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# Bacteriophage-like Particles Induced by Mitomycin C in *Bacillus circulans* F-2

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# Mitomycin C에 의해 유도되는 *Bacillus circulans* F-2의 Bacteriophage-like 입자

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**To detect prophages and bacteriocins, twenty strains of *Bacillus circulans* were treated with mitomycin C. The resulted lysates were subjected to electron microscopy, and also examined for killing and plaque-forming activities. Fifteen strains showed killing activity on two or more strains of *Bacillus circulans*. 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 (FA9, FA5) or without fiber (FA1, FA6), T even phage-like particle with a head of 50 nm in diameter and a tail of 140 nm long (FA7), or T7 phage-like particle with a head of 70 nm in diameter and a tail of 20 nm long (FA17). The killing agent of FA17 showed phage-forming activity on several strains different from killing sensitive strains of *Bacillus circulans*.**

*Bacillus curculans* F-2 is a powerful raw starch-digesting amylase producing bacterium (1). We had been studying on production, characterization, purification and industrial application of the enzymes including raw starch-digesting amylase (2,3) and a bifunctional amylase-pullulanase enzyme (Kim *et al.*,Ref. 4,5). Furthermore, to study the molecular structures of those enzymes and elucidate the gene expression system of *Bacillus circulans*, we cloned the genes and the enzymes were succesfully expressed in *Escherichia coli* (Kim *et al.*, Ref. 6,7). This bacterium is unique in that it produces the amylase only when it is cultivated on a medium containing raw starch as carbon source. Only a small amount of amylase was produced when it was grown on soluble starch or maltose media (3). The amylase production of this bacterium is highly sensitive to glucose repression (3). With the background of this strain, we have studied the development of the new host-vector system using *B. circulans* F-2 and its phage produced. Therefore, in the course of a search for a phage of *B. circulans* F-2, a temperate phage was isolated together with structured bacteriocins of various morphologies.

Phages have been useful as a source of perturbations in the bacterial cell. Defective phages enable one to apply smaller perturbations to a bacterial system

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and, in so doing, to study phenomena which are normally masked behind the biochemical and genetic activity of a replicating phage. In particular, the careful choice of defective phages, whether intentionally induced or fortuitously detected, may allow a highly specific probe into some aspect of bacterial physiology. Defective phages are generally found by induction and lysis of supposedly nonlysogenic bacterial strains. Since they are noninfective, only observation under the electron microscope or fractionation of such phage lysate may show their existence. Production of defective phages may be due to spontaneous reversion of the detective genes. Although a number of particulate and soluble bacteriocins from different sources have been shown to possess killing activity on a narrow range of related bacteria, little is known about bacteriocin like killing action of a complete phage (12).

To use the unique properties of *B. circulans* F-2 as a host for the gene expression and study its phage produced, isolation and characterization of the phage are essential. In this report, we describe morphological aspects of newly found particulate bacteriocins, and a temperate phage (Sigma FA2) in *Bacillus circulans* F-2.

## Materials and Methods

### Microorganisms

The origin of ten strains of *B. circulans* used in this study were our collection. Fifteen strains of other *Bacilius* species included seven strains (preserved in this laboratory) of *B. brevis* NCIB7577, NCIB8803, ATCC8185, ATCC8186, ATCC10027, ATCC10068 and other two strains (preserved in this laboratory), *B. circulans* NCIB9374, *B. laterosporous* NCIB8213 and *B. subtilis* ATCC6633.

### Media and buffer

The media used in this study were NBY and HIBY. NBY contains 1% polypeptone (Diago eiyo Co., Tokyo, Japan), 0.5% meat extract (Wako Co., Tokyo, Japan) and 0.1% yeast extract (Difco, Detroit, USA). HIBY contains 2.5% heart infusion broth (Nissui Co., Tokyo, Japan) and 0.1% yeast extract (Difco). For agar plate, 1.5% or 0.7% (for soft top agar) agar was added to NBY. Sigma 80 buffer containing 20 mM Tris-HCl (pH 7.4), 0.1 M, NaCl, 1 mM $MgSO_4$ and 0.01% gelatine was used to dilute killing agents and prepare sucrose gradient.

### Mitomycin C induction

Each strain was grown in NBY at 37°C with vigorous shaking and their optimal density at A660 were monitered by spectrophotometer (Shimadzu Co., Tokyo, Japan). At optimal densisty of about 0.1 to 0.2, mitomycin C (Kyowa Hakko Co., Tokyo, Japan) was added to the desired final concentration. The cells were further incubated under the same condition until cell density declined. The resulted lysates were treated with DNase and (Sigma, 1 $\mu$g/m*l* each) at 37°C for 30 min, 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 (8). Killing titer was expressed by the highest dilution of the 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 (9).

### Sucrose gradient

After 10 m*l* lysates were clarified by the above mentioned methods, released particles were pelleted at 120,000×g for 1 hr at 4°C, suspended in 0.2 m*l* of sigma 80 buffer, and layed on a linear 5 to 20% (w/v) sucrose gradient (5 m*l*) and centrifuged at 37,000×g for 30 min at 4°C. Fractionation of ten drops each collected from the bottom of the centrifuge tube, and their killing and plaque-formating activities were as above.

### Electon microscopy

All preparations were carried out on carbon-coated copper grids. A drop of crude lysates or fration after sucrose or CsCl density gradient was washed with Sigma 80 buffer or with distilled water to temove contaminant of medium, sucrose, or CsCl. The gride were negatively stained with 1% uranyl acetate, and were examined with JEOL 200CX electron microscope (JEOL Co., Tokyo, Japan) at 80 kV.

## Results

### Inducibility of *B. circulans* F-2

Spontaneous production of phage by lysogenic bacteria occurs at a probability $10^{-2}$ to $10^{-5}$ per bacterium on generation (10). Although the rate of

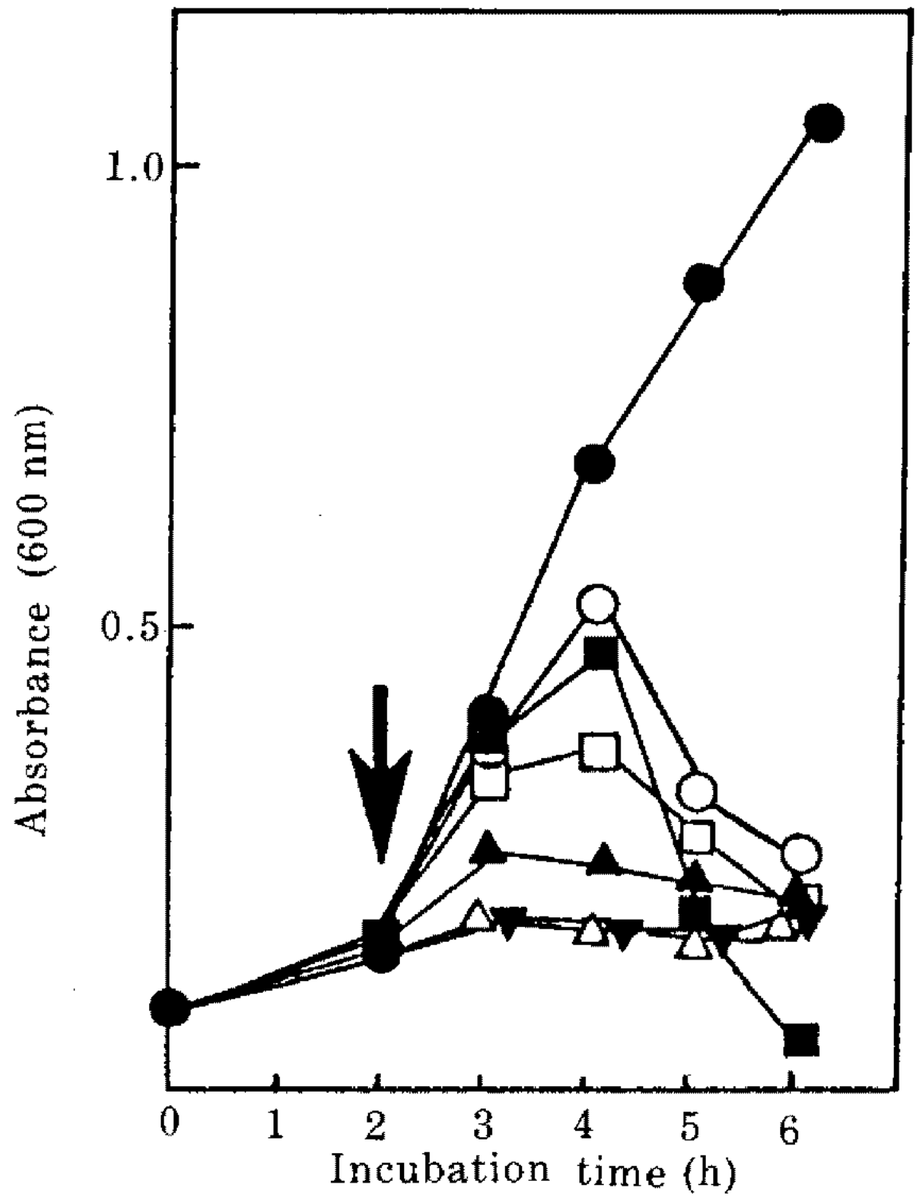


**Fig 1. Induction of *B. circulans* F-12 by mitomycin C.**
The strain was grown in NBY at 37°C with shaking. Various amounts of mitomycin C were added at the early log phage indicated by arrow. See text for further details. ●; no addition control; ○, 0.1 μg/ml ■, 0.25μg/ml; □, 0.5 μg/ml; ▲, 1 μg/ml; △, 5 μg/ml; ▽, 10μg/ml.

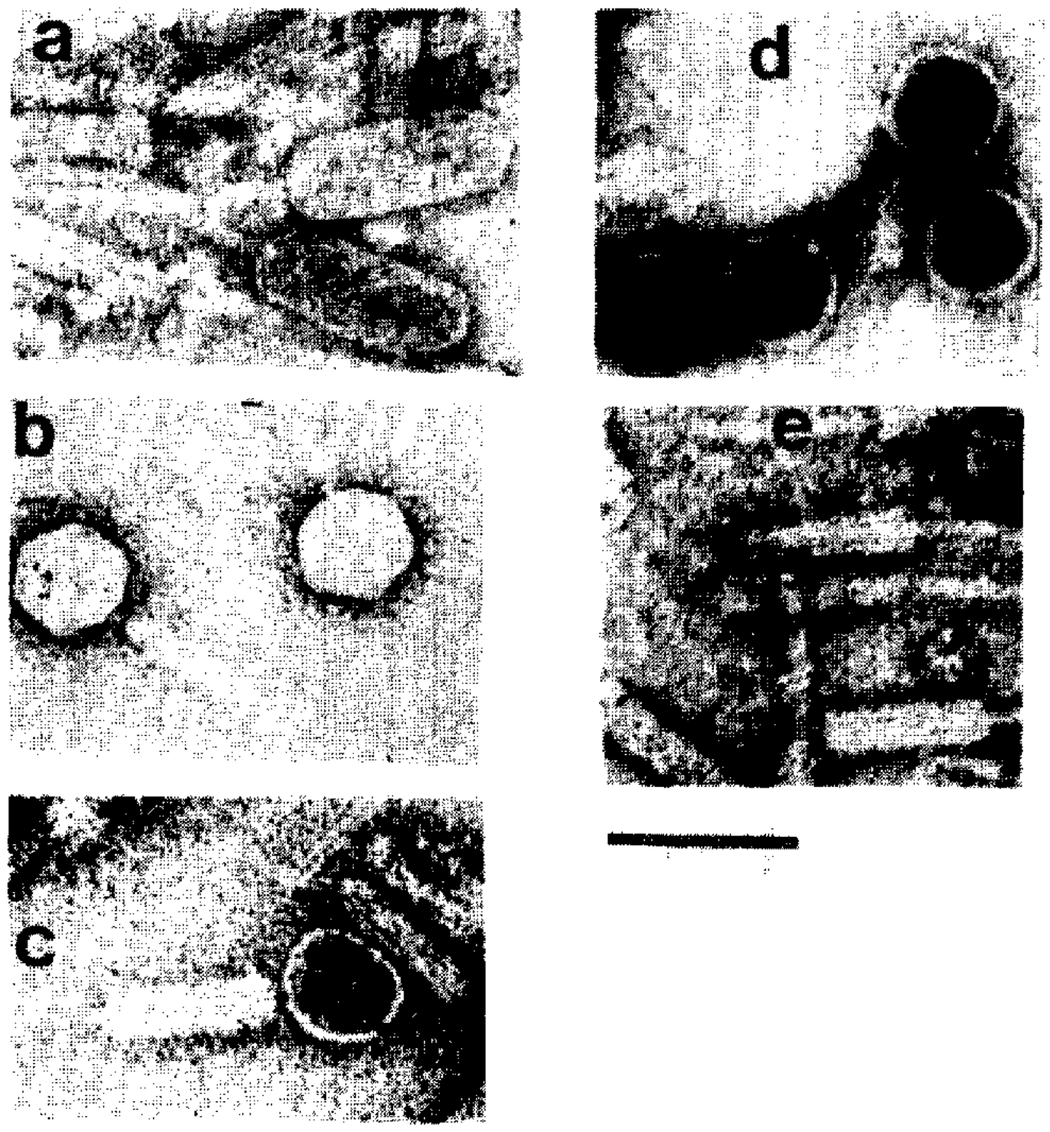


**Fig. 2. Electron micrographs of mitomycin C inducible particles of *B. circulans*.**
Mitomycin C-induced lysates of FA1 (a), FA3 (b), FA4, (c), FA5 (d), and FA7 (e) were negatively stained with 1% uranyl acetate. All bars represent 100 nm.

spontaneous production of lysogenized phages of *B. circulans* F-2 is not known, phage-like particles were rarely shown by electron microscopy of sedimentable 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. circulans* in NBY mitomycin C was added to a concentration ranging from 0.1 to 10 μg/ml, and the optical density at 660 nm of the culture was measured in the course of time. Representative results are shown in Fig. 1. Under this condition, a concentrations of 0.1-0.25 μg of mitomycin C caused maximal lysis of *B. circulans* F-2. Although it has been reported that maximal lysis occurred at 5-10 μg/ml mitomycin in *Clostridium tetani* (11), both the growth and the induction were prevented by 5 μg/ml or higher concentration of mitomycin C in *B. circulans* F-2. 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 particle, such as intact phage (Fig. 2a, b), intact phage with empty head (Fig. 2c), empty phage of head (Fig. 2d), or tail of phage (Fig. 2e) in their morphology.

**Killing activity**

It has been known that the some of inducible particles structually related to bacteriophage components could act bacteriocidally (12). Therefore, the killing activity of lysates obtained above was examined by cross streak methods (data not shown). Except for FA2, FA13, and FA12, twelve lysates out of 15 strains were able to kill two or more of the other strains of *B. circulans*, and no sensitive strain was detected in *Bacillus* species tested. Killing activity was found only 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. 3). Typical particles were summarized in Table 1. The sheath of tail-like particles showed extended or contracted form (Fig. 4). The tail sheath of a particle was examined and found that the

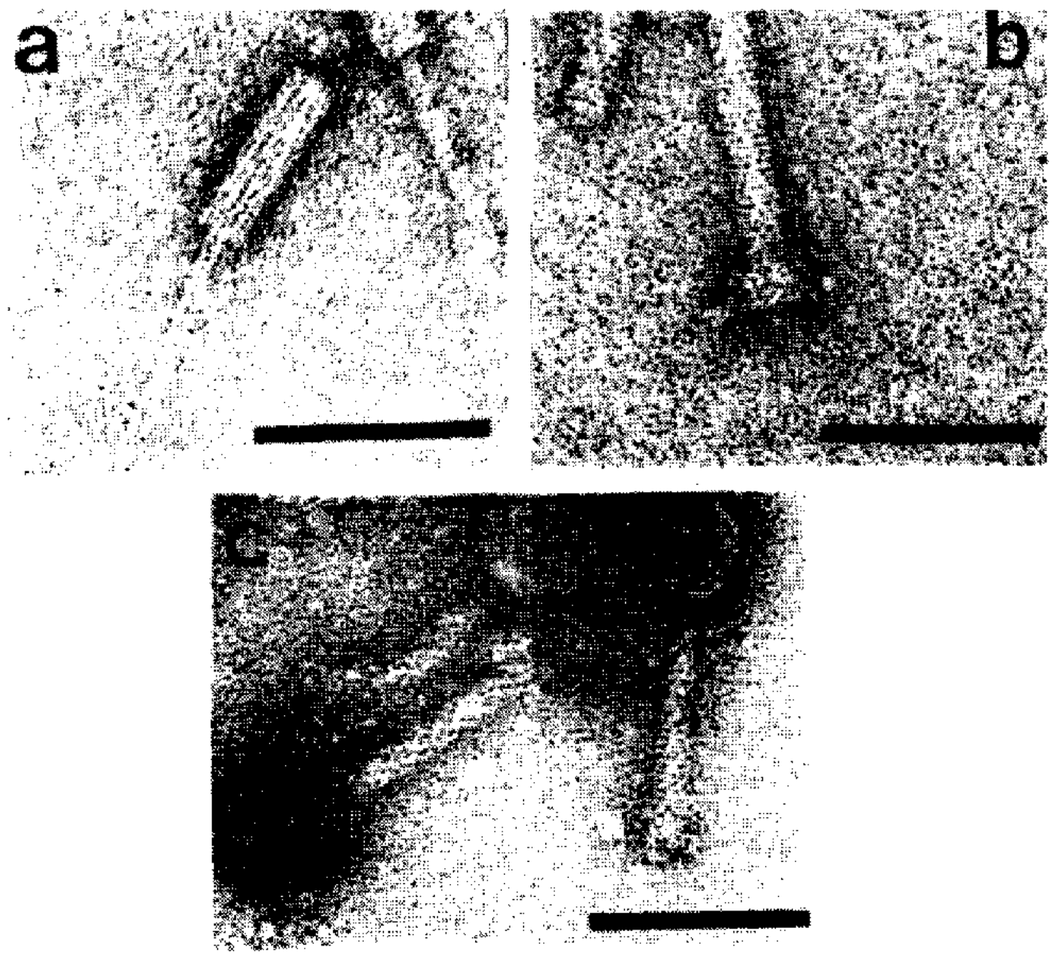


**Fig. 3. Electron micrograph of ciricin.**
Samples were taken from the peak fraction of sucrose gradient centrifugation, and negatively stained with 1% uranyl acetate.Ciricin particles shown are obtained from FA6 (a), FA10 (b), FA13 (c). All bars represent 100 nm.

**Fig. 4. Electron micrograph showing contracted form of ciricin of FA10.**
Negative staining was with 1% uranyl acetate. The bars represent 100 nm.

extended form showed about striatious 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 *Bacillus subtilis* (12). When contracted, the diameter (about 24 nm) of the sheath was a little greater than that of the extendede one (about 20 nm), and the subunits seemed to be rearranged into longitudinal rows (Fig. 4). 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. 5). Six fibers at tail tip were observed in preparations of tail-like particles from FA10 ang FA5 (Fig. 6). These last two structures have also been reported in PBSX and in INCO particles of

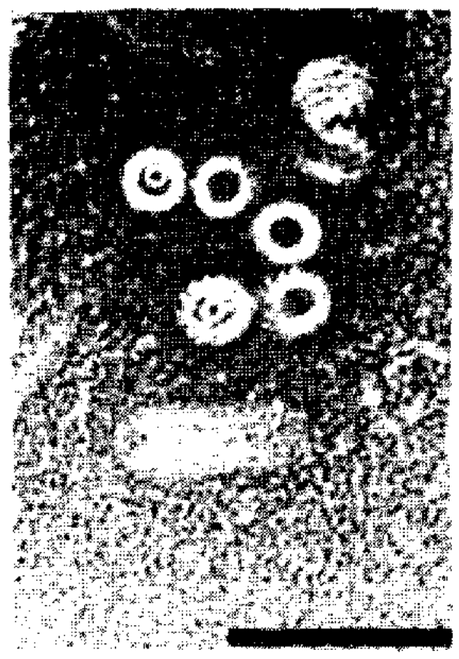

**Fig. 5. Electron micrograph of contracted ciricin of FA8 in endon view.**
Negative staining was with 1% uranyl acetate.The bar represents 100 nm.

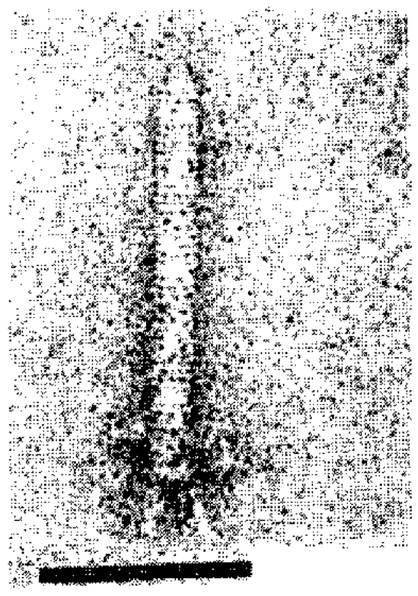

**Fig. 6. Electron micrograph of FA9 ciricin showing six tail fibers.**
Negative staining was with 1% uranyl acetate.The bar represents 100 nm.

*Rhizobium* (13).

**Temperative phage**

Together with the killing activity, plaque-forming activity was also found in the lysate of FA12. After sucrose gradient centrifugation, it was found that peak fraction for both killing (against FA10, 13, 14, 11, and 15) and the plaque-forming (against FA1, 7, and 9) activities coincined with each other (data not shown). Electron microscopy showed morphologically singular and homogeneous particles in the peak and the neighbouring fractions (Fig. 7a, b). The particle was called Sigma FA2. Sigma FA2 made turbid plaques with a center colony (Fig. 8). The size of plaques on FA7 or FA9 was about 1 to 1.5 nm in diameter. Although two types (large and small) of plaque are seen in Fig. 8, small plaques showed the same morphology as large plaques if additional incubation was carried out. The plaques on FA2 were very turbid and small,

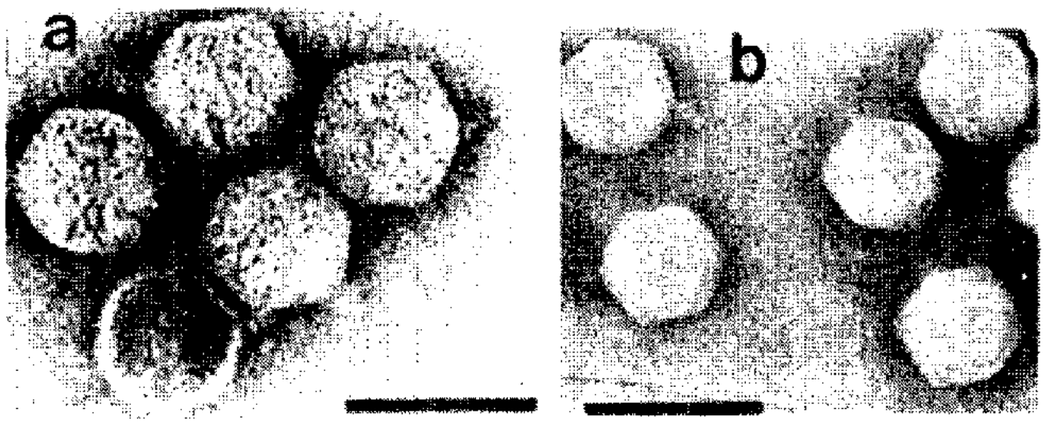


**Fig. 7. Electron micrograph of Sigma FA2. Samples as taken from the peak fraction of killing and plaque-forming activities.**
Negative-staining was with 1% uranyl acetate. The bar represents 100 nm.

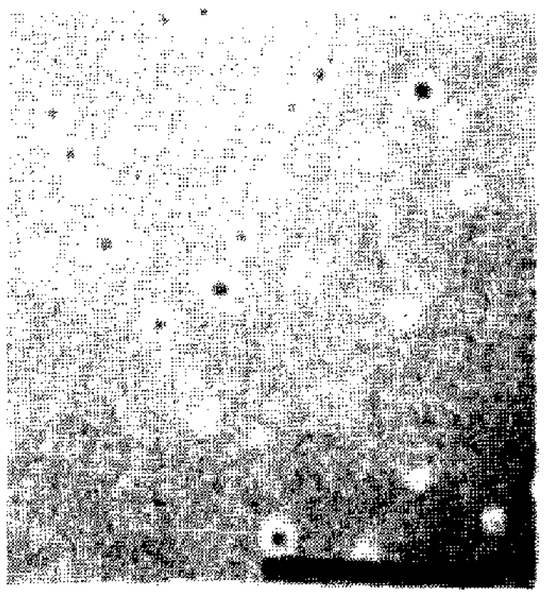

**Fig. 8. Plaque induced by purified Sigma FA2 on NBY plates with some lysogenized cells in it.**
The bar represents 10 nm.

and hard to count. Sigma FA2 multiplied on FA7 were centriguged 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-indeced lysate of K17, killing and plaque-forming activities coincined 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 K17 (Table 1).

## Discussion

A large number of diverse bacterial species have been showed to produce particles which contained either all or some of the normal phage conponents, but they failed to form plaque on any known host. These particles have been called as a defective phage (14). Present report shows that *B.circulans* F-2 produce such defective phages. Although a few reports sugested 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 order defective prophage 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 aeroginosa* 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. circulans* F-2, 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.

Morphologically, two killing agents (from FA7 and FA17) appeared to be intact phage (Fig. 3c, 7). Indeed, one of these showed plaque-forming activity on hosts. Therefore, this particle is not a defective phage but a complete phage. There is possibility that known defective phage which looked like complete phage in their morphology may have plaque-forming activity

**Table 1. Killing agents of *B. circulans***

| Producer (strain) | Morphology | Size (nm) | Density (sucrose %) | Sensitive strain |
|---|---|---|---|---|
| FA1 | Tail | 20×170 | 6-7 | 3,4,K17 |
| 6 | Tail | 20×170 | 6-7 | 10,13, K17 |
| 10 | Tail with fibers | 20×170 | 8-9 | 3,4 |
| 5 | Tail with fibers | 20×170 | 8-9 | 4,10 |
| 13 | T-even phage type | Head: 50 (diam)<br>Tail: 140×120 | 10-11 | 4,10 |
| K17 | T7 phage type | Head: 50 (diam)<br>Tail: 10×30 | 13-14 | 10,13,14 |

on unknown host. The trait of the particle (Sigma FA2) is of interest in relationship between bacteriocin and bacteriophage, and it is needed to be investigated further. Some of the biochemical results observed will be reported in somewhere.


## 요 약

*Bacillus circulans* F-2를 포함한 *B. circulans*의 prophage와 bacteriocin을 검출하기 위하여, mitomycin C를 처리하고 전자현미경상, plaque 형성능(plaque-forming activity), 세균세포살균능(killing activity)을 시험했다. killing activity 양성균으로부터 서당밀도균배원심(sucrose gradient centrifugation)을 통해 bacteriophage-like particle의 존재를 확인했다. 이들 입자들은 형태학적으로 phage tail 또는 T4 phage와 닮은 구조를 가지고 있었다.



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