Studies on the Marine Bacteria—III. On the Effect of Minerals on the Lysis of Bacteria in Hypotonic Medium*

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In the previous reports^{1),2)}, the authors found that in comparison with terrestrial bacteria, the marine bacteria require not only NaCl, but also some amount of K-, Mg-, and Ca-salt for their growth. The minerals required by bacteria may be an activator of the enzymes by which many metabolisms are carried out during the bacterial growth. On the other hand, the minerals may play as a controller of permeation of metabolites passing through the surface structure of bacteria, and the minerals also may concern with the maintenance of bacterial body. In this study, the experiment was mainly carried out to resolve the relationships between the body maintenance and some minerals.

Many species of marine bacteria are lysed in the suspension of sea water diluted extremely with distilled water. Early workers ascribed the effect to the lowered osmotic pressure of the suspending medium^{3),4)}. In a study of 96 isolates of marine bacteria, TYLER *et al.*⁵⁾ observed that in major cases, the suspensions of cells were susceptible to a loss of optical density in distilled water. MACLEOD and MATULA⁶⁾ found that five marine bacteria differed considerably in the lytic susceptibility. They noted differences in the capacities of different salts to prevent lysis of marine bacteria. SUD *et al.*⁷⁾ described that the cell walls of marine bacteria may be different in nature from those of non-marine bacteria and such differences reflect variations in the chemical nature of the walls. In this experiment, using three different mineral requiring bacteria, the authors studied the effect of minerals on the lysis of bacteria.

Materials and Methods

Different types of bacteria²⁾; 1055-1 (Marine type), *Vibrio parahaemolyticus* (Halophilic type), and *Pseudomonas fluorescens* (Terrestrial type) were used for comparison of lytic susceptibility, and their mineral requirement was shown in Table 1. The culture media of these bacteria were as follows; Medium 2216E agar⁸⁾ for 1055-1, 3% NaCl-nutrient agar for *V. parahaemolyticus*, and nutrient agar for *P. fluorescens*.

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All of the bacteria tested were subcultured at 25°C for 24 hours. The cells were harvested from the agar plates and were heavily suspended in the artificial sea water (ASW) diluted three fold. Prior to measurement of the lytic susceptibility, the heavy suspensions of test bacteria were adjusted to give an optical density of 0.8 at 540 $m\mu$ in a spectrophotometer when diluted 1:99 in ASW. Successively, for determination of lytic susceptibility, the heavy suspensions of 0.1 ml were added to 9.9 ml of each test solution, and then, the test suspensions were incubated at 25°C for one hour. The lytic susceptibility was observed by the turbidity decrease, the restoration of turbidity, and the release of ultraviolet-absorbing materials from the cells. The turbidity as optical density at 540 $m\mu$ of the suspensions were measured

Table 1. Test organisms and their mineral requirements.

Organism	Source	Range of NaCl concn. (M) for growth*			Salts required for growth	Type
		min.**	opt.***	max.**		
1055-1 (<i>Pseudomonas</i> sp.)	Sea water 48°00'N-170°00'E; Depth, 694m)	0.04	0.8	2.5	Na- and Mg-or Ca-salt	Marine (M-)
<i>Vibrio</i> <i>parahaemolyticus</i> (0-5)	Feces of the patients of raw fish meat poisoning	0.05	0.5	1.7	Na-salt	Halophilic (H-)
<i>Pseudomonas</i> <i>fluorescens</i>	Fresh-water	0.00	0.17	1.0	none	Terrestrial (T-)

*: With the other salts at ASW level.

**: Minimal and maximal salt concentrations tolerating recognizable growth during 10 days incubation at 25°C.

***: Optimal salt concentration for growth during 3 days incubation at 25°C.

Composition of ASW (HERBST's artificial sea water): NaCl, 0.51 M; KCl, 9.8 mM; MgCl₂, 0.052 M; MgSO₄, 0.021 M; CaCl₂, 7.3 mM; pH 8.0. Total molarity of salts=600 mM)

at zero hour incubation (*T-0*) and again were measured after one hour incubation (*T-1*). After reading the final turbidity of the suspension, the restoration of turbidity above mentioned was observed in the suspensions that mineral concentration was adjusted to ASW level and the final volume was prepared to 20 ml. The lysis of bacteria in such suspensions, of course, stopped. The turbidity of stopped suspension (*T-stop*) was compared with that of control which has been suspended in ASW. Next, the suspensions were used for measuring the release of U.V.-absorbing materials from the cells. That is, the suspensions were then centrifuged at 18,000×*G* for 10 minutes. The U.V.-absorption of the clear supernatant obtained was measured at 260 $m\mu$. In this study, all of the ASW used were prepared according to HERBST's formula (See Table 1).

Results

Turbidity changes of bacteria in ASW and distilled water. The cells of each

bacterium were suspended in the both ASW and distilled water respectively, and the suspensions were subjected to the observation of turbidity changes during the suspending. In the successive experiments, these suspensions were used for a control in order to determine the lytic susceptibility of bacteria in various dilutions of different salts and the relationships between susceptibility and hypotonicity. The results obtained were shown in Fig. 1.

The turbidimetric difference observed in the both suspensions was remarkable as shown in Fig. 1. Namely, in ASW the turbidity decrease was not observed within one hour incubation in all bacteria tested. On the other hand, in distilled water most remarkable decrease of turbidity was observed at the onset of suspending in all bacteria tested, and successive decreases during one hour incubation were also observed to be variable according to bacterial species. Practically, the turbidity of *P. fluorescens* did not decrease, although that of the both *V. parahaemolyticus* and 1055-1 decreased with the lapse of incubation time.

Lytic susceptibility of bacteria in various concentrations of ASW. The lytic susceptibility of bacteria suspended in various concentrations of ASW was observed in the suspensions after one hour incubation at 25°C. The results obtained were shown in Fig. 2.

In all bacteria tested, the suspensions showed a decrease in turbidity as the ASW concentration decreased. In suspensions of *P. fluorescens*, the both *T-0* and *T-1* were completely identical under the various concentrations, and the *T-stop* was also identical in all concentrations, in the same way, the release of U.V.-absorbing materials from the cells could not be observed. As compared with the lytic susceptibility of *P. fluorescens*, those of the both *V. parahaemolyticus* and 1055-1 were distinguishable between the *T-0* and the *T-1* at 10 mM or less. Furthermore, the *T-stop* was partially decreased below 10 mM after one hour incubation. This decrease seemed to be associated with an increase in the U.V.-absorbance of the supernatant.

Lytic susceptibility of bacteria in various concentrations of NaCl. The lytic susceptibility of bacteria suspended in various concentrations of NaCl was assessed by the method previously described. The results were shown in Fig. 3.

The effect of NaCl solution on the lysis of *P. fluorescens* was similar to that of ASW, that is, the bacterium was not susceptible to lysis in all concentrations. But

Fig. 1. Turbidity changes in bacterial suspensions with both ASW and distilled water during incubation at 25°C.

V. parahaemolyticus was markedly affected by the lower concentration than 100 mM. In this case, noticeable difference was observed between the *T-0* and the *T-1*.

Fig. 2. Effect of ASW concentration on the lysis of bacteria after one hour incubation at 25°C.

- T-0: Turbidity (O.D. at 540 $m\mu$) of suspension at 0 hour incubation.
- T-1: Turbidity (O.D. at 540 $m\mu$) of suspension after 1 hour incubation.
- T-stop: Turbidity (O.D. at 540 $m\mu$) of suspension stopping the lysis.
- U.V.: U.V.-absorption (O.D. at 260 $m\mu$) of supernatant.

These symbols are applied from Fig. 2 to Fig. 4.

Fig. 3. Effect of NaCl concentration on the lysis of bacteria after one hour incubation at 25°C.

Cont.: Control (ASW).

Symbols: See Fig. 2.

Furthermore, the turbidity of the suspensions was not restored by increasing the salt concentration. The behaviors of *V. parahaemolyticus* above mentioned demonstrate the loss of restorable ability of the turbidity caused by suspending in dilute solution. Similarly, the release of the U.V.-absorbing materials from the cells also increased according with the decrease of NaCl concentration in the suspension. At the same dilution, as compared with ASW higher release of the U.V.-absorbing materials was observed in NaCl solution. In case of *1055-1*, noticeable lysis was ascertained by the turbidity decrease, the restoration of turbidity, and the U.V.-absorbance of the supernatant was observed in concentrations of 100 mM or less, the highest lysis being observed at 10 mM. And their lysis at 10 mM of NaCl was much greater than that in distilled water. Therefore, the lysis of the bacterium in NaCl solution may be due not only to hypotonic tension, but also to other physicochemical effects. In this experiment, the lytic effect of KCl was identical with that of NaCl.

Lytic susceptibility of bacteria in various concentrations of $MgCl_2$. The lytic susceptibility was observed using various concentrations of $MgCl_2$ solution. The

turbidity decrease, the restoration of turbidity, and the U.V.-absorbance of the supernatant were shown in Fig. 4. As shown in the figure, the decrease of the *T-0* in various concentrations of $MgCl_2$ were similar to that of ASW in all bacteria tested. The *T-1* seemed to be higher than the *T-0* in various concentrations with a few exceptions. In case of the both *V. parahaemolyticus* and 1055-1, the relative value of the both *T-0* and *T-1* was converse at 1 mM or less. In such solutions, the *T-stop* also decreased. Similarly, the increase of the U.V.-absorbance of the supernatant was observed in 1 mM or less. It is interesting to suppose the following presumption that $MgCl_2$ solution of 1 mM concentration over has a preventive effect on the lytic susceptibility of bacteria. In this experiment, the protective effect was demonstrated not only in $MgCl_2$, but also in $CaCl_2$.

Fig. 4. Effect of $MgCl_2$ concentration on the lysis of bacteria after one hour incubation at 25°C.
Cont.: Control (ASW).
Symbols: See Fig. 2.

Discussion

In the present experiment, it was ascertained that the turbidity of bacterial suspension in hypotonic medium decreased more markedly in accordance with the environmental hypotonicity. In all bacteria examined, the highest decrease of turbidity was observed at the onset of suspending in hypotonic medium. To the contrary, successive decrease during the incubation might be variable according to bacterial species. The observation of turbidity decrease occurred initially only by transferring the cells into the hypotonic medium may be available in assuming the following notion; the bacterial cells suspended in a hypotonic medium swell by absorbing water and their volume increase, and simultaneously the swelled cells begin to lose their own reflex. The phenomena above mentioned are introduced only the decrease of refractive index by the osmotic swelling. Accordingly, for estimation of the lytic degree of bacteria in hypotonic medium, it might not be appropriate to apply the turbidimetry, since the apparent turbidity decrease distrubs the real measurement of lysis. In this experiment, the extent of the difference between the *T-0* and the *T-1* in each suspension seemed to be proportional to the degree of the *T-stop* decrease and the release of U.V.-absorbing materials from the cells. Furthermore, it was found that there are two types of restoration of the lost turbidity, one is the complete restoration, and the other is partial restoration. In the former, the turbidity decrease

is a decrease of refractive index caused by osmotic swelling of bacterial cells in a suspending medium, but not the lysis of the cells, therefore, if the salt concentration was restored to ASW level, the optical density of it was also restored to that of control which has been suspended in ASW. In the latter, the decrease of turbidity may be due to the lysis of cells by physicochemical action, the activity of an enzyme, or the both activity, therefore, the decrease is not able to restore completely. By the notion above mentioned, the lysis of bacterial cells in hypotonic medium is correlated to the extent of the difference between the *T-0* and the *T-1*.

Among three bacteria, the lysis of *P. fluorescens* could not be observed in any concentrations of minerals used. In comparison with the lysis of *P. fluorescens*, that of *V. parahaemolyticus* was affected by mineral concentrations, being more increased in the decrease of mineral concentration. The lytic behavior was generally observed in various minerals, however, there were differences between monovalent and divalent cations. In NaCl or KCl, the lysis occurred in 100 mM concentration or less, but in MgCl₂ or CaCl₂, it occurred below 1 mM concentration. The 1055-1 was more susceptible to lysis in hypotonic medium than other two types of bacteria tested. The bacterium was lysed slightly in 600 mM of NaCl or KCl solution, and the lytic activity in these solutions was observed below 100 mM, the highest being in 10 mM. The lytic activity of 10 mM gives rather higher activity than distilled water. The lytic activity of the salts at the concentration above mentioned may presumably be due to stimulative action on the lysis of marine bacteria. Furthermore, the both solutions, MgCl₂ and CaCl₂ of 1 mM or more were rather protective against the lysis of bacteria. It was ascertained that sea water diluted 50 to 100 fold was much effective to prevent the lysis of bacteria examined.

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