DNA protection ability of intracellular highly concentrated salts on *Halobacterium salinarium*, an extremely halophilic bacterium

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Halobacterium salinarium is an extremely halophilic archaebacterium, which lives in the surface of salt lake and salt plant. In these environments, *H. salinarium* is usually exposed by intense irradiation of sunlight including ultraviolet light (UV). UV produces DNA lesions, and possesses mutagenic and/or lethal effects on organisms. *H. salinarium* can tolerant various DNA-damaging agents such as UV and gamma rays. *H. salinarium* contains saturated concentrations of K^+ and Cl^- in the cell. Since Cl^- is known to be a radical scavenger, the intracellular highly accumulated ions seem to be involved in the DNA protection. *In vitro*, highly concentrated KCl inhibited the generations of cyclobutane pyrimidine dimer (CPD) and DNA strand break, the DNA lesions induced by UV and gamma rays, respectively. These protection efficiencies are larger than that shown by carotenoid, an abandon antioxidant in the cell of *H. salinarium*. Therefore the intracellular highly concentrated salts play effective role not only to tolerant against osmotic pressure, but also to maintain the genetic information in the extremely severe environment.

1. *HALOBACTERIUM SALINARIUM* SHOWS HIGH RESISTANCE TO DNA-DAMAGING AGENTS

Halobacterium salinarium is an extremely halophilic archaebacterium, which lives in the surface of salt lake and salt plant. In these environments, *H. salinarium* is constantly confronted with highly concentrated salts. *H. salinarium* accumulates 5.3 M potassium cation (K^+) and 3.3 M chlorine anion (Cl^-) in the cell (1). These highly concentrated salts can work to resist against highly osmotic pressure of the environment. Simultaneously, this species is usually exposed by intense irradiation of sun light including ultraviolet light (UV) in this environment. UV produces DNA lesions, and possesses mutagenic and/or lethal effects on organisms (2). Therefore *H. salinarium* needs some potentials to tolerant any DNA damage such as UV damage.

Another aspect of *H. salinarium* leads its expected high resistance against DNA damage. The cell of *H. salinarium* shows bright red color as same as most halophilic archaebacteria (3). This red color is based on its carotenoids. Bacterioruberin is a major species of the carotenoids in the cell of *H. salinarium* (4). Bacterioruberin is also found in the cell of *Rubrobacter radiotolerans*, a radio-resistant bacterium, and plays a role to protect DNA against ionizing radiation on the species (5, 6). This fact suggests that bacterioruberin plays same role in the cell of *H. salinarium*, and this species shows some resistance against DNA-damaging agents such as ionizing radiation.

To examine the resistance of *H. salinarium* against DNA damage, we investigated the survival frequencies against various DNA-damaging agents. The cells were exposed by UV or gamma ray, and cultured on nutrient agar plates to score viable colonies. Using dose required to reduce viability to 37% (D_{37}), *H. salinarium* showed 21.2 and 4.27 times more resistant than *Escherichia coli* to UV and gamma rays, respectively (Fig. 1) (7). UV mainly produces cyclobutane pyrimidine dimer (CPD) as DNA lesion, and oxidative damage is major DNA lesion induced by ionizing radiation (2, 8). As mentioned above, *H. salinarium* contains a large amount of carotenoid in its cell. Carotenoids

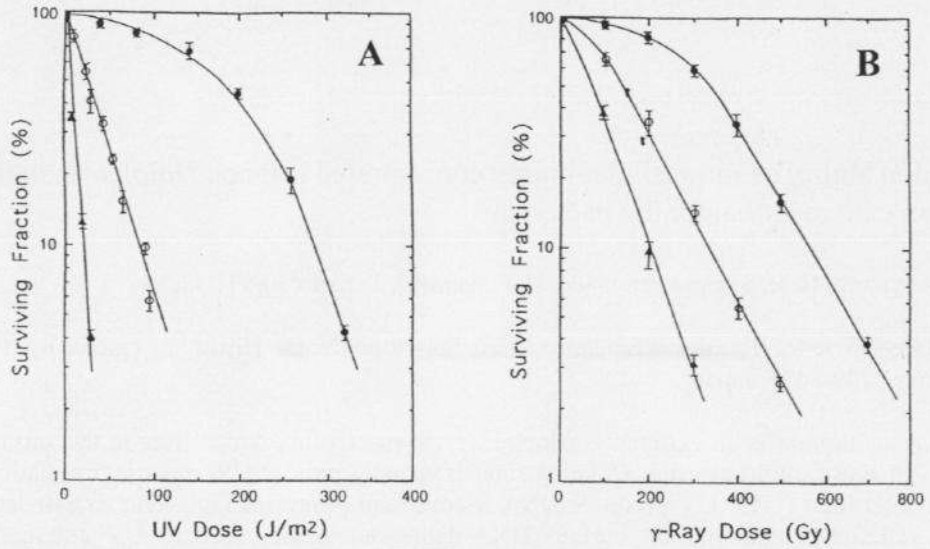

Figure 1. Sensitivities of carotenoids-deficient mutant of *H. salinarium* (open circle), its red-pigmented wild type (closed circle), and *E. coli* (closed triangle) to UV (A) and gamma rays (B). The data is based on five independent experiments.

can scavenge reactive oxygen species (ROS) produced by ionizing radiation, and lead resistance against ionizing radiation (9). On the other hand, carotenoids play roles two major functions; light-harvesting in photosynthesis and protection of cell against the harmful photooxidation effects in photosynthetic plants and bacteria (9). Thus carotenoids can protect DNA against UV. Carotenoids in the cell of *H. salinarium* also seem to protect the cell against both UV and ionizing radiation.

To estimate the protective effect of intracellular high concentrated salts *in vivo*, we must remove the effect of carotenoid from the cell. Therefore, we subjected the cells of *H. salinarium* with MNNG (10), and constructed the mutant lacking carotenoid and showing white colony (11). This lacking mutant became more sensitive than the wild type to tested DNA-damaging agents (Fig. 1). Thus D_{37} of the mutant reduced 0.4 and 0.2 times to UV and gamma rays, respectively. However, the mutant was still highly resistant than *E. coli* (Fig. 1) (11). D_{37} of the mutant showed 4.0 and 2.0 times higher resistant than *E. coli* to UV and gamma rays, respectively. This result suggests that other factors, *i. e.* highly concentrated salt, instead of carotenoid, play more effective role to protect DNA in the cell of *H. salinarium*. However, we cannot examine the resistance ability of *H. salinarium* against DNA-damaging agents without any salt, because it cannot survive under this condition. Therefore we need *in vitro* examination of the salt effect on DNA-protection.

2. *IN VITRO* DNA-PROTECTION OF HIGHLY CONCENTRATED SALT AGAINST DNA-DAMAGING AGENTS

As mentioned above, *H. salinarium* contains saturated concentrations of K^+ and Cl^- in the cell (1). Between them, Cl^- is known to be a radical scavenger (12). To solve the involvement of the intracellular highly accumulated ions in DNA protection against UV and ionizing radiation, we estimated the amount of DNA lesions induced by UV and gamma rays under the condition of highly concentrated salt.

2.1. Concentrated salt shows *in vitro* DNA-protection against UV

UV produces various DNA lesions including cyclobutane pyrimidine dimer (CPD), (6-4) photoproduct and pyrimidine hydrates (2). Among these UV lesions, CPD is specifically recognized by T4 endonuclease V (DenV), which can incise DNA at CPD site (13). Thus CPD is available to be

analyzed as single strand break (SSB) by DenV. SSB on DNA changes the conformation of circular plasmid DNA from closed circular form (type I) to open circular form (type II). Plasmid DNA showing Type II form can be separated from type I by its slower migration feature on agarose gel electrophoresis. pDEL19 plasmid DNA (4.8 kbp) was irradiated by various doses of UV without or with 2 M KCl, and then treated with an excess amount of DenV. As mentioned above, DenV convert CPD to SSB on pDEL19. The relationship between the irradiation dose and the amount of CPD as type I is shown in Fig. 2A (14). D_{37} are 18.9 and 33.7 J/m^2 without and with 2 M KCl, respectively. Thus 2 M KCl decreased DNA damage induced by UV. The factor of reducing effect of 2 M KCl for UV damaging is 1.78. Thus the highly accumulated salts in the cell of *H. salinarium* seems to protect DNA against UV *in vivo*. However, the protection mechanism of 2 M KCl still has been unclear in the present system, since UV directly deposits energy to DNA without via ROS unlike the case of ionizing radiation.

2.2. Concentrated salt shows *in vitro* DNA-protection against ionizing radiation

Plasmid DNA was also exposed by gamma rays in absence and presence of 2 M KCl. Ionizing radiation including gamma rays can produce many kinds of DNA lesion as modified base, loss of base, and single and double strand breaks (2). Among these lesions, SSB is a specific and detectable lesion on analysis of agarose gel electrophoresis. pDEL19 was irradiated by gamma rays without or with 2 M KCl, and directly subjected on agarose gel electrophoresis. SSB were detected after irradiations under both conditions, and lower amount of SSB were shown with 2 M KCl. The relationship between the irradiation dose and the amount of type I is shown in Fig. 2B (14). The doses yielding 37% of type I (D_{37}) are 1.1 and 50.4 Gy without and with 2 M KCl, respectively. Thus 2 M KCl reduced generation of SSB as 45.8 times. This value is 25.7 times higher than that for UV. The major effect of ionizing radiation appears via its producing ROS (8). Since Cl^- is a well-known radical scavenger (12), Cl^- seems to scavenge these ROS and reduce the amount of SSB. The result suggests that the highly accumulated Cl^- in the cell of *H. salinarium* protects DNA against ionizing radiation *in vivo*. Thus the intracellular highly concentrated salts play effective role to maintain the genetic information in the extremely severe environment. These results are summarized in Table 1.

2.3. The effect of cations to DNA-protection against ionizing radiation

As mentioned above, 2 M KCl shows DNA protection ability, and it might be derived from the

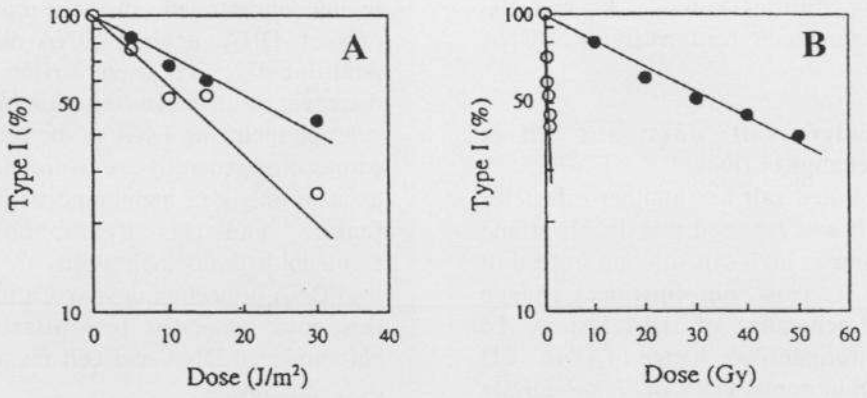

Figure 2. Relationship between the irradiation dose and the amount of type I plasmid. The amounts of type I and II in the ethidium bromide-stained gel were measured on an image analyzer, and the percent of type I was plotted against the irradiation dose. A, UV-irradiated DNA in the absence and presence of 2 M KCl; B, gamma-irradiated DNA in the absence and presence of 2 M KCl. The data points are the average of two independent experiments. Open circle, without KCl; closed circle, with 2 M KCl

Table 1
D_{37} for CPD and SSB formations in the absence and presence of salts

	D_{37}		
Salt	None	KCl[a]	NaCl[a]
CPD (J/m^2)	18.9	33.7	ND[b]
SSB (Gy)	1.1	50.4	10.4

[a][KCl] = [NaCl] = 2 M
[b]Not determined

radical scavenging ability of its Cl^-. The reason of using KCl in the present study is K^+ and Cl^- are two major ions concentrated in the cell of *H. salinarium* (1). We also evaluated the protection ability of same concentration of NaCl. Surprisingly, NaCl showed less protection than KCl (14). D_{37} against SSB induced by gamma rays were 10.4 Gy with 2 M NaCl (Table 1). This values was 4.85 times lower than that of 2 M KCl. If all of the protection are derived from only the radical scavenging of Cl^-, this result was not confirmed. One expected mechanism of different effects between KCl and NaCl is the different affinities to DNA backbone. The phosphate groups of DNA in aqueous solution bind some coordinated cations such as K^+ and Na^+, and these binds caused electrostatic shielding of the phosphate groups of DNA that leads DNA is dissolved into solvent. Na^+ binds to the phosphate group of DNA more tightly than K^+ (15). These different binding abilities between K^+ and Na^+ might cause different some conformations of DNA (16).

2.4. Concentrated salt does not affect conformational change of DNA

Highly concentrated salt has another effect for DNA molecule. It was reported that double strand DNA shows z-form in high salt solution instead of usual b-form (17). This conformational change might affect the generation of DNA lesions. To elucidate the conformational change of DNA, CD spectra of the chromosomal DNA of *H. salinarium* were measured with various concentrations of KCl (11). The chromosomal DNA was extracted and purified by Marmur's method from the cells of *H. salinarium*. The purified chromosomal DNA of *H. salinarium* was dissolved in 10 mM phosphate buffer (pH 7.0) to 0.12 mM concentration. With various concentrations of KCl (up to 3.5 M), the CD spectra were taken at room temperature, and there were no significant difference among these various conditions (11). This result suggests that the intracellular high concentrated salt has no effect of the conformational change of DNA. Alternatively, highly concentrated salt simply plays a role of radical scavenger in DNA-protection against ionizing radiation in the present study.

3. OTHER DNA-PROTECTIONS ON *H. SALINARIUM*

3.1. DNA protection of bacterioruberin

On the other hand, we must evaluate the effect of bacterioruberin against DNA damage induced by UV and ionizing radiation. With similar procedure for 2 M KCl, CPD and SSB on pDEL19 were estimated after irradiations of UV and gamma rays, respectively. Bacterioruberin was extracted and purified by method as previous study (14). D_{37} for CPD formation without and with 0.1 mM bacterioruberin were 16.3 and 21.6 J/m^2, respectively (14). On the other hand, D_{37} for SSB formation in the absence and presence of 0.1 mM bacterioruberin were 5.2 and 11.2 Gy, respectively (14). The protection factors for CPD and SSB were 0.75 and 0.46, respectively. These values were lower than those of 2 M KCl (0.56 and 0.02, respectively). Especially, the protection ability of 2 M KCl against gamma rays was the significant highest. These results suggest that the intracellular highly concentrated salt is the most major factor to protect DNA against DNA-damaging agents. Additionally, we used Brij56, a commercial detergent, to dissolve bacterioruberin into aqueous solution including DNA in the present study, since carotenoids generally are hydrophobic. Carotenoids exist in only cell membrane with its hydrophobic feature, and this hydrophobic property of carotenoids limits their ability to scavenge ROS to lead DNA protection *in vivo*. Carotenoid might play this role via only few attachments between chromosomal DNA and cell membrane in the cell of *H. salinarium*.

3.2. DNA repair on *H. salinarium*

Considering of DNA repair abilities contributing in the surviving of halophilic bacteria is also necessary. However, there are very few reports about the DNA repair, since the adjustments of the optimal salt condition for any halophilic enzymes

are difficult (3). Photolyase of *H. halobium* was only reported as DNA repair enzyme of halophilic archaebacteria, but it only showed the cloning data but not its enzymatic activity (18).

5. CONCLUSION AND PERSPECTIVE

Since *H. salinarium* faces severe environment fulfilling DNA-damaging agents in its habitat, it shows highly tolerant abilities against DNA-damaging agents. The present study shows that 2 M KCl effectively inhibits DNA damaging induced by UV and ionizing radiation *in vitro*. This result suggests that the intracellular highly concentrated salt plays important role to protect DNA in the cell of *H. salinarium*. This effect of salt for DNA-protection seems to be higher than that of other factors such as bacterioruberin. Thus the roles of the intracellular concentrated salt is not only to tolerant the stress of environmental high salt pressure, but also to protect the genetic information against environmental DNA-damaging agents. Of course, we need further elucidation for the detailed molecular mechanisms of salt on DNA protection, especially that for UV. We must also elucidate other protection manners such as DNA repair on halophilic bacteria. The present study can lead an expected application to preservation procedure against the contamination of halophilic bacteria on field of food or agricultural industry.

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