Studies on the Marine Bacteria—IV. Comparison of Dissolutions of Bacteria in Hypotonic Medium*

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(Received April 15, 1968)

It is well known that in Gram-negative bacteria, there is a spectrum of susceptibility to lysis ranging from organisms requiring high salt concentration to prevent disruption of the cells to those maintaining their integrity in distilled water. Many species of marine bacteria when suspended in distilled water may lyse, while terrestrial bacteria do not. This may be at least one criterion enabling marine bacteria to distinguish from most land forms.

The lytic susceptibility of those bacteria was practically manifested by the turbidity decrease and the release of cell materials resulting from their brief exposure to hypotonic medium.¹⁻⁴⁾ BUCKMIER and MACLEOD⁵⁾ demonstrated that the suspension of a marine pseudomonad showed a decrease in turbidity as the salt concentration lowered, and that the turbidity decrease occurred mostly within 10 seconds after suspending, and there was only a slight decrease in turbidity in 30 minutes. In the previous report,⁴⁾ the authors ascertained that the turbidity of several Gram-negative bacteria decreased distinctly as soon as the bacterial cells were suspended in the hypotonic medium, and discussed that the turbidity decrease was due to the change in refractive index resulting from osmotic swelling of bacterial cells in the suspensions. Furthermore, the authors found that the turbidity once lost in the hypotonic medium was restored by adjusting salt concentration in the medium to isotonic concentration, and that such a restoration of turbidity was variable according to bacterial species. In this experiment, the authors made a detailed study of the relationship between the restoration of turbidity and the dissolution of bacterial cells in the hypotonic medium.

Experimentals

The bacteria used were exactly the same as those in the previous report,⁵⁾ and they are 1055-1 (Marine type), *Vibrio parahaemolyticus* (Halophilic type), and *Pseudomonas fluorescens* (Terrestrial type). In order to ascertain the dissolution of bacteria suspended in the hypotonic medium, the turbidity decrease, the release of ultraviolet-absorbing materials from cells, the restoration of turbidity, and the decline of via-

* This study was partly supported by a grant from the Scientific Research Found of the Ministry of Education.

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bility were measured by the methods previously reported.⁵⁾ Especially, in this experiment, above-mentioned four parameters were examined in the suspensions which were incubated for varying periods of time at 25°C after suspending each of the bacteria in distilled water, and the results were compared with those of a control suspension in artificial sea water (ASW). The restoration of turbidity was observed in each suspension whose mineral concentration was adjusted to ASW level or 0.6 M of NaCl by adding the same volume of double-strength ASW or 1.2 M NaCl solution to the bacterial suspensions in distilled water. The number of viable cells was estimated by the plate count method in the suspensions which were adjusted to ASW level. The media used for plate counting were the optimal agar for the growth of respective bacteria. The cultures were carried out at 25°C for 6 days before colony counts were made.

Results

Dissolution of bacterial cells in distilled water during incubation. Each of the test bacteria was suspended in distilled water according to the method described in

Fig. 1. Changes in parameters with relation to dissolution of 1055-1 suspended in distilled water during incubation at 25°C.

C: Control suspension in ASW

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 in ASW = 0.6 M)

$$\oplus: \text{Turbidity (\%)} = \frac{\text{O.D. at } 540 \text{ m}\mu \text{ of test suspn.}}{\text{O.D. at } 540 \text{ m}\mu \text{ of control suspn.*}} \times 100$$

$$\odot: \text{Viable count (\%)} = \frac{\text{Viable count in test suspn.}}{\text{Viable count in control suspn.**}} \times 100$$

$$\bullet: \text{Release of cell materials (\%)} = \frac{\text{O.D. at } 260 \text{ m}\mu \text{ of the supernatant fluid of test suspn.}}{\text{Total O.D. at } 260 \text{ m}\mu \text{ of control suspn.***}} \times 100$$

*: It was read 0.8 in test tube having a diameter of 18 mm.

**: It was counted 10⁹ cells in one ml of the suspension.

***: It was measured with Multipurpose Spectrophotometer (Shimadzu Seisakusho Ltd., Japan) and was read a total of 1.25.

the previous section, and the dissolution of bacterial cells was also observed by the three methods already described. The results obtained are shown in Fig. 1 through 3.

As shown in Fig. 1, in the suspension of 1055-1, the turbidity decreased rapidly just after suspending; then it decreased gradually with the lapse of incubation time. The suspended cells released a part of cell materials soon after suspending, and the release increased in proportion to the lapse of incubation time. The extent of the leakage in supernatant fluid after 5 hours of incubation amounted nearly to 65 per cent of the total content in the suspension. The viable counts did not decrease in the suspensions during the first 10 minutes of incubation, followed by a marked decrease in the number with the lapse of incubation time, and almost all cells were killed after incubating for 5 hours.

As shown in Fig. 2, the changes of the three parameters with relation to dissolution of *V. parahaemolyticus* were similar to those of 1055-1, but their extents were less distinguished than those of 1055-1. Namely, the U.V.-absorbance of the supernatant fluid of test suspension after 5 hours of incubation was about 40 per cent of the total absorbance in the suspension, and the viable counts at the same time reached to 0.01 per cent or so of initial count.

Fig. 2. Changes in parameters with relation to dissolution of *V. parahaemolyticus* suspended in distilled water during incubation at 25°C.
Symbols; See Fig. 1.

Fig. 3. Changes in parameters with relation to dissolution of *P. fluorescens* suspended in distilled water during incubation at 25°C.
Symbols: See Fig. 1.

In Fig. 3, the turbidity of *P. fluorescens* decreased remarkably just after suspending, but successive changes of the parameters with relation to dissolution of the bacterium were not observed during 5 hours of incubation. These results suggested that *P. fluorescens* did not received any harm in distilled water.

Correlation between the dissolution of cells and the restoration of turbidity during incubation. The restoration of turbidity was measured in the suspensions of the test bacteria in distilled water during incubation. The results obtained are

Table 1. Restoration rates of turbidity by adjusting mineral concentration in the bacterial suspensions to ASW level and 0.6 M of NaCl.

The suspensions were incubated for varying periods at 25°C after suspending each of bacteria in distilled water.

Incubation time (min.)	Restoration of turbidity (%)*					
	1055-1		<i>V. parahaemolyticus</i>		<i>P. fluorescens</i>	
	Adjusting the conc. of mineral in the suspns. to					
	ASW	0.6 M NaCl	ASW	0.6 M NaCl	ASW	0.6 M NaCl
0	100	100	100	100	100	100
10	100	85	100	100	100	100
30	94	75	92	92	100	100
60	77	62	78	78	100	100
120	65	35	67	67	100	100
180	42	27	58	58	100	100
(Control)	100	—	100	—	100	—

* Restoration of turbidity (%) = $\frac{\text{O.D. at } 540 \text{ m}\mu \text{ of test suspn. adjusted}}{\text{O.D. at } 540 \text{ m}\mu \text{ of control suspn. adjusted}} \times 100$

Control was suspended in ASW.

shown in Table 1.

In case of 1055-1, a marked difference with regard to the restoration rates of turbidity was observed between the suspensions adjusted to ASW level and those adjusted to 0.6 M of NaCl. That is, if the mineral concentration in the bacterial suspension was adjusted to ASW level within 10 minutes of incubation, its restoration rate would be 100 per cent. However, in the suspension adjusted to 0.6 M of NaCl, the same rate was only obtained at zero minute of incubation. The restoration rates of the bacterial suspensions incubating for varying periods decreased with the lapse of incubation time. These decreases were more remarkable in the suspensions adjusted to 0.6 M of NaCl than those adjusted to ASW level. In case of *V. parahaemolyticus*, the restoration rates of the bacterial suspensions incubating within 10 minutes were 100 per cent, while after incubating for 10 minutes, they decreased gradually with the lapse of incubation time. No appreciable difference was observed in the restoration rates between in the suspensions adjusted to ASW level and to 0.6 M of NaCl. On the other hand, the restoration rates of *P. fluorescens* incubating for varying periods were 100 per cent at every incubation time.

Discussion

Bacteriolysis, the dissolving of bacterial cells, is demonstrated in various bacteria. ABRAM and GIBBONS⁶⁾ proposed that sodium was required to maintain the cell walls of the halophile *Halobacterium cutirubrum*. BUCKMIRE and MACLEOD⁵⁾ concluded that a polyelectrolyte in the surface of cells of a marine pseudomonad was settled by

cross linking with divalent cations, therefore the deficiency of the divalent cations in the suspending medium might cause the bacteriolysis. As an illustration of terrestrial bacteria which do not lyse in distilled water, ASBELL and EAGON⁷⁾ reported that *Pseudomonas aeruginosa* could be induced to lyse in the incubation mixture containing the metal-binding agent ethylenediaminetetraacetic acid, and that the divalent cations were important for maintenance of rigid surface structure. It may safely be assumed that the divalent cations play an important role in the bacteriolysis. On the other hand, BROWN⁸⁾ emphasized the enzymatic reaction on the bacteriolysis.

In the present examination, the bacterial turbidity in distilled water decreased rapidly just after suspending as compared with that in ASW, nevertheless, the turbidity once decreased was restored completely by adjusting mineral concentration in the suspension to ASW level, and the decrease in viable counts could not be observed in the same suspension. Therefore, the rapid decrease in turbidity just after suspending in hypotonic medium do not show the degree of bacteriolysis. In both of the suspensions of 1055-1 and *V. parahaemolyticus* in distilled water, a part of cell materials was released immediately after suspending. The released cell materials might be derived from the surface structure of cell in which the materials were contained as an accessory, because such a release did not affect the viability of cells in the suspension. The release of cell materials just after suspending was limited to the both 1055-1 and *V. parahaemolyticus*, but it was not observed in *P. fluorescens*. The authors considered that the former bacteria are easy to receive a damage from the osmotic shock since their habitat is the hypertonic environment. Thus, a hypotonicity may make their surface structure loose, and the U.V.-absorbing materials in the structure may diffuse into the hypotonic environment. The viable counts in the both suspensions of 1055-1 and *V. parahaemolyticus* began to decrease after incubating for 10 minutes from suspending, successively a distinct decrease being observed with the lapse of incubation time. Such a tendency during incubation was also found in the restoration rates which observed on the bacterial suspensions in distilled water.

From the observation on the restoration of turbidity, the restoration effect of ASW was more conspicuous than that of 0.6 M of NaCl when applied to the suspensions of 1055-1, while similar effects were noted on the suspensions of *V. parahaemolyticus* with either ASW level or 0.6 M of NaCl. Only with living whole-cells, the following phenomenon may be seen; the turbidity of bacterial suspension in a hypotonic medium was restored by adjusting the medium to isotonic with mineral solutes⁹⁾. In case of 1055-1, the restoration rates of turbidity showed a decided difference between the suspensions adjusted to ASW level and those adjusted to 0.6 M of NaCl. The difference in restoration rate between the two adjustings may be due to partial damage to the cytological structure of the bacterium.

Seemingly the bacteriolysis are complicated; in general, it is caused by physicochemical reaction accompanied with enzymatic reaction. The dissolution of 1055-1 in distilled water may be due to a damage of an individual cell inflicted by the deficiency of the divalent cations in the medium, while in case of *V. parahaemolyticus* it may rather be due to the plasmoptysis of cells in the hypotonicity. The dissolution of cells in distilled water could not be observed in *P. fluorescens*.

Summary

The dissolutions of cells of marine and terrestrial bacteria in distilled water were manifested by the turbidity decrease, the release of cell materials, the restoration of turbidity, and the decline of viability. The three test bacteria showed a rapid decrease of turbidity immediately after suspending. The rapid decrease in turbidity did not mean the degree of bacteriolysis. The restoration rates of turbidity by adjusting the mineral concentration in the suspending medium to ASW level were correlated with the viability of cells in the suspensions indicating the degree of damage of cells. The dissolution of 1055-1 in distilled water may be due to a damage of an individual cell inflicted by the deficiency of the divalent cations in the medium, while in case of *V. parahaemolyticus* it may rather be due to the plasmoptysis of cells in the hypotonicity. The dissolution of cells in distilled water could not be observed in *P. fluorescens*.

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