Studies on the Interaction between \( \phi {BA1} \) of Bacillus aneurinolyticus and Its Killing-Sensitive Host

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Abstract In order to investigate the mechanism of killing of \( \phi {BA1} \) against several strains of Bacillus aneurinolyticus, the possibility of \( \phi {BA1} \) to propagate on Bacillus aneurinolyticus KA10, which was one of the killing-sensitive hosts of the phage, was examined. Electron microscopy exhibited no propagation of mature particles of \( \phi {BA1} \) in KA10 cells examined. Endogenous high molecular weight bacteriocin lysogenized in KA10 neither conferred immunity against \( \phi {BA1} \) nor was induced in the course of killing by \( \phi {BA1} \). Phenomena similar to Weigle-reactivation in Escherichia coli of \( \phi {BA1} \) in UV-irradiated productive host (KA23) could be observed, whereas no enhancement of killing activity was observed in the killing-sensitive host. Those results taken together, there was no indication of the expression of \( \phi {BA1} \) genome in the cell of killing sensitive host KA10. Thus the possibility of propagation of \( \phi {BA1} \) in B. aneurinolyticus KA10 was ruled out in the present experimental condition.

Key words: Bacillus aneurinolyticus, bacteriocin, bacteriophage, immunity, reactivation.

Introduction

As reported in a previous paper\(^1\), a temperate phage (\( \phi {BA1} \)) of Bacillus aneurinolyticus showed killing activity together with plaque-forming activity with strain specificity. Since the killing by a phage such like \( \phi {BA1} \) showing two activities described above has not been known, it is of interest to make clear any event that occurs during the course of killing even though a large number of investigation have been reported on interaction between bacteriophage and host cell\(^2\)-\(^4\). On the other hand, B. aneurinolyticus strains which show killing sensitivity carried one or more lysogenized bacteriocins of high molecular weight\(^\circ\). Although the role of a bacteriocin in its host cell is not understood in any bacteria, a possibility concerning with the resistance of host cell to an invading phage has been suggested in some bacteria\(^9\). In this study, the interaction between \( \phi {BA1} \) and its killing-sensitive (KA10) or phage-productive (KA23) host cells were examined morphologically and biochemically.
It was also studied whether the lysogenized high molecular weight bacteriocin had some inhibiting effect on the propagation of \( \Phi \) B A1 in the cell of killing-sensitive host.

**Methods**

Microorganisms

*Bacillus aneurinolyticus* KA2 and KAS232 were used as sensitive strains to the high molecular weight bacteriocin produced by *B. aneurinolyticus* KA10 (aneurinol 10). KA10 was used as the sensitive strain to the killing action of \( \Phi \)BA1, and KA23 was used as phage productive host of \( \Phi \)BA1. These bacterial cells were grown aerobically at 37°C in the media shown below.

**Media and buffers**

NBY, HIBY, and \( \Phi \)80 buffer are described in the proceeding paper¹. Kimura C medium² contains, per liter: 1.0g KH₂PO₄, 2.0g NaH₂PO₄, 1.0g NaCl, 0.7g MgSO₄/7H₂O, 0.03g FeSO₄/7H₂O, 20g glycerol, 20g sodium glutamate, 5g sodium citrate, and 100 \( \mu \)g thiamin. The pH was adjusted to 7.2. SM buffer is 0.05M Tris-HCl pH 7.6, 0.1M NaCl, 0.008M MgSO₄ and 0.01% gelatin. TMS buffer contains 0.01M NaCl, 0.01M MgCl₂, and 0.05M Tris-HCl pH 8.0.

**Phage preparation**

After repeated single plaque isolation on the productive host KA23, a crude phage stock was prepared by plating phage with KA23 on NBY agar. The crude preparation was treated with DNase and RNase (10 \( \mu \)g/ml each) at 37°C for 60 min, and clarified of cell debris by centrifugation at 3,000\( \times \)g for 10 min. The supernatant fluid was centrifuged at 30,000\( \times \)g for 60 min at 4°C. The pellets were slowly dispersed in SM buffer, and centrifuged into a gradient of 20% sucrose (w/v) and three layers of CsCl (\( \rho \)=1.3, 1.5, and 1.7 g/ml) from top to bottom in an RPS50 rotor (Hitachi Co.) at 50,000\( \times \)g for 90 min. The opalescent phage band was collected, suspended in SM buffer containing CsCl (0.8 g/ml), and centrifuged for 24 h at 150,000\( \times \)g. The opalescent band was collected and dialyzed against TMS buffer overnight at 4°C.

**One-step growth experiment**

*B. aneurinolyticus* KA23 or KA10 was grown to a density of about 5\( \times \)10⁷ cells/ml. The bacteria were spun down and resuspended in 0.5 volume of NBY contained 0.002M KCN. \( \Phi \)BA1 were then added at a multiplicity of infection (MOI) of 1. After 10 min of adsorption, the culture was centrifuged (3,000\( \times \)g, 10 min) and diluted to 10⁴ into pre-warmed NBY and incubated at 37°C with shaking. The numbers of infectious center were assayed at intervals.

Electron microscopy

Exponentially growing KA10 or KA23 cells in NBY were infected with \( \Phi \)BA1 at MOI 20(KA10), or MOI 0.2, 1, and 20 (KA23). At various times after infection, 2 ml of infected culture was withdrawn and prefixed with 2.5% (v/v) glutaraldehyde, 0.1 M sodium phosphate buffer of pH 7.2 for 10 h, and postfixed with 1% (v/v) osmium tetroxide in 0.1 M sodium phosphate buffer of pH 7.2 for 5 h. Fixed cells were embedded in agar, dehydrated in a graded series of acetone solution, and embedded in Spurr resin. Thin sections were cut with a glass knife on an ultramicrotome (LKB), mounted on copper grids, stained with uranyl acetate and lead citrate, and examined in a JEOL 200CX electron microscope operating at 80 kV.

Infection of mitomycin C-induced cells with \( \Phi \)BA1.

To exponentially growing KA10 cells (3\( \times \)10⁷ cells/ml), mitomycin C (Kyowa hakkô Co, 0.2 \( \mu \)g/ml) was added, and incubated further. At various times after the addition of mitomycin C, 1 ml each of the culture was separated apart, infected with constant amount of \( \Phi \)BA1 (3.5\( \times \)10⁶ PFU), and incubated for 5 h. After addition of 20 \( \mu \)l of chloroform at the end of the incubation, cells were vigorously stirred to liberate intracellular \( \Phi \)BA1 into the medium and centrifuged at 3,000\( \times \)g for 10 min. The supernatant was examined on plaque-forming and killing (bacteriocin) activities as described in the proceeding paper¹.

Bacteriocin production of killing-sensitive cell after infection of \( \Phi \)BA1.

Exponentially growing KA10 cells (6\( \times \)10⁶ cells/ml) in NBY or HIBY were spun down, resuspended in NBY or HIBY at a density of 8\( \times \)10⁶ cells/ml, and infected with \( \Phi \)BA1 at MOI 12. After incubation for 3 h, cells were centrifuged at 3,000

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¹ Kimura C medium: A medium used for growing bacteria, containing essential nutrients and salts.

² SM buffer: A buffer solution commonly used in molecular biology to maintain pH and ionic strength.

³ TMS buffer: A buffer solution used for the centrifugation of viral particles.

⁴ JEOL 200CX: A type of transmission electron microscope.

⁵ Mitomycin C: A antibiotic used in genetics to induce DNA damage, which can lead to cell death or changes in DNA sequence.
×g for 10 min, the supernatant fluid was further centrifuged at 100,000×g for 45 min, and bacteriocin activity of the precipitate was determined. On the other hand, the cell pellet after low speed centrifugation (3,000×g) was treated with 0.1 ml of 20 mM Tris-HCl containing lysozyme (5 mg/ml), DNase (20 μg/ml), and RNase (20 μg/ml) at 37°C for 1 h. The lysate was centrifuged at 5,000×g for 8 min, the supernatant was examined on a bacteriocin activity.

Weigle like reactivation

Exponentially growing KA10 or KA23 cells were spun down and resuspended in 2.8 ml of ×4 diluted Kimura C medium at a density of 3×10⁸ cells/ml, and irradiated with 15 W germicidal lamp (Toshiba Co.) at a distance of 92 cm. For measuring the reactivation of plaque-forming activity, 0.2 ml of UV-irradiated KA23 cells were mixed with φBA1, plaque-forming activity of which had been reduced to 0.03% by an independent UV irradiation procedure similar to the above irradiation of KA23 cells. After 20 min of incubation, plaque-forming activity was measured according to Adams 27. For the enhancing of killing activity, UV irradiated KA10 cells were mixed with φBA1 which were irradiated with various dose of UV, and survival cells were counted on NBY plate as the measure of killing activity. All procedures described above were carried out under the condition in which photoreactivation could be excluded.

Result

Growth of φBA1

One-step growth curves of φBA1 on KA10 and KA23 are shown in Fig. 1. The latent period of φBA1 on KA23 at 37°C was 90 min and the burst size was about 70 in the medium used. No growth of φBA1 was seen on KA10, and this was obviously the same even when the host was infected with lower (3×10⁷ PFU) or higher (1.2×10⁹ PFU) concentrations of φBA1. Inability of φBA1 to produce progeny particle in KA10 cells was observed with electron microscopy (Fig. 2). Particles which seemed to be progeny of φBA1 were observed in KA23 cell at 90 min after infection (Fig. 3-b). Electron less dense structures which could be considered as granules were almost disappeared from KA10 cell at 40 min after infection of the phage. At 180 min after infection ghost cells, that were inferred to have lost their cellular contents with the release of mature phage particles, were observed at a fairly high frequency among KA23 cells, but not among KA10 cells (Fig. 2-c, Fig. 3-c).

Possibility of participation of lysogenized bacteriocin in φBA1–KA10 interaction

Goldberg 28 reported that nonpermissive host for a phage SP10 was rendered phage sensitive after treatment with UV light or mitomycin C, and suggested that inability of the phage to propagate is the result of an immunity conferred by the bacteriocin lysogenized in the host. Since KA10 is known to carry a high molecular weight bacteriocin (named aneuricin 10), we examined whether aneuricin 10 participated in the inhibition of the propagation of φBA1 on KA10. If lysogenized aneuricin 10-determined immunity is responsible for it, φBA1 should be able to propagate on the depressed (namely aneuricin 10 induced) KA10 cell. Typical pattern of induction of KA10 with mitomycin
C was shown in Fig. 4. At various times after addition of mitomycin C, cells were infected with φBA1. As shown in Table 1, however, φBA1 couldn't multiply on induced KA10 cells. The values presented are assumed to indicate the number of unadsorbed φBA1. The decrease of values with increasing incubation time is interpreted as increased adsorption according to the increase of host cell as shown in Fig. 4. No growth of φBA1 was also observed on KA10 induced with UV irradiation (data not shown). φB A1 inhibited the production of aneuricin 10 when they infected within 2 h after addition of mitomycin C (Fig. 5).

On the other hand, MacCorquodale et al suggested participation of intracellular colicin Ib in the killing of E. coli when phage BF23
Fig. 3 Electron micrographs of *B. aneurinolyticus* KA23 infected with ϕBA1. Samples were taken at 90 min (b), and 180 min (c) after infection. Uninfected control (a) was taken at 180 min. All bars represent 1µm.

<table>
<thead>
<tr>
<th>Time after addition of mitomycin C (min)</th>
<th>No addition control (min)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Infectious centers (±10^6 PFU/ml)</td>
<td>143</td>
</tr>
</tbody>
</table>
Fig. 4 Typical pattern of induction of *B. aneurinolyticus* KA10 with mitomycin C. KA10 cells were grown in NBY at 37°C with shaking. 0.2 μg/ml mitomycin C was added at time zero. ○; mitomycin C, ●; no addition control.

**Table 2** Aneuricin 10 activity of KA10 culture

<table>
<thead>
<tr>
<th></th>
<th>Sensitive strains</th>
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<tbody>
<tr>
<td></td>
<td>Φ BA1+</td>
<td>KA2</td>
<td>KAS 232</td>
</tr>
<tr>
<td>Ppt of extracellular fraction</td>
<td>+ 64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Sup of extracellular fraction</td>
<td>+ 2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Intracellular fraction</td>
<td>+ 0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a: infected at MOI 12  
b: expressed by the highest dilution of sample which could inhibit the growth of sensitive strain by spot test on NBY agar plate.  
c: Precipitate obtained by ultracentrifugation of the low speed-supernatant.  
d: Supernatant obtained by ultracentrifugation of the low speed-supernatant.  
e: Cell pellet after low speed centrifugation was lysed by lysozyme, the lysate was further centrifuged. The supernatant was used as intracellular fraction.

Fig. 5 Effect of Φ BA1 on production of aneuricin 10. KA10 cells (3 × 10⁶ cells/ml) were induced with the addition of 0.2 μg/ml mitomycin C at time zero. At indicated times, 1 ml culture was taken, infected with Φ BA1 at MOI 12, and incubated for 5h. The titer of produced aneuricin was measured by serial dilution method. 0% inhibition corresponds to the aneuricin 10 titer of 1024.

Fig. 6 Weigle like reactivation of Φ BA1 on KA23. UV-irradiated Φ BA1 (the viability was reduced to 0.03%) were added to UV-irradiated bacteria of various doses. The number of infectious particles was measured by double agar plate method. Weigle like reactivation was expressed by dividing the number of survival phage at given UV dose by the number of active phage at zero UV dose.
infected *E. coli* harbouring ColIb plasmid. In order to examine the possibility that aneuracin 10 is the responsible agent for killing of KA10, we examined on stimulated production of aneuracin 10 to the level adequate for the killing of host after infection of φBA1. As shown in Table 2, the amount of aneuracin 10 was similar regardless of the infection of φBA1. These low values obtained indicate that spontaneous induction has occurred.

Weigle like reactivation of *B. aneurinolyticus*

Weigle like reactivation of φBA1 on UV-irradiated KA23 (phage propagation host) is shown in Fig. 6. The extent of increase in survival was somewhat low compared with the results reported in *E. coli* or *B. subtilis*. Although no φBA1 particle was produced in KA10 as described above, φBA1-DNA has been proved to be injected into KA10 cell indicating the possibility to be open that early gene of φBA1 might be expressed in KA10 cell. If early gene product(s) participate in the killing phenomenon, enhancing of killing of UV-damaged φBA1 should also be expected to be observed in the UV-irradiated host. However, no enhancing of killing was observed under any condition of UV-irradiation (data not shown).

**Discussion**

As no φBA1 particle could be detected in *B. aneurinolyticus* KA10 cell after infection of the phage φBA1 by electron microscopy, the possibility was denied that mature particles were produced but could not burst the host cell because of the defectiveness of the phages in their lysis mechanism. This finding is consistent with the results that φBA1-DNA was degraded rapidly after injection into KA10 cell and that killing could occur in heavily UV-irradiated φBA1. φBA1 could not replicate in induced and derepressed KA10 cell. Hence, unlike the reported result, it appears that lysogenized aneuracin 10 does not share common repressor (immunity) with the phage (φBA1) in KA10 cell. Further, since the aneuracin 10 was not induced after infection of φBA1, the intracellular aneuracin 10 was unlikely to participate in the killing. These two results suggest that aneuracin 10 and the killing of φBA1 are independent with each other. The fact that the induction of aneuracin 10 by mitomycin C was inhibited by the infection of φBA1 suggests the inhibitory action of φBA1 on macromolecular synthesis. The possibility is inferred that the inhibition of synthesis of proteins such like rec A protease resulted in the interruption of SOS function. Ineffectiveness of φBA1 infected later than 2 h after addition of mitomycin C indicates the presence of a point of no return in the induction of aneuracin.

In this study, Weigle like reactivation was newly found on *B. aneurinolyticus* (KA23). As the Weigle reactivation is believed to be a manifestation of the host’s inducible error-prone repair pathway, it is suggested that this organism *B. aneurinolyticus* also has error-prone repair mechanism(s) like those found in *E. coli*. On the other hand, the result that no enhancing of killing activity was observed suggests little possibility of expression of φBA1 DNA in the course of killing of KA10.

**References**

5) Goldberg, I.D. and Bryan, T.: Productive in-


