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Energy dependency on the salt-resistance of *Staphylococcus aureus*: Effects of various inhibitors on the growth in high salinity condition

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Abstract

It has previously been shown that the barrier system for high environmental salinity is closely related to the salt-resistance of *Staphylococcus aureus*. The present investigation was undertaken to clarify the energy dependency for the maintenance of intracellular univalent cation contents in cells grown on high concentration of salt containing medium. The results are summarized as follows: (1) The growth of 10% NaCl-Staph which was grown in the 10% NaCl containing nutrient broth was more sensitive to NaN₃ than Normal-Staph which was grown only on nutrient broth. The anaerobic conditions in both media demonstrated a more powerful effect on growth inhibition of 10% NaCl-Staph than Normal-Staph. Therefore, 10% NaCl-Staph must have a higher energy dependency than Normal-Staph. (2) The high sensitivity to uncouplers, such as DNP and FCCP in 10% NaCl-Staph, also suggested an energy dependency which was probably related to respiration and not to anaerobic glycolysis. (3) The intracellular Na⁺ contents of Normal-Staph and 10% NaCl-Staph were 12.0 and 152.9 mmoles per Kg wet weight of cells respectively, and the content of K⁺ in 10% NaCl-Staph (90.2 mmoles per Kg wet weight) was lower than that of Normal-Staph (215.8 mmoles per Kg wet weight). These intracellular Na⁺ and K⁺ contents were strongly affected by the addition of various inhibitors to the medium. The measurements of intracellular univalent cation contents indicated the existence of an adaptively developed barrier system in 10% NaCl-Staph and the existence of energy-dependent transport mechanisms for efflux of Na⁺ in Normal-Staph and for the influx of K⁺ in 10% NaCl-Staph.

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**ENERGY DEPENDENCY ON THE SALT-RESISTANCE OF
Staphylococcus aureus: EFFECTS OF VARIOUS
INHIBITORS ON THE GROWTH IN HIGH
SALINITY CONDITION**

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Abstract: It has previously been shown that the barrier system for high environmental salinity is closely related to the salt-resistance of *Staphylococcus aureus*. The present investigation was undertaken to clarify the energy dependency for the maintenance of intracellular univalent cation contents in cells grown on high concentration of salt containing medium. The results are summarized as follows: (1) The growth of 10% NaCl-Staph which was grown in the 10% NaCl containing nutrient broth was more sensitive to NaN_3 than Normal-Staph which was grown only on nutrient broth. The anaerobic conditions in both media demonstrated a more powerful effect on growth inhibition of 10% NaCl-Staph than Normal-Staph. Therefore, 10% NaCl-Staph must have a higher energy dependency than Normal-Staph. (2) The high sensitivity to uncouplers, such as DNP and FCCP in 10% NaCl-Staph, also suggested an energy dependency which was probably related to respiration and not to anaerobic glycolysis. (3) The intracellular Na^+ contents of Normal-Staph and 10% NaCl-Staph were 12.0 and 152.9 mmoles per Kg wet weight of cells respectively, and the content of K^+ in 10% NaCl-Staph (90.2 mmoles per Kg wet weight) was lower than that of Normal-Staph (215.8 mmoles per Kg wet weight). These intracellular Na^+ and K^+ contents were strongly affected by the addition of various inhibitors to the medium. The measurements of intracellular univalent cation contents indicated the existence of an adaptively developed barrier system in 10% NaCl-Staph and the existence of energy-dependent transport mechanisms for efflux of Na^+ in Normal-Staph and for the influx of K^+ in 10% NaCl-Staph.

Staphylococcal cells are more resistant to sodium chloride than the other bacteria. This resistance may depend on the barrier systems for the influx of sodium ions, on the pump systems of intracellular sodium ions, or on both of these systems.

On the mechanism of the barrier system of cells, many investigators (1, 2, 3) have pointed out changes in lipid compositions of the cells growing in

high salinity conditions (1), and the thermosensitivity of halo-resistance (2, 3) of the cells. KANEMASA *et al.* (1) reported that cardiolipin of *Staphylococcus aureus* markedly increased and phosphatidylglycerol and lysylphosphatidylglycerol diminished in proportion to the increase of sodium chloride concentration of the culture medium, and that the major change in the fatty acid composition is directly proportional to the quantity of branched acids. ERWIN *et al.* (2) and HURST *et al.* (3) independently reported and discussed that sublethal heating led to the degradation of lipid and to the decrease in the salt-resistance. These data suggested the importance of lipid components in the cell membrane for the barrier to high osmotic environment, but they did not provide direct information on the obstruction of sodium ion permeation in the growing cells and the lethal effect of salt.

The present investigation was carried out to clarify the energy dependency for the maintenance of intracellular univalent cation contents in the cells grown on the high concentration of salt containing medium and suggested that the energy dependency may be chiefly concerned with the active efflux of intracellular sodium ions in Normal-Staph and the influx of extracellular potassium ions in 10% NaCl-Staph.

MATERIALS AND METHODS

Organism, Growth Media and Conditions of Cultivation: *Staphylococcus aureus* 209P was cultured by gentle shaking at 37°C in the nutrient broth containing NaCl in 145 mM or 1,900 mM. The cells grown in the medium of 145 mM or 1,900 mM NaCl were abbreviated as Normal-Staph and 10% NaCl-Staph respectively. Growth was followed by measuring the absorbance at 650 nm. The anaerobic cultures were performed under the nitrogen gas in the presence or absence of 1% glucose.

Inhibitors: The inhibitors used for this experiment were sodium azide (NaN_3 ; Katayama Chemical Co., Ltd.), monoiodo acetic acid (IAA; Katayama Chemical Co., Ltd.), 2,4-dinitrophenol (DNP; Katayama Chemical Co., Ltd), carbonylcyanide p-trifluoromethoxy phenylhydrazone (FCCP; kindly supplied by Dr. L. Packer, Univ. Calif. Berkely), N-ethylmaleimide (NEM; Nakarai Chemical Co., Ltd.), p-chloromercuri benzoic acid (PCMB; Katayama Chemical Co., Ltd.), 5,5'-dithiobis (2-nitro-benzoic acid) (DTNB; Nakarai Chemical Co., Ltd.) and antibiotics such as gramycidine B (Gra; Calbiochem. Co., Ltd.) and valinomycin (Val; Sigma Chemical Co., Ltd.).

Assay Method for Inhibitors' Effects: Determinations of the per cent growth inhibition were made by the following equation, $100 - \frac{\text{ICM}}{\text{IFM}} \times 100$. ICM and IFM show the integration values of the growth curves of the cells in the media with and without inhibitor in each instance.

Measurements of Intracellular Univalent Cation Contents: The cells on the late-logarithmic stage were harvested, washed twice with physiological saline

solution and resuspended at the concentration of 10 mg wet cells per ml in the nutrient broth which contained 145mM NaCl and 36.7 mM K^+ or in the nutrient broth which contained 1,900 mM NaCl and 36.7 mM K^+ . Cell suspensions were adjusted to pH 7.0 by the addition of NaOH. Cells which were incubated at 37°C for one hr. under the gentle shaking condition were washed at an appropriate time with distilled water and were ashed with 10 ml of 60% perchloric acid. The ashed solutions were diluted and offered for flame analysis. Flame photometry was performed by C_2H_2 -air gas system for flame analysis and by the analytical wavelengths at 5890 Å for Na^+ and at 7664.9 Å for K^+ respectively.

RESULTS

Effect of NaN_3 for the Cell Growth in the Medium Containing High Concentration of NaCl: Growth inhibition with NaN_3 for Normal-Staph and 10% NaCl-Staph is shown in Fig. 1a and 1b, respectively. Sodium azide, an inhibitor for energy metabolism at the concentrations used this investigation, strongly suppressed the growth of 10% NaCl-Staph. Fig. 2 reveals the concentration dependency of NaN_3 for the growth inhibition of Normal-Staph and 10% NaCl-Staph. The concentrations of NaN_3 for 50% growth inhibition

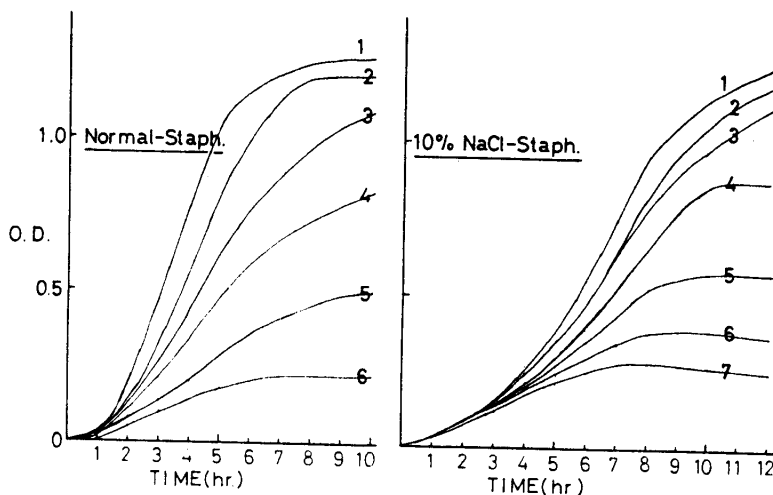


Fig. 1. Growth patterns of Normal-Staph (a) and 10% NaCl-Staph (b) in nutrient broth media containing various concentrations of NaN_3 .

- a) 1: Control containing no inhibitor.
 2: 1 mM NaN_3 , 3: 2 mM NaN_3 , 4: 4 mM NaN_3 ,
 5: 6 mM NaN_3 , 6: 8 mM NaN_3 .
 b) 1: Control containing no inhibitor,
 2: 0.1 mM NaN_3 , 3: 0.2 mM NaN_3 ,
 4: 0.4 mM NaN_3 , 5: 0.6 mM NaN_3 ,
 6: 0.8 mM NaN_3 , 7: 1.0 mM NaN_3 .

were 5.2 and 0.75 mM for Normal-Staph and 10% NaCl-Staph, respectively.

Role of Glycolysis on the Growth in the Medium Containing High Concentration of NaCl: Growth patterns shown in Fig. 3 indicated that both of

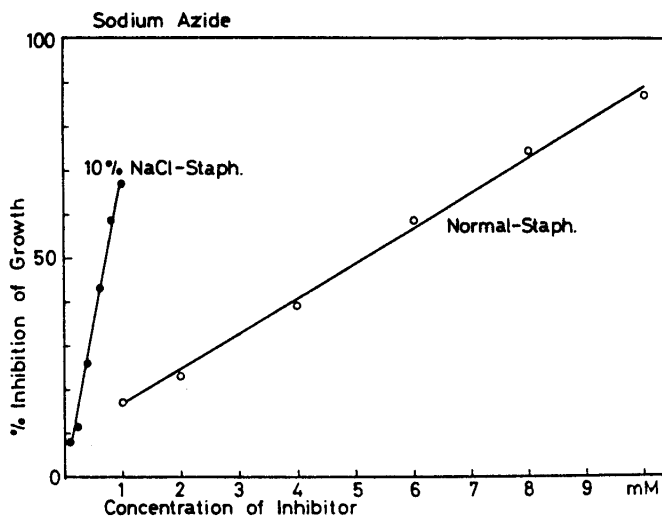


Fig. 2. Inhibitory effect of NaN_3 on the growth of Normal-Staph and 10% NaCl-Staph.

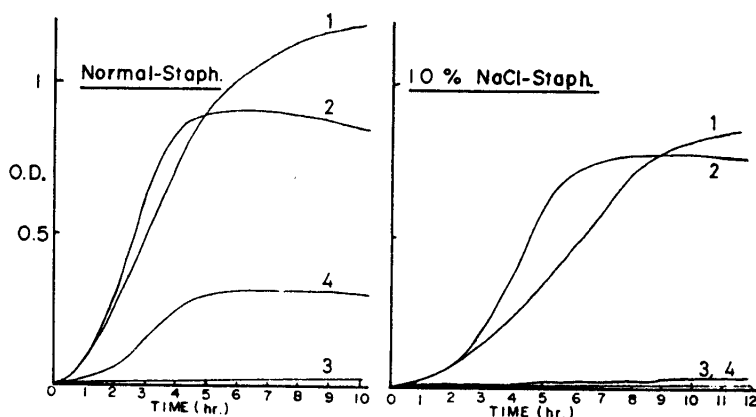


Fig. 3. Growth patterns of Normal-Staph (a) and 10% NaCl-Staph (b) under the aerobic and anaerobic conditions.

- 1: Cultured in the nutrient broth medium under aerobic condition.
- 2: Cultured in the 1% glucose containing nutrient broth medium under aerobic condition.
- 3: Cultured in the nutrient broth medium under anaerobic condition.
- 4: Cultured in 1% glucose containing nutrient broth medium under anaerobic condition.

Normal-Staph and 10% NaCl-Staph could not grow in nutrient broth medium under the anaerobic conditions. But a slight growth inhibition was observed on addition of glucose in the case of Normal-Staph but not with 10% NaCl-Staph. However, the growth of Normal-Staph under this anaerobic condition was very small, and the growth maximum at the stationary phase was also kept low (Fig. 3a). In the medium containing IAA which is particularly active in inhibiting 3-phospho-glyceraldehyde dehydrogenase, 50% inhibition of growth of both Normal-Staph and 10% NaCl-Staph was observed by IAA at the concentration of 100 μ M.

Effects of the Uncouplers on the Growth in the Medium Containing High Concentration of NaCl: The rate of growth was compared between Normal-Staph and 10% NaCl-Staph in the presence of uncouplers such as 2,4-dinitrophenol (DNP) and carbonyl cyanide p-trifluoromethoxy phenylhydrazone (FCCP) in varying concentrations. The sensitivity of 10% NaCl-Staph to these uncouplers was much higher than that of Normal-Staph, and the concentrations of DNP for 50% inhibitions of the growth were 249 μ M and 50.2 μ M for Normal-Staph and 10% NaCl-Staph, respectively. Growth of 10% NaCl-Staph was five times as susceptible to DNP as that of Normal-Staph (Fig. 4). FCCP which was more powerful uncoupler also showed the same the results, in brief the optimal concentrations for 50% inhibition of the growth were 0.65 μ M for 10% NaCl-Staph and 2.35 μ M for Normal-Staph.

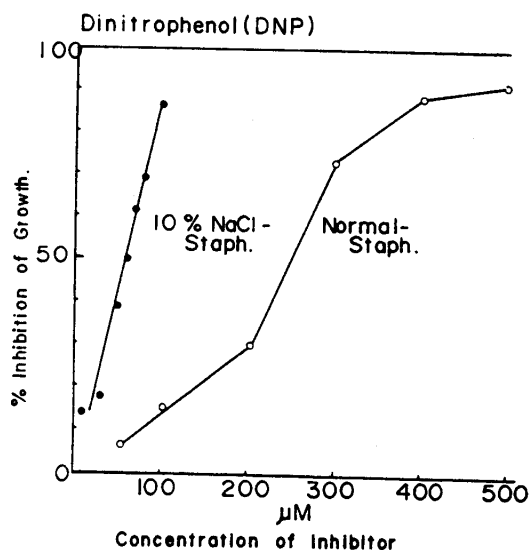


Fig. 4. Inhibitory effects of DNP on the growth of Normal-Staph and 10% NaCl-Staph.

Effects of the Blocking Agents of SH-Groups on the Growth in the Medium Containing High Concentration of NaCl: The 5, 5'-dithiobis (2-nitro-benzoic acid) (DTNB), N-ethylmaleimide (NEM), and p-chloromercuribenzoic acid (PCMB) were used for comparing the effects of SH-group blocking on the growth of Normal-Staph and 10% NaCl-Staph. DTNB did not reveal any effect on the growth of Normal-Staph and 10% NaCl-Staph even at the concentrations of 400 μ M or more. However, NEM and PCMB revealed a marked inhibitions on the growth of Normal-Staph and 10% NaCl-Staph. The concentrations of NEM for the 50% inhibition of growth were 64 μ M and 68 μ M for Normal-Staph and 10% NaCl-Staph respectively as shown in Fig. 5. But the concentration dependency of NEM for the inhibition of growth was not linear. At high concentrations of NEM, the degrees of growth inhibitions were relatively low (Fig. 5) and 100% growth inhibition was not observed even by addition of the inhibitor at more than 400 μ M NEM. The inhibitory pattern of PCMB for the cell growth remarkably differed from the NEM inhibition profile: the growth of 10% NaCl-Staph was inhibited by 23% at a concentration of 30 μ M, and the complete inhibition was observed at 40 μ M PCMB (Fig. 6). On the other hand, Normal-Staph sustained the growth inhibition in proportion to the concentration of

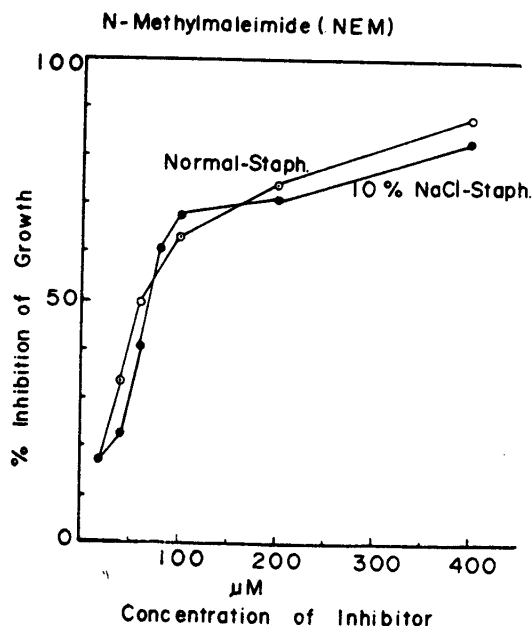


Fig. 5. Inhibitory effects of NEM on the growth of Normal-Staph and 10% NaCl-Staph.

inhibitor and the 10% NaCl-Staph was about twice as sensitive to PCMB treatment as with Normal-Staph.

Effects of Ionophorous Antibiotics on the Growth of Normal-Staph and 10% NaCl-Staph: Ionophorous antibiotics, valinomycin and gramycidin, indi-

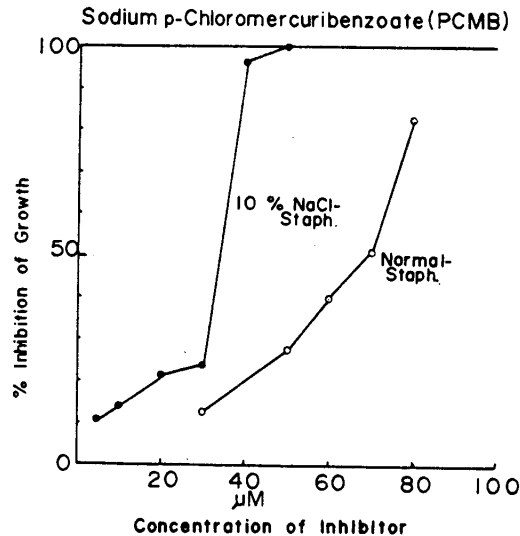


Fig. 6. Inhibitory effects of PGMB on the growth of Normal-Staph and 10% NaCl-Staph.

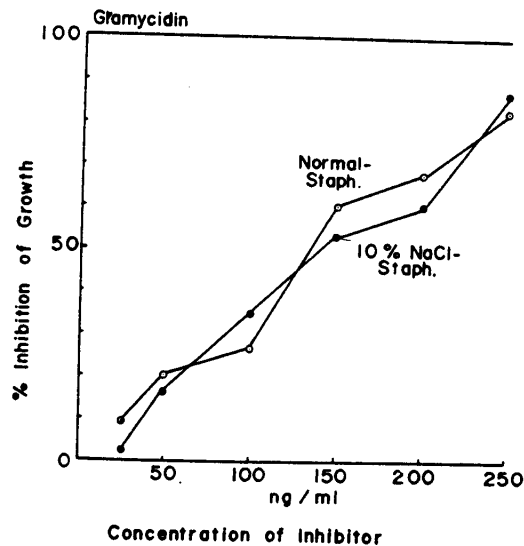


Fig. 7. Inhibitory effects of gramycidin on the growth of Normal-Staph and 10% NaCl-Staph.

cated the peculiar inhibitory effects on the growth of Normal-Staph and 10% NaCl-Staph. Gramycidin revealed similar inhibitory effects under both normal and 10% salinity conditions: namely, 50% growth inhibitions for Normal-Staph and 10% NaCl-Staph were attained at the concentrations of 135 and 140 ng/ml (Fig. 7) respectively. On the other hand, the inhibitory effects of valinomycin on the growth of Normal-Staph and 10% NaCl-Staph were extremely different; there was no inhibition of the growth of 10% NaCl-Staph at the concentration of 300 ng/ml valinomycin which inhibited 50% of Normal-Staph growth as shown in Fig. 8.

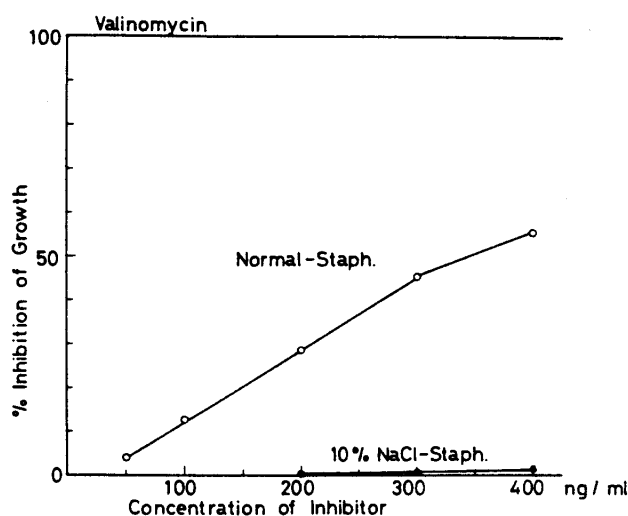


Fig. 8. Effects of valinomycin on the growth of Normal-Staph and 10% NaCl-Staph.

Effects of Various Treatments on the Intracellular Univalent Cation Contents: The intracellular Na^+ contents of Normal-Staph and 10% NaCl-Staph were 12.0 and 152.9 mmoles per Kg wet weight of cells, respectively. And the content of K^+ in 10% NaCl-Staph (90.6 mmoles per Kg wet weight) was lower than that of Normal-Staph (215.8 mmoles per Kg wet weight) as described in Table 1. These intracellular Na^+ and K^+ contents were strongly affected by the addition of various inhibitors, especially the inhibitors of energy transfer reaction. In the case of Normal-Staph, the Na^+ contents were increased 5–13 times, whereas the content of K^+ was slightly decreased by the treatment with these energy transfer inhibitors (Table 1).

Generally it was considered that the intracellular Na^+ is pumped out to the outside of the cell by using the mechanism of active transport coupled

Energy Dependency on Salt-resistance of *S. aureus*

TABLE 1 UNIVALENT CATION CONTENTS OF CELLS AFFECTED BY VARIOUS TREATMENTS

Cells	Normal-Staph					10% NaCl-Staph				
	145 mmoles Na ⁺		36.7 mmoles K ⁺		K ⁺ /Na ⁺	1,900 mmoles Na ⁺		36.7 mmoles K ⁺		K ⁺ /Na ⁺
Treatment	A	R ₁	B	R ₂		A	R ₁	B	B ₂	
Before Treat.	12.0	0.08	215.8	5.88	18.0	152.9	0.08	90.6	2.47	0.60
Control 37°C, 1 hr.	8.5	0.06	215.9	5.88	22.9	107.8	0.06	215.4	5.87	2.00
+DNP (3.75 mM)	48.5	0.33	168.4	4.59	3.5	182.4	0.10	5.3	0.14	0.03
+PCMB (500 μM)	119.9	0.83	134.6	3.67	1.1	121.8	0.06	4.6	0.13	0.04
+Gra (200 ng/ml)	65.6	0.45	112.5	3.07	1.9	225.8	0.12	5.4	0.15	0.02
55°C, 30 min	66.4	0.46	136.9	3.37	2.1	179.5	0.09	22.0	0.60	0.12

A: Na⁺ mmoles per Kg wet weight of cells.B: K⁺ mmoles per Kg wet weight of cells.R₁: Intracellular Na⁺ contents to extracellular Na⁺ contents.R₂: Intracellular K⁺ contents to extracellular K⁺ contents.

with the uptake of K⁺ from the outside to the inside. Therefore, calculations were made of the ratio of intracellular cation contents to extracellular cation contents (R) concerned with K⁺ and Na⁺, expressed as the energy dependency of intracellular ion contents. The ratio of intracellular Na⁺ contents to extracellular Na⁺ contents (R₁) in Normal-Staph increased by the treatment of the cell with various inhibitors for energy transfer reaction. Against remarkable changes of R₁ value, the decrement of R₂ (intracellular K⁺ contents to extracellular K⁺ contents) values were quite small. Of course, the K⁺/Na⁺ was decreased by such a treatment because of the increased content of intracellular Na⁺. In the case of 10% NaCl-Staph, the tendency of the change in these values of R₁ and R₂ was reversed: i. e. no change in the value of R₁ and a remarkable decrease in R₂ by the disturbances in the energy transfer reaction. Therefore, the ratio of K⁺/Na⁺ also decreased by these treatments because of the decrement in R₂ or intracellular K⁺ content. From these data it was concluded that energy dependency to pump out the Na⁺ from the inside to the outside is very high in the case of Normal-Staph but for 10% NaCl-Staph the dependency is high for active accumulation of K⁺.

Incubation of the cells at 55°C for 30 min, which was used for the implication of barrier systems, also increased the intracellular potassium ions in both treated Normal-Staph and 10% NaCl-Staph (Table 1).

DISCUSSION

Staphylococcal cells have resistance to a high concentration of saline environments so that they can grow in such a high osmotic condition. Such a salt tolerance has been discussed by many investigators, but the explanations for the mechanism of the cell growth at a high concentration of salt were focused on the permeation barrier systems of the cell membrane (1, 2, 3). With this point in mind, the present investigation was carried out to clarify the energy dependency concerning the maintenance of intracellular univalent cation contents of cells to explain such resistant mechanisms.

The energy dependency of the growth of *S. aureus* under high salinity condition was disclosed by comparing the cell sensitivity to the various kind of inhibitors for energy transfer reactions or by comparing the cell growth rates under the anaerobic condition. In the comparisons of Normal-Staph and 10% NaCl-Staph, it was observed that the 10% NaCl-Staph had a high sensitivity to the various inhibitors such as NaN_3 , DNP, FCCP and PCMB (Fig. 1, 2, 4, and 6). These inhibitors are particularly active in inhibiting the energy transfer reaction, thus the above-mentioned results suggest that *S. aureus* has a strong energy dependency in the case of the growth under highly concentrated saline conditions. By the consideration of the classes of inhibitors and of concentration of inhibitors, it is indicated that the energy sources of these energy dependency lie on the respiration (Figs. 1, 2, 4, and 6). And the similar observations were obtained in the comparison of the growth of Normal-Staph and 10% NaCl-Staph under the anaerobic glycolytic condition (Fig. 3).

KATAYAMA *et al.* (4) previously reported that *S. aureus* cells grown in the semi-synthetic medium containing the high concentration of NaCl maintained constantly by the intracellular univalent cation. In the present investigation, however, 10% NaCl-Staph cultured in the medium of nutrient broth containing 10% NaCl revealed a high intracellular Na^+ content as compared with that of Normal-Staph. But the values of R_1 in both Normal-Staph and 10% NaCl-Staph were 0.10 and 0.09, respectively (Table 1). This suggested that Staphylococcal cells had a relative constancy of intracellular cation against the environmental salinity. CHRISTIAN and WALTHO (5) reported a high Na^+ content in Normal-Staph (98 ± 20 mmoles/Kg cell water) grown in the brain plus heart infusion broth. Then, the high Na^+ contents in 10% NaCl-Staph may be said to aid in maintaining the level of Na^+ contents constant.

In both Normal-Staph and 10% NaCl-Staph, the disturbances in the intracellular cation constancy by ionophorous antibiotics such as gramicidin and valinomycin (Figs. 7 and 8) induce the death of cells, because under

these conditions intracellular univalent cation contents are changed to have higher Na^+ and lower K^+ contents (Table 1) except in the case of valinomycin in high salinity conditions (Fig. 8). The ineffectiveness of valinomycin in 10% NaCl-Staph suggests the inactivation of valinomycin action by the composition changes of membrane lipids in 10% NaCl-Staph (1) or the interference of K^+ leakage through the valinomycin channel from the cell by high concentration of Na^+ in medium. Thus, the existence of constancy of such intracellular cation content is essential for the growth in the high concentration of salt containing medium, and the maintenance of this constancy probably depends on the energy derived from the respiration.

As shown in Table 1, 10% NaCl-Staph has an adaptively developed barrier for the environmental Na^+ and has a small energy dependency for the pumping-out of Na^+ , because the intracellular Na^+ contents are slightly increased by the treatment of the cell with various inhibitors for energy transfer reaction. On the other hand, the comparisons of intracellular K^+ contents between Normal-Staph and 10% NaCl-Staph suggest that a large amount of energy is required for the maintenance of intracellular K^+ contents in 10% NaCl-Staph, because cessation of energy supply by treatment of various inhibitors brought about the K^+ release from the inside of the cell to the outside. Then, these results may be interpreted that the two types of ion active transport as demonstrated by the treatment of various inhibitors chiefly consist of Na^+ transport in Normal-Staph and K^+ transport in 10% NaCl-Staph, and that 10% NaCl-Staph also possesses a barrier system against the extracellular Na^+ .

The significance of high contents of K^+ has been reported and the discussions have been carried on the mechanisms of the accumulation by many investigators. HAROLD *et al.* (6, 7, 8, 9) reported the usage of the energy produced by the hydrolysis of ATP for the accumulation of K^+ and the extrusion of Na^+ and H^+ in the glycolyzing cells of *Streptococcus faecalis*. And they also stated that K^+ accumulation is essential for the growth. In *S. aureus*, GROSS *et al.* (10) reported the adenosine triphosphatase activity which was Mg^{++} -dependent and K^+ or Na^+ -stimulated. However, they did not clearly demonstrate the ATPase related to the active transports. Sensitivity to the SH-blocking agents observed in Normal-Staph and 10% NaCl-Staph was discussed in relation to the K^+ transport as reported by CHO *et al.* (11) in *Mycoplasma laidlawii*. Their studies indicated that K^+ transport was energy dependent and appeared to be mediated by sulfhydryl-containing proteins. For the other mechanisms of ion transport, JEACOCKE *et al.* (12) measured the respiration-driven proton translocation and they stated that transmembrane potential observed in the respiring cells was utilized for the formation of a

resultant K^+ -gradient across the membrane. This mechanism is very attractive for the explanations of outflux of Na^+ and influx of K^+ in maintaining the intracellular cation constancy. The results in author's investigation also suggested the possibility of the existence of this mechanism, because 10% NaCl-Staph proved to be very sensitive to the uncouplers as compared with Normal-Staph.

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