Bull Yamaguch Med Sch 32, 27-34, 1985

Inducible Particles of *Bacillus aneurinolyticus*.

Shin-ichi Ito

Department of Microbiology, Yamaguchi University School of Medicine, Ube, Yamaguchi 755, Japan

(Received September 4, 1985)

Abstract To detect prophages and bacteriocins, twenty strains of *Bacillus aneurinolyticus* were treated with mitomycin C. The resulted lysates were subjected to electron microscopy, and also examined for killing and plaque-forming activities. Seventeen strains showed killing activity on two or more strains of *Bacillus aneurinolyticus*. Killing agents were centrifuged in linear 5 to 20% sucrose gradient, and studied with electron microscopy which revealed the presence of particles. They looked morphologically like phage tail of 190 nm long with fiber (KA10, KA17) or without fiber (KA1, KA6), T even phage-like particle with a head of 50 nm in diameter and a tail of 140 nm long (KA23), or T7 phage-like particle with a head of 70 nm in diameter and a tail of 20 nm long (KAS232). The killing agent of KAS 232 showed plaque-forming activity on several strains different from killing sensitive strains of *Bacillus aneurinolyticus*.

Key words: *Bacillus* bacteriocin, bacteriophage, mitomycin C, electron microscopy.

Introduction

Bacillus aneurinolyticus is a group of thiamin-decomposing bacteria[1]. We have been studying on taxonomy[2], method for detection[3], and the ultrastructure of cell wall[4,5] of those organisms.

Because of the production of thiaminase II [EC. 3.5.99.2; thiamin hydrolase], the physiological meaning of which is still an enigma, a number of studies on this organism have been reported[6,7]. As far as we are aware, however, there is no previous report on the prophage and bacteriocin of *Bacillus aneurinolyticus*. In this report, we describe morphological aspects of newly found particulate bacteriocins (aneuricin), and a temperate phage (ϕBA1) in *B. aneurinolyticus*.

Methods

Microorganisms

The origin of twenty strains of *B. aneurinolyticus* used in this study were described elsewhere[8]. Nineteen strains of other *Bacillus* species included eight strains (preserved in this laboratory) of *B. thiaminolyticus*, *B. brevis* NCIB7577, NCIB8803, ATCC8185, ATCC8186, ATCC10027, ATCC10068 and other two strains (preserved in this laboratory), *B. circulans* NCIB9374, *B. laterosporus* NCIB8213

and *B. subtilis* ATCC6633. These were examined on their sensitivity to killing agents and to phage produced by *B. aneurinolyticus*.

Media and buffer

The media used in this study were NBY and HIBY. NBY contains 1% polypeptone (Daigo Eiyo Co.), 0.5% meat extract (Wako Co.) and 0.1% yeast extract (BBL). HIBY contains 2.5% Heart infusion broth (Nissui Co.) and 0.1% yeast extract (BBL). For agar plate, 1.5% or 0.7% (for soft top agar) agar was added to NBY. ϕ80 buffer containing 20 mM Tris-HCl pH 7.4, 0.1 M NaCl, 1 mM $MgSO_4$ and 0.01% gelatin was used to dilute killing agents and prepare sucrose gradient.

Mitomycin C induction

Each strain was grown in NBY at 37°C with shaking and their optical density at A_{600} were monitored by spectrophotometer (Bosche and Lomb). At optical density of about 0.1 to 0.2, mitomycin C (Kyowa Hakko Co.) was added to the desired final concentration. The cells were further incubated under the same condition until optical density declined. The resulted lysates were treated with DNase and RNase (Sigma, 1 μg/ml each) at 37°C for 1 h, centrifuged at 3,000×g to remove cell debris and unlysed cells. The supernatant fluids were tested for killing and plaque-forming activities, and subjected to electron microscopy.

Assay for killing activity

The killing activity was detected by the cross-streak method and assayed semi-quantitatively by the serial dilution method[9]. Killing titer was expressed by the highest dilution of sample which could inhibit the growth of indicator bacteria by the spot test on agar plate. Assay for plaque formation was performed by double agar layer method [10].

Sucrose gradient

After 10 ml lysates were clarified by the above mentioned methods, released particles were pelleted at 120,000×g for 1 h at 4°C, suspended in 0.2 ml of ϕ80 buffer, and layered on a linear 5 to 20% (w/v) sucrose gradient (5 ml) and centrifuged at 37,000×g for 30 min at 4°C. Fractions of ten drops each were collected from the bottom of the centrifuge tube, and their killing and plaque-forming activities were examined as above.

Electron microscopy

All preparations were carried out on carbon-coated copper grids. A drop of crude lysates or fractions after sucrose or CsCl density gradient centrifugation was placed on the grid, dried, and washed with ϕ80 buffer or with distilled water to remove contaminant of medium, sucrose, or CsCl. The grids were negatively stained with 1% uranyl acetate, and were examined with JEOL 200CX electron microscope at 80 kV.

Results

Inducibility of *B. aneurinolyticus*

Spontaneous production of a phage by lysogenic bacteria occurs at a probability of 10^{-2} to 10^{-5} per bacterium per one generation[10]. Although the rate of spontaneous production of lysogenized phages of *Bacillus aneurinolyticus* is not known, phage-like particles were rarely shown by electron microscopy of sefdimentable material from the supernatants of cultures of some strains of this organism (data not shown). To increase the rate of production of those particles, mitomycin C which was effective inducer was used in this study.

To exponentially growing cells of *B. aneurinolyticus* in NBY mitomycin C was added to a concentration ranging from 0.1 to 10 μg/ml, and the optical density at 600 nm of the culture was measured in the course of time. Representative results are shown in Fig. 1. Under this condition, a concentration of 0.1–0.25 μg of mitomycin C caused maximal lysis of *B. aneurinolyticus*. Although it has been reported that maximal lysis occured at 5–10 μg/ml mitomycin C in *Clostridium tetani*[11], both the growth and the induction were prevented by 5 μg/ml or

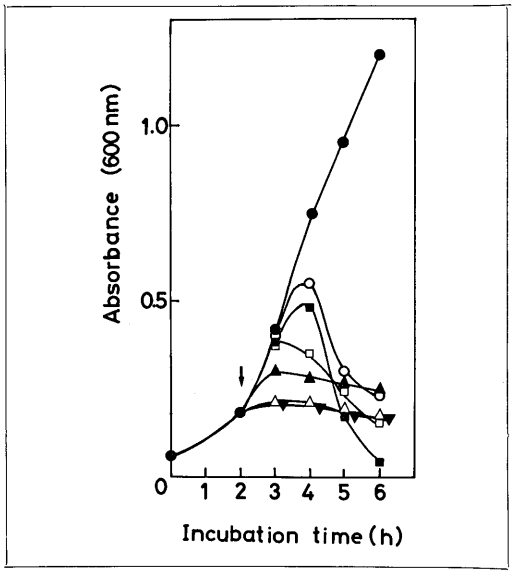

Fig. 1 Induction of *B. aneurinolyticus* by mitomycin C. *B. aneurinolyticus* strainKA5 was grown in NBY at 37°C with shaking. Various amounts of mitomycin C were added at the early log phase indicated by arrow. ●; no addition control, ○; 0.1 μg/ml, ■; 0.25 μg/ml, □; 0.5 μg/ml, ▲; 1 μg/ml, △; 5 μg/ml, ▼; 10 μg/ml

higher concentration of mitomycin C in *B. aneurinolyticus*. After a low-speed centrifugation, those induced lysates were examined for the presence of released particles with electron microscope. All lysates contained one or more types of particles, such as intact phage (Fig. 2 a, b), intact phage with empty head (Fig. 2 c), empty head of phage (Fig. 2 d), or tail of phage (Fig. 2 e) in their morphology.

Killing activity

It has been known that some of inducible particles structurally related to bacteriophage components could act bactericidally[12]. Hence ,the killing activity of lysates obtained above was examined by cross streak methods (Fig. 3). Except for KA2, KA13, and KAS23, seventeen lysates out of 20 strains were able to kill two or more of the other strains of *B. aneurinolyticus* but not themselves. Killing was observed only among the strains of *B. aneurinolyticus*, and no sensitive strain was detected in other *Bacillus* species tested. Killing activity was found not in the supernatants by the detection method used, but in the precipitate after ultracentrifugation (120,000×g, 60 min) indicating the high molecular nature of killing agent.

Structure of killing agent

Electron microscopic observation of almost entire fractions of linear 5 to 20% (w/v) sucrose gradient centrifugation revealed that the peak fraction of killing activity was the richest in phage-like or phage tail-like particles in all cases (Fig. 4). Typical particles were summarized in Table 1. The sheath of tail-like particles showed extended or contracted form (Fig. 5). The tail sheath of a particle was examined and found that the extended form showed about 55 striations 3 nm apart with each other. The dimension of those particles was 190 nm long and 20 nm in diameter, which was similar to the tail of PBSX, a defective phage of *B. subtilis*[12]. When contracted, the diameter (about 24 nm) of the sheath was a little greater than that of the extended one (about 20nm), and the subunits seemed to be rearranged into longitudinal rows (Fig. 5). Electron microscopy showed a cog wheel structure which was interpreted as end-on view of contracted sheath, and the 12 teeth was assumed to be corresponding to the longitudinal rows (Fig. 6). Six fibers at tail tip were observed in preparations of tail-like particles from KA10 and KA17 (Fig. 7). These last two structures have also been reported in PBSX and in INCO particles of *Rhizobium*[13].

Temperate phage

Together with the killing activity, plaque-

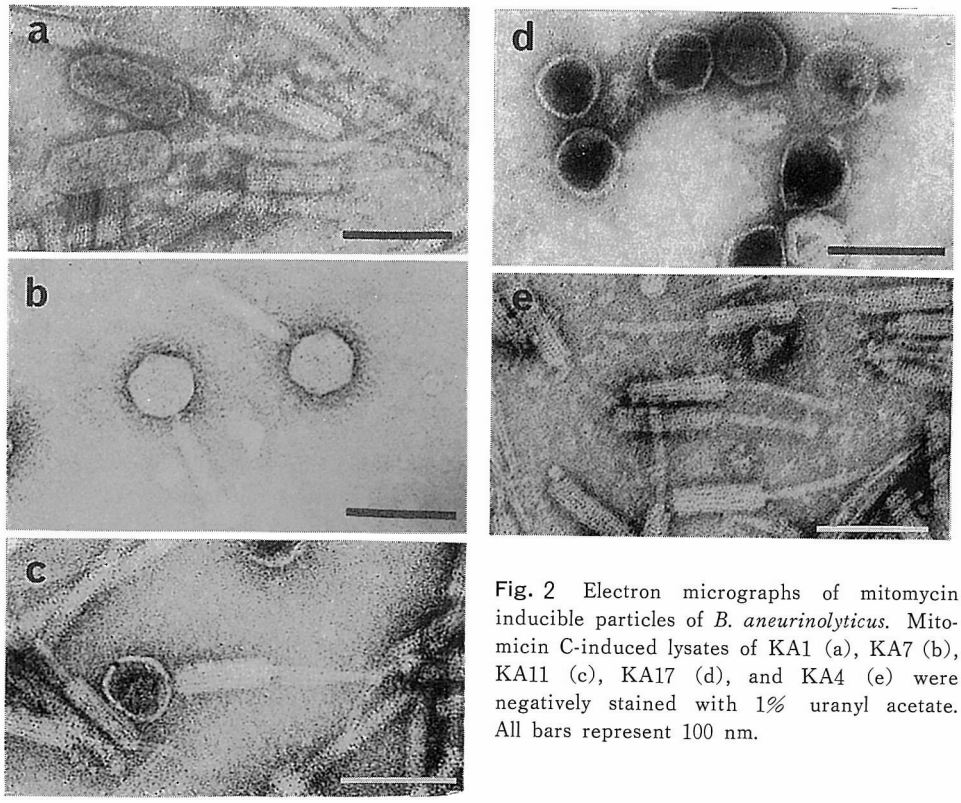

Fig. 2 Electron micrographs of mitomycin inducible particles of *B. aneurinolyticus*. Mitomicin C-induced lysates of KA1 (a), KA7 (b), KA11 (c), KA17 (d), and KA4 (e) were negatively stained with 1% uranyl acetate. All bars represent 100 nm.

forming activity was also found in the lysate of KAS232. After sucrose gradient centrifugation, it was found that peak fraction for both killing (against KA10, 13, 14, 17, and 22) and the plaque-forming (against KA1, 23, and 24) activities coincided with each other (data not shown). Electron microscopy showed morphologically singular and homogeneous particles in the peak and the neighbouring fractions (Fig. 8-a,b). The particle was called ϕBA1. ϕBA1 made turbid plaques with a center colony (Fig. 9). The size of plaques on KA23 or KA24 was about 1 to 1.5 mm in diameter. Although two types (large and small) of plaque are seen in Fig. 9, small plaques showed the same morphology as large plaques if additional incubation was carried out. The plaques on KA1 were very turbid and small, and hard to count. ϕBA1 multiplied on KA23 were centrifuged in CsCl equilibrium gradient, fractionated, and killing and plaque-forming activities were examined for each fraction. As with the result of sucrose gradient centrifugation of mitomycin C-induced lysate of KAS232, killing and plaque-forming activities coincided again with each other. The morphology of purified particles could not be distinguished from that of the particle observed in the mitomycin C-induced lysate of KAS232 (Table 1).

Discussion

A large number of diverse bacterial species have been shown to produce particles

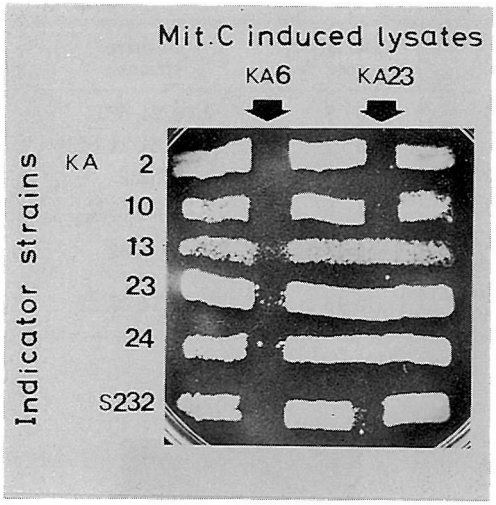

Fig. 3 Cross streak test. Exponentially growing cells of indicator strains were cross-streaked over mitomycin C-induced lysates pre-streaked on NBY plate, and incubated at 37°C for 12 h. Growth inhibition observed at the crosses indicate the killing activity of mitomycin C-induced lysate.

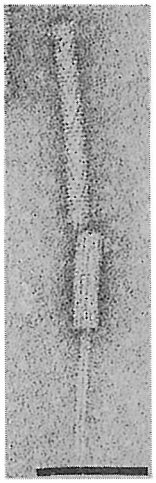

Fig. 5 Electron micrograph showing extended or contracted form of aneuricin of KA 10. Negative staining was with 1% uranyl acetate. The bar represents 100 nm.

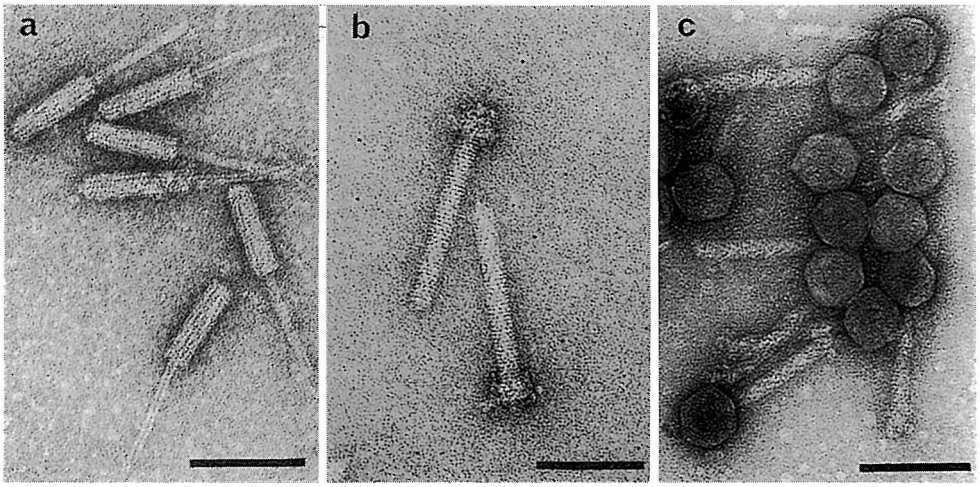

Fig. 4 Electron micrograph of aneuricin. Samples were taken from the peak fraction of sucrose gradient centrifugation, and negatively stained with 1% uranyl acetate. Aneuricin particles shown are obtained from KA6 (a), KA10 (b), and KA23 (c). All bars represent 100 nm.

Table 1 Killing agents of *B. aneurinolyticus*

Producer atrsins	Morpho logy	Size (nm)	Density (sucrose %)	Sensitive strains
KA 1	Tail	20×190	6- 7	2, 23, 24, S232
6	Tail	20×190	6- 7	2, 10, 13, 23, 24, S232
10	Tail with fiber	20×190	7- 8	2, S232
17	Tail with fiber	20×190	7- 8	2, 23, 24, S232
23	T-even phage type	Head: 50 (diam) Tail : 14×140	9-10	2, 10, S232
S232	T7 phage type	Head: 70 (diam) Tail : 10×20	12-13	10, 13, 14, 17, 22

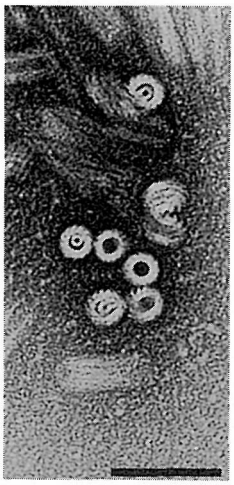

Fig. 6 Electron micrograh of contracted aneuricin of KA1 in endon view. Negative staining was with 1% uranyl acetate. The bar represents 100 nm.

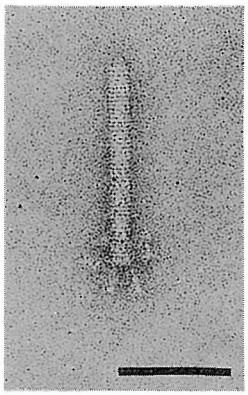

Fig. 7 Electron micrograph of KA10 aneuricin showing six tail fibers. Negative staining was with uranyl acetate.The bar represents 100 nm.

which contained either all or some of the normal phage components, but they failed to form plaques on any known host. These particles have been called as a defective phage[14]. Present report shows that *B. aneurinolyticus* produce such defective phages. Although a few reports suggested the participation of a defective prophage (PBSX) in host cell wall metabolism[15] or in resistance of host to a specific phage[16], the role of the other defective prophages in their host cell has been unclear. On the other hand, it has been suggested that a phage and R-type pyocins (bacteriocins of *Pseudomonas aeruginosa* which have a structure very similar to tails of T-even phage) have been derived from some common ancestor[17,18]. In the case of *B. aneurinolyticus*, phage-tail like particle similar to R-type pyocin was the type most frequently observed and most of them showed bacteriocin activity. But no intact phage which showed morphological similarity to those tail-like particles was observed.

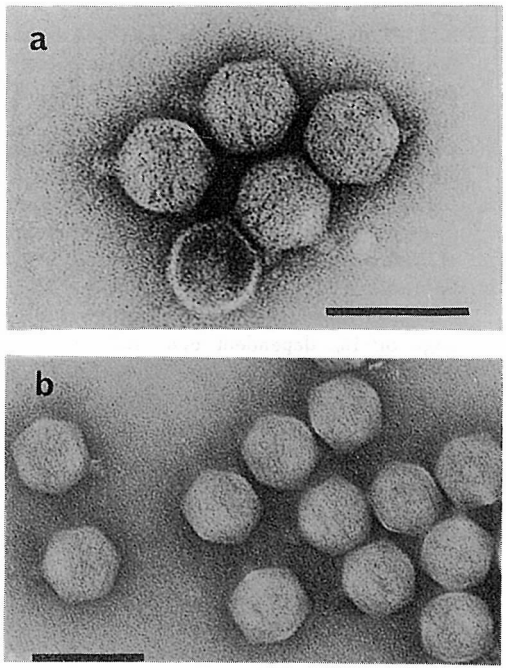

Fig. 8 Electron micrograph of φBA1. Sample was taken from the peak peak fraction of killing and plaque-forminig activities. Negatively staining was with 1% uranyl acetate. The bar represents 100 nm.

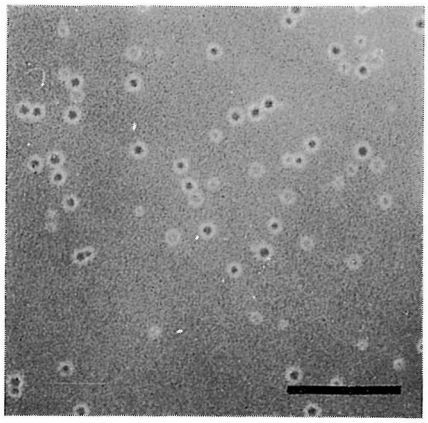

Fig. 9 Plaques produced by purified φBA1 on NBY plates with some lysogenized cells in it. The bar represents 10 nm.

Morphologically two killing agents (from KA23 and KAS232) appearred to be intact phage (Fig. 4-C, 8). Indeed, one of these showed plaque-forming activity on hosts different from the killing sensitive hosts. Therefore, this particle is not a defective phage but a complete phage. There is a possibility that known defective phages which looked like complete phage in their morphology may have plaque-forming activity on unknown host. The trait of the particle (φBA1) is of interest in the relationship between bacteriocin and bacteriophage, and it is needed to be investigated further. Some of the biochemical results observed have been reported in the proceedings[19].

References

1) Parry, J.M., Turnbull, P.C.B. and Gibson, J.R.: *A color atlas of Bacillus Species*, Wolfe Medical Publications, London, 1983, p. 196.
2) Hayashi, R., Yoshii, Z., ¦Harada, T., Nigota, K., Tsubota, Y., Shibutake, M., Sasaki, M., Suzuki, S. and Takagi, T.: Studies on thiaminase of Clostridia. (I) Thiaminase of *Clostridium sporogenes* (in Japanese). *Vitamins*, **27**: 342–344, 1963.
3) Yoshii, Z., Harada, T., Tsubota, Y., Sasaki, M., Miura, D. Sera, Y. and Nigota, K.: Studies on the detection of thiamine decomposing Bacilli. (I) Introduction and enriching cultivation (in Japanese). *Jpn. J. Bacteriol.*, **22**: 490–499, 1967.
4) Yoshii, Z., Yamataka, S., Suedomi, K., Miura, D. and Hayashi, R.: Ultrastructure of the cell wall of *Bacillus aneurinolyticus*. *J. Electron. Microsc.*, **17**: 67–73, 1968.
5) Abe, M. and Yoshii, Z.: Isolation and chemical characterization of regular arrayed macromolecular structure in the cell wall of *Bacillus aneurinolyticus* (Kimura et Aoyama) (in Japanese). *Jpn. J. Bacteriol.*, **38**: 695–701, 1983.
6) Yoshii, Z., Nigota, K., Nishimune, T., Kobayashi, S., Konishi, H., Takao, K., Maeda, H., Watanabe, K., Mii, H., Mizuno, A., Utsunomiya, S., Akitomi, S., Oie, S., Sugiyama, K. and Okada, M.: Thiaminase-producing microbes. (I) Compilation a list of references in Japanese (in Japanese). *Yamaguchi Med. J.*, **30**:

401-437, 1981.
7) Yoshii, Z., Nigota, K., Kobayashi, S., Konishi, H., Takao, K., Maeda, H., Watanabe, K., Akitomi, H., Utsunomiya, S., Mii, H., Mizuno, A., Sugiyama, K., Harada, T. and Okada, M.: Thiaminase-producing microbes. (Ⅱ) Compilation of a list of references in foreign languages (in Japanese), *Yamaguchi Med. J.*, 31 : 717-767, 1982.
8) Abe, M. and Kimoto, M.: Distribution of two types of regular-array particles in the cell wall of *Bacillus aneurinolyticus* (Kimura et Aoyama). *Microbiol. Imminol.*, 28 : 841-846, 1984.
9) Kageyama, M. and Egami, F.: On the purification and some properties of a pyocin, a bacteriocin produced by *Pseudomonas aeruginosa*. *Life Sci.*, 9 : 471-476, 1970.
10) Adams, M.H.: *Bacteriophages*, Interscience Publishers, New York, 1959.
11) Hara, T., Matsuda, M. and Yoneda, M.: Isolation and some properties of nontoxigenic derivatives of a strain of *Clostridium tetani*. *Biken J.*, 20 : 105-115, 1977.
12) Bradley, D.E.: Ultrastructure of bacteriophages and bacteriocins. *Bacteriol. Rev.*, 31 : 230-314, 1967.
13) Lotz, W. and Mayer, F.: Isolation and characterization of a bacteriophage tail-like bacteriocin from a strain of *Rhizobium*. *J. Virol.*, 9 : 160-173, 1972.
14) Garro, A.J. and Marmur, J.: Defective bacteriophages. *J. Cell. Biol.*, 7 : 253-264, 1970.
15) Mauel, C. and Karamata D.: Characterization of proteins induced by mitomycin C treatment of *Bacillus subtilis*. *J. Virol.*, 49 : 806-812, 1984.
16) Goldberg, I.D. and Bryan, T.: Productive infection of *Bacillus subtilis* 168, with bacteriophage SP-10, dependent upon inducing treatments. *J. Virol.*, 2 : 805-812, 1968.
17) Kageyama, M., Shinomiya, T., Aihara, Y. and Kobayashi, M.: Characterization of a bacteriophage related to R-type pyocins. *J. Virol.*, 32: 951-957, 1979.
18) Shinomiya, T.: Phenotypic mixing of pyocin R2 and bacteriophage PS17 in *Pseudomonas aeruginosa* PAO. *J. Virol.*, 49 : 310-314, 1984.
19) Ito, S., et al.: Bacteriocin-like killing mechanism of a temperate phage ϕBA1 of *Bacillus aneurinolyticus*. *Jpn. J. Bacteriol.*, 40 : 398, 1985.