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Studies on the Interaction between φBA1 of *Bacillus aneurinolyticus* and Its Killing-Sensitive Host

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Abstract In order to investigate the mechanism of killing of φBA1 against several strains of *Bacillus aneurinolyticus*, the possibility of φBA1 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 φBA1 in KA10 cells examined. Endogenous high molecular weight bacteriocin lysogenized in KA10 neither conferred immunity against φBA1 nor was induced in the course of killing by φBA1. Phenomena similar to Weigle-reactivation in *Escherichia coli* of φBA1 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 φBA1 genome in the cell of killing sensitive host KA10. Thus the possibility of propagation of φBA1 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 (φBA1) of *Bacillus aneurinolyticus* showed killing activity together with plaque-forming activity with strain specificity. Since the killing by a phage such like φBA1 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[1]. 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[5]. In this study, the interaction between φBA1 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 ϕB 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 (aneuricin 10). KA10 was used as the sensitive strain to the killing action of ϕBA1, and KA23 was used as phage productive host of ϕBA1. These bacterial cells were grown aerobically at 37°C in the media shown below.

Media and buffers

NBY, HIBY, and ϕ80 buffer are described in the proceeding paper[1]. Kimura C medium[6] contains, per liter: 1.0g KH_2PO_4, 2.0g Na_2HPO_4, 1.0g NaCl, 0.7g $MgSO_4 7H_2O$, 0.03g $FeSO_4 7H_2O$, 20g glycerol, 20g sodium glutamate, 5g sodium citrate, and 100 μg thiamin. The pH was adjusted to 7.2. SM buffer is 0.05M Tris-HCl pH7.6, 0.1M NaCl, 0.008M $MgSO_4$ and 0.01% gelatin. TMS buffer contains 0.01M NaCl, 0.01M $MgCl_2$, and 0.05M Tris-HCl pH8.0.

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 μg/ml each) at 37°C for 60 min, and clarified of cell debris by centrifugation at 3,000×g for 10 min. The supernatant fluid was centrifuged at 30,000×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 (ρ=1.3, 1.5, and 1.7 g/ml) from top to bottom in an RPS50 rotor (Hitachi Co.) at 50,000×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×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 5×10^7 cells/ml. The bacteria were spun down and resuspended in 0.5 volume of NBY contained 0.002M KCN. ϕBA1 were then added at a multiplicity of infection (MOI) of 1. After 10 min of adsorption, the culture was centrifuged (3,000×g, 10 min) and diluted to 10^{-4} 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 ϕBA1 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 ϕ BA1.

To exponentially growing KA10 cells (3×10^7 cells/ml), mitomycin C (Kyowa hakko Co., 0.2 μg/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 ϕBA1 (3.5×10^8 PFU), and incubated for 5 h. After addition of 20 μl of cloroform at the end of the incubation, cells were vigorously stirred to liberate intracellular ϕBA1 into the medium and centrifuged at 3,000×g for 10 min. The supernatant was examined on plaque-forming and killing (bacteriocin) activities as described in the proceeding paper[1].

Bacteriocin production of killing-sensitive cell after infection of ϕBA1.

Exponentially growing KA10 cells (6×10^7 cells/ml) in NBY or HIBY were spun down, resuspended in NBY or HIBY at a density of 8×10^8 cells/ml, and infected with ϕBA1 at MOI 12. After incubation for 3 h, cells were centrifuged at 3,000

×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 (5mg/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 3×10^8 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 [2]. 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 (3×10^7 PFU) or higher (1.2×10^9 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

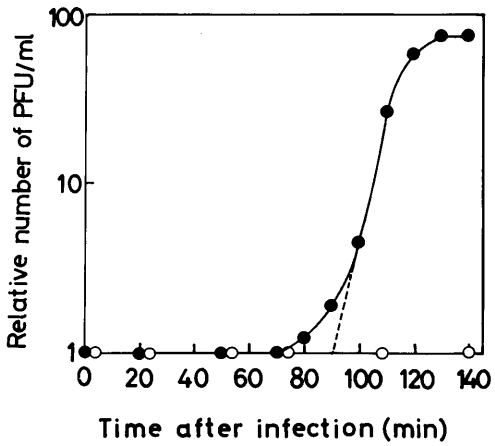

Fig. 1 One-step growth of ϕBA1 on *B. aneurinolyticus* KA10 and KA23. At indicated times, samples were withdrawn and assayed for the number of infectious centers on KA23 (for details, see methods).

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[5] 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

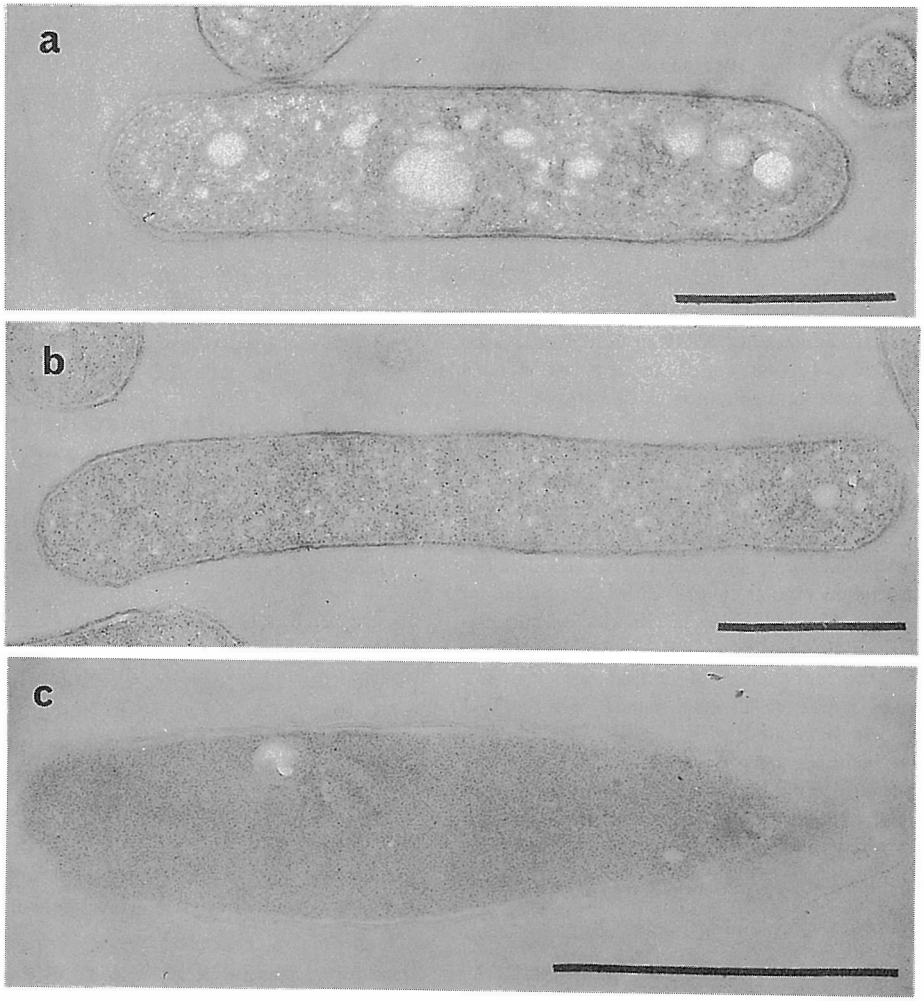

Fig. 2 Electron micrographs of *B. aneurinolyticus* KA10 infected with ϕBA1. Samples were taken at 40 min (b), and 180 min (c) after infection. Uninfected control (a) was taken at 180 min. All bars represent 1 μm.

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). ϕBA1 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[7] suggested participation of intracellular colicin Ib in the killing of *E. coli* when phage BF23

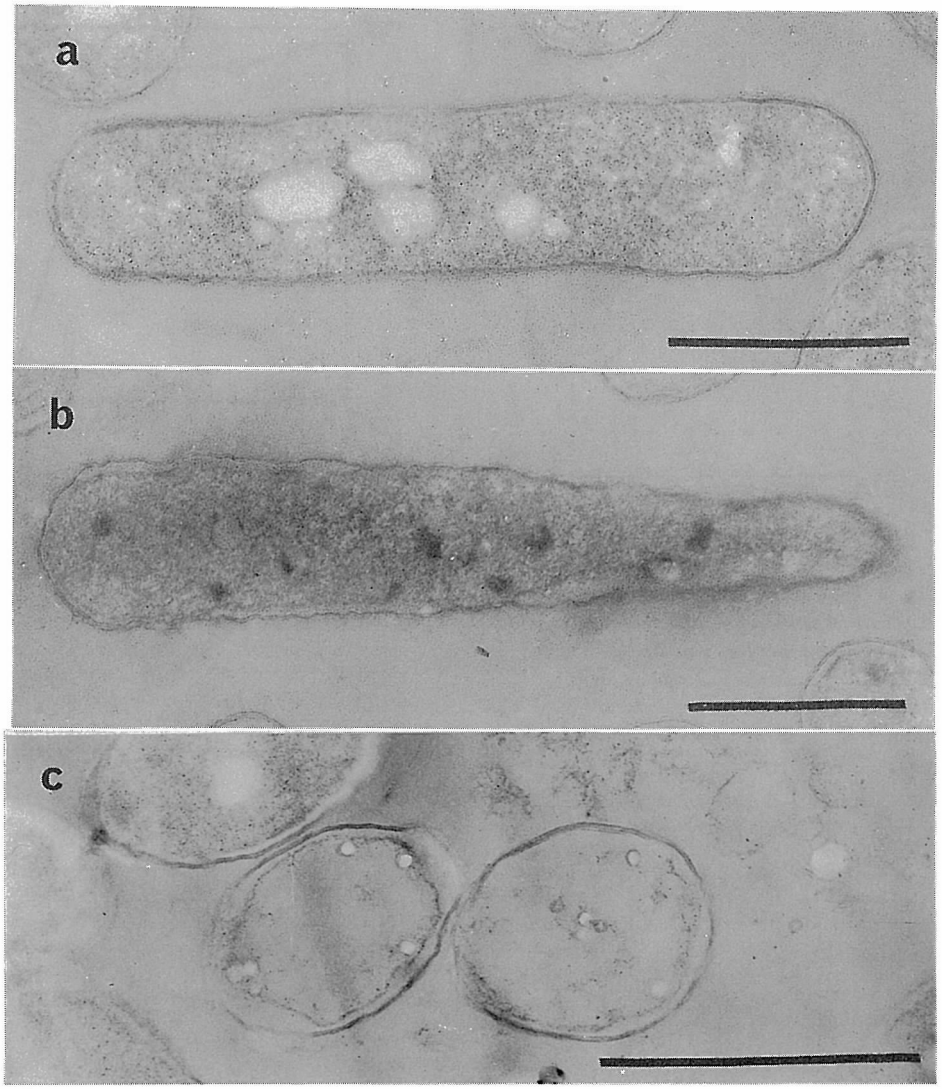

Fig. 3 Electron micrographs of *B. aneurinolyticus* KA23 infected with ϕBA1. Samples were taken at 90 min (b), and 180 min (c) after infection. Uninfected control (a) was taken at 180 min. All bars represent 1μm.

Table 1 Propagation of ϕBA1 on mitomycin C-induced KA10

	Times after addition of mitomycin C (min)						No addition control (min)	
	0	30	60	120	150	180	0	180
Infectious centers ($\pm 10^6$ PFU/ml)	143	153	137	135	112	88	149	70

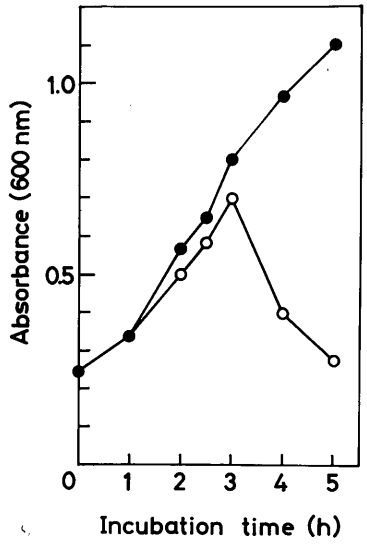

Fig. 4 Typical pattern of induction of *B.aneurinolyticus* KA10 with mitomycin C. KA10 cells were grown in NBY at 37°C with shaking. 0.2 μg/ml mitomycin C was added at time zero. ○; mitomycin C, ●; no addition control.

Table 2 Aneuricin 10 activity of KA10 culture

	φBA1[a]	Sensitive strains	
		KA2	KAS 232
		[b]	
Ppt of extracellular fraction[c]	+	64	64
	−	64	64
Sup of extracellular fraction[d]	+	2	2
	−	2	2
Intracellular fraction[e]	+	0	0
	−	0	0

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.

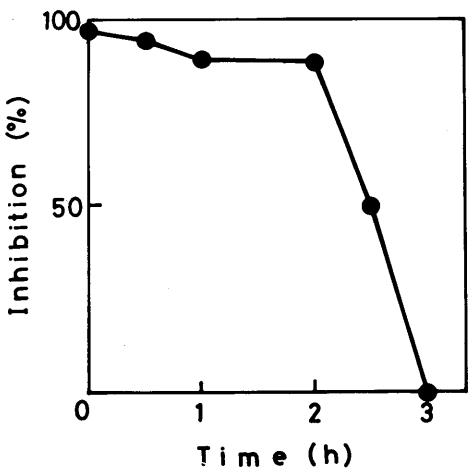

Fig. 5 Effect of φBA1 on production of aneuricin 10. KA10 cells (3×10^7 cells/ml) were induced with the addition of 0.2 μg/ml mitomycin C at time zero. At indicated times, 1 ml culture was taken, infected with φBA1 at MOI 12, and incubated for 5h. The titer of produced aneuricin was measured by serial dilution method. 0% inhibition corresponds to the aneuricin 10 titer of 1024.

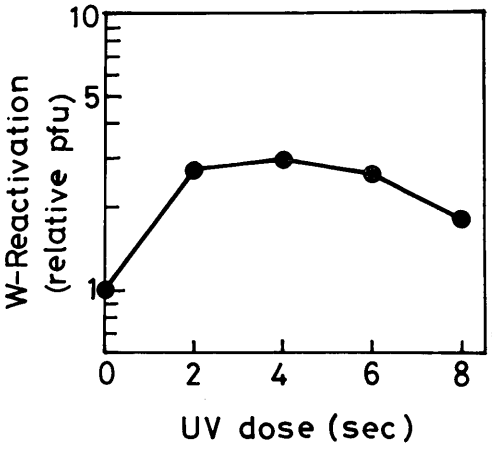

Fig. 6 Weigle like reactivation of φBA1 on KA23. UV-irradiated φBA1 (the viability was reduced to 0.03%) were added to UV-irradiated bacteria of various doses. The number of infectious particles was measured by double agar layer method. Weigle like reactivation was expressed by dividing the number of suvival 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 aneuricin 10 is the responsible agent for killing of KA10, we examined on stimulated production of aneuricin 10 to the level adequate for the killing of host after infection of ϕBA1. As shown in Table 2, the amount of aneuricin 10 was similar regardless of the infection of ϕBA1. These low values obtained indicate that spontaneous induction has occured.

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*[8] or *B. subtilis*[9]. Although no ϕBA1 particle was produced in KA10 as described above, ϕBA1-DNA has been proved to be injected into KA10 cell[10] 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[10]. ϕBA1 could not replicate in induced and derepressed KA10 cell. Hence, unlike the reported result[5], it appears that lysogenized aneuricin 10 does not share common repressor (immunity) with the phage (ϕBA1) in KA10 cell. Further, since the aneuricin 10 was not induced after infection of ϕBA1, the intracellular aneuricin 10 was unlikely to participate in the killing. These two results suggest that aneuricin 10 and the killing of ϕBA1 are independent with each other. The fact that the induction of aneuricin 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[11]. Ineffectiveness of ϕBA1 infected later than 2 h after addition of mitomycin C indicates the presense of a point of no return in the induction of aneuricin.

In this study, Weigle like reactivation was newly found on *B. aneurinolyticus* (KA23). As the Weigle reactivation[12] is believed to be a manifestation of the host's inducible errror-prone repair pathway[9], it is suggested that this organism *B. aneurinolyticus* also has error-prone repair mechanism(s) like those found in *E. coli*[8]. 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.

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