halomonas eurihalina*. comb. nov. *Int. Syst. Bacteriol.* **45**: 712-716.
23. Mojica, F. J. M., E. Cisneros, C. Ferrer, F. Rodriguez-Valera, and G. Juez. 1997. Osmotically induced response in representatives of halophilic prokaryotes: the bacterium *Halomonas elongate* and the Archaeon *Haloferax volcanii*. *J. Bacteriol.* **179**: 5471-5481.
24. Neumann, S., U. Matthey, G. Kaim, and P. Dimroth. 1998. Purification and properties of the F_1F_o ATPase of *Ilyobacter tartaricus*, a sodium ion pump. *J. Bacteriol.* **180**: 3312-3316.
25. Ono, H., K. Sawadas, N. Khunajakr, T. Tao, M. Yamamoto, M. Hiramoto, A. Shinmyo, M. Takano, and Y. Murooka. 1999. Characterization of biosynthetic enzymes for ectoine as a compatible solute in a moderately halophilic eubacterium. *Halomonas elongata*. *J. Bacteriol.* **181**: 91-99.
26. Peterson, B. N. J. and M. L. Salin. 1993. Purification of a catalase-peroxidase from *Halobacterium halobium*: characterization of some unique properties of the halophilic enzyme. *J. Bacteriol.* **175**: 4197-4202.
27. Peterson, B. N. J. and M. L. Salin. 1995. Purification and characterization of a mesohalic catalase from the halophilic bacterium *Halobacterium halobium*. *J. Bacteriol.* **177**: 378-384
28. Prowe, S. G., Jack L. C. van de Vossenberg, A. J. M. Driessen, G. Antranikian, and W. N. Konings. 1996. Sodium-coupled energy transduction in the newly isolated thermoalkaliphilic strain LBS3. *J. Bacteirol.* **178**: 4099-4104.
29. Quesado, E., V. Bejar, M. J. Valderrama, and A. Ramons-Cormenzana. 1990. *Vocaniella eurihalina* gen. Nov., sp. nov., a moderately halophilic nonmotile gram-negative rod. *Int. J. Syst. Bacteriol.* **40**: 261-267.
30. Ryu H. J., Y. J. Jeong, and D. H. Park. 2004. Growth and physiological properties of wild type and mutants of *Halomonas subglaciescola* DH-1 in saline environment. *J. Microbiol.* **42**: 174-180.

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국문초록

Halomonas subglaciescola DH-1의 생장에 미치는 염화나트륨의 영향

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호염성세균 *H. subglaciescola* DH-1은 염화나트륨이 없거나 0.8 M 이하로 존재하는 환경에서 생장하지 못한다. 이 호혐성세균은 2.0 M의 염화나트륨이 존재하는 조건에서는 최적온도(30°C) 보다 높은 40°C에서 생장이 가능하였으나, 0.8 M의 염화나트륨이 존재하는 조건에서는 생장이 크게 저하되었다. 세포추출물을 염화나트륨이 존재하는 조건에서 50°C로 1시간 동안 열처리하였을 때 세포내 효소의 활성이 유지되었으나, 염화나트륨이 없는 조건에서 열처리하였을 때 효소의 활성은 유지되지 않았다. 반면, 대장균의 세포추출물의 효소활성은 1.0 M 이상의 염화나트륨이 존재할 때 온도 또는 pH와 관계없이 측정되지 않았다. *H. subglaciescola* DH-1은 pH 7~10의 범위에서 생장하였고, 생장을 위한 최적 pH는 8이었다. 이러한 생리적인 특성으로부터 염화나트륨은 *H. subglaciescola* DH-1의 물질대사를 위한 필수적인 무기영양소라는 사실을 유추할 수 있다.