Kor. J. Appl. Microbiol. Biotechnol.
Vol. 19, No. 6, 553-560 (1991)

Killing Activity and Molecular Properties of Bacteriophage Sigma FA1 of *Bacillus circulans*

Kim, Cheorl-Ho* and Dong-Soo Kim[1]

Genetic Engineering Research Institute, Korea Institute of Science and Technology, P.O. Box 17, Daeduk Science Town, Yusung-ku, Taejeon 305-606, Korea

[1]Department of Food Science and Technology, Faculty of Engineering Kyungsung University, Pusan 608-736, Korea

Bacteriophage Sigma FA1의 치사활성과 구조특성

김철호* · 김동수[1]

한국과학기술연구원 유전공학연구소

[1]경성대학교 식품공학과

Abstract — In the previous paper (10), a new temperate phage, Sigma FA1 had been isolated from *B. circulans*. Sigma FA1 had an icosahedral head with a diameter of about 70 nm, and a tail about 15 nm long, and beared a circularly permuted, linear duplex DNA. Sigma FA1 killed sensitive cells by a single-hit process. Phage DNA injected into the cell immediately after infection was degraded slowly. Our results indicate that the killing action of Sigma FA1 is different from the phenomenon of abortive infection and suggest that the killing might be caused by a proteinaceous component of Sigma FA1.

Bacillus circulans is a species closely related to *Bacillus subtilis* and also shares many properties with *Bacillus brebis* and some with *B. laterosporous*. The most specific biochemical trait of this strain is the productivity of raw starch-digesting amylase (EC 3.2.1.1) (1). Production, characterization, purification and industrial application of the enzymes, including raw starch-digesting amylase (2,3) and a bifunctional amylase-pullulanase enzyme (4,5), have long been studied by the author and co-workers. In addition, to study the molecular structures of those enzymes and to elucidate the gene expression system of *Bacillus circulans*, Kim *et al.* cloned the genes, and the enzymes were succesfully expressed in *Escherichia coli* (Kim *et al.*, 6). Furthermore, a new enzyme, C1-amylase which needs chloride-ion in it's expression of activity, was cloned and sequenced, and the enzyme was exclusively characterized (7-9).

On the other hand, in the course of search for a prophage of *B. circulans*, a temperate phage was isolated together with structured bacteriocins of various morphologies (10). This phage, called Sigma FA1, exhibited either (nonproductive) killing activity against several closely related strains or plaque formation on sensitive cells. These killing sensitive hosts were found to be nonlysogenic for any detectable complete phage. 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 (for high molecular weight bacteriocins, little is known about bacteriocin-like killing action by a complete phage which does not accompany the expression of the phage genome (11). The limited cases of killing by a complete phage include the abortive infection of *Escherichia coli* phage (12, 13), *Salmonella* phage (14), and *Bacillus subtilis* phage (15, 16) onto sensi-

Key words: *Bacillus* bacteriocin, bacteriophage, mitomycin C

*Corresponding author

tive hosts which are lysogenic for another prophage or carry a plasmid. We are not aware of any previous examples of killing activity by a complete phage which is independent of the expression of phage DNA or which is active on an apparently prophage-free host.

In this article, the authors describe an inducible phage of *B. circulans*, the characterization of its bacteriocin-like killing activity, and evidence which differentiates the killing action of Sigma FA1 from abortive infections like those listed above.

Materials and Methods

Microorganisms, media and buffers

The origin of ten strains of *B. circulans* used in this study was described in a previous paper (10). Fifteen other strains of *Bacillus* species included six strains (preserved in this laboratory) of *B. brevis* NCIB7577, NCIB8803, ATCC8185, ATCC8186, ATCC10027, ATCC10068 and three other strains (preserved in this laboratory), *B. circulans* NCIB 9374, *B. laterosporus* NCIB8213 and *B. subtilis* ATCC6633. These were examined for their sensitivity to killing agents and their production of phage by *B. circulans*.

The media used in this study were NBY and HIBY, which were described in a previous paper (10). 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), 0.1 M NaCl, 1 mM $MgSO_4$ and 0.01% gelatin, was used to dilute killing agents and to prepare sucrose gradients. SM buffer, which was used to dilute the phage and to prepare sucrose or CsCl gradients, contained 0.05 M Tris-HCl (pH 7.6), 0.1 M NaCl, 0.08 M $MgSO_4$, and 0.01% gelatin. TMS buffer contained 0.01 M NaCl. 0.01 M $MgCl_2$ and 0.05 M Tris-HCl (pH 8.0). TES contained 0.01 M Tris-HCl (pH 8.0), 0.15 M NaCl, and 2 mM EDTA. SSC [1XSSC is 0.15 M NaCl plus 0.015 M sodium citrate (17)], Sigma 80 buffer (18) and Kimura medium (19) were prepared as described.

Screening of killing agents and phages

Exponentially growing *B. circulans* was supplemented with 0.2 μg of Mitomycin C per m*l* and incubated further at 37℃ for 6 hr with shaking. The resulting lysates were treated with DNase and RNase (1 μg/m*l* each at 37℃ for 1 hr) and the supernant of low-speed centrifugation (5,000×g) was tested for killing activity by the cross-streak test and assayed semi-quantitatively by the serial dilution method (20). Results were expressed as the highest dilutions of samples giving positive responses. The low-speed supernatant was also assayed for plaque formation by the double agar layer method (21) with strain FA23 as the host.

Preparation of killing agents

After lysates were clarified by the method above, killing agents were pelleted at 120,000×g for 60 min at 4℃, suspended in 0.2 m*l* of Sigma 80 buffer, and purified through at linear 5% to 20% (wt/vol) sucrose gradient at 37,000×g for 30 min at 4℃. The killing activity of the fractions was examined as above.

Purification of Sigma FA1

After repeated single-plaque isolations, a phage stock was prepared by plating of FA23. The crude preparation was treated with DNase and RNase (10 μg/m*l* each, 37℃, 1 hr), and the pellet produced by centrifugation at 30,000×g was suspended and centrifuged down into a gradient of 20% sucrose (wt/vol) and three layers of CaCl (ρ=1.3, 1.5, and 1.7 g/cm) at 50,000×g for 90 min. The opalescent band obtained was centrifuged for 24 hr at 150,000 ×g in SM buffer containing CsCl (0.8 g/m*l*) and then dialyzed against TMS buffer. Phage DNA was isolated from the CsCl-purified Sigma FA1 by phenol extraction (22).

Electron microscopy

All preparations were carried out on carbon-coated copper grids. A drop of crude lysates or fraction after sucrose or CsCl density gradient, was washed with Sigma 60 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 a JEOL 200CX electron microscope (JEOL Co., Tokyo, Japan) at 80 kV.

Uptake of radioactive precursors by Sigma FA1 infected cells

Cells were grown in Kimura C to a concentration of 5×10^6 cells per m*l*, supplemented with radiolabeled precursors, and infected with Sigma FA1 at a multiplicity of infection (MOI) of 25. At the indicated time, 50 μ*l* samples were transfered into tubes of 1 m*l* of cold 10% trichloroacetic acid (TCA). The acid precipitate was collected on a membrane filter (pore size, 0.45 μm), washed with cold 5% TCA, dried, and counted. In experiments measuring (^{3}H) thymidine incorporation, the medium was supplemented with deoxyadenosine (1 mg/m*l*) and thymine (2 μg/m*l*). The radiolabeled precursors were added immediately before infection.

Preparation of radiolabeled phage

FA23 cells (4×10^8 cells/m*l*) were infected with Sigma FAI at an MOI of 5×10^{-5}. After adsorption for 20 min, deoxyadenosine (1 mg/m*l*) and thymine (2 μg/m*l*) were added along with (^{3}H) thymidine (450 μci/m*l*) and incubated for confluent soft agar plaques on an NBY agar plate. Phage particles were purified as described above.

Immunological methods

Anti-Sigma FA1 serum was raised by injecting purified Sigma FA1 ($\times10^{11}$ in 0.5 m*l* of gelatin-free SM buffer) into a BALBc mouse (male) three times at one-week intervals. Immunodiffusion in 0.6% agar was done by the Ochterlony technique (24). Immunoelectrophoresis (25) was performed in 0.6% agar containing 0.19 M Tris-HCl (pH 8.6) at 50 V for 4 hr.

Analysis of phage DNA after infection

FA10 cells (10^8 cells/m*l*) growing in HIBY were infected with DNA labeled Sigma FA1 at an MOI of 13. At various times, a 1 m*l* sample was chilled and spun down, and the precipitate was analyzed through slot-lysis electrophoresis by the method of Gonzaloz *et al.* (26) with 0.7% agarose. To measure the conversion of radiolabeled phage DNA into acid-soluble material, FA10 cells (10^9/m*l*) were infected with Sigma FA1 at an MOI of 0.1. At various times, a 0.1 m*l* sample was taken and placed into 1 m*l* of cold 10% TCA and kept at 0℃ for 1 hr. The precipitates were collected on membrane filters (pore size, 0.45 μm), washed with 5% TCA, and counted.

Results

Developmental production of killing agents and phage

All the strains of *B. circulans* were lysed within about 3 hr at 37℃ after treatment with mitomycin C. Electron microscopic observations showed that all lysates contained a wide variety of particles, such as apparently complete phage, phage heads, or phage tails. Out of 15 lysates, 13 were able to kill two or more other strains of *B. circulans*, but not the producer strain itself. Killing was observed only among strans of *B. circulans*, and no sensitive strains were detected in the other *Bacillus* species tested, including *B. brevis*, *B. laterosporus*, and *B. subtilis*.

When killing agents were purified by sucrose gradient centrifugation, they were found in fractions of different sucrose concentration, and were specific for each particle (Fig. 1). Interestingly, it was found that the peak fraction for both the killing (against FA10) and the plaque-forming activities coincided in the F-2 lysate. A phage particle of *B. circulans* was called Sigma FA1. Sigma FA1 made turbid pla-

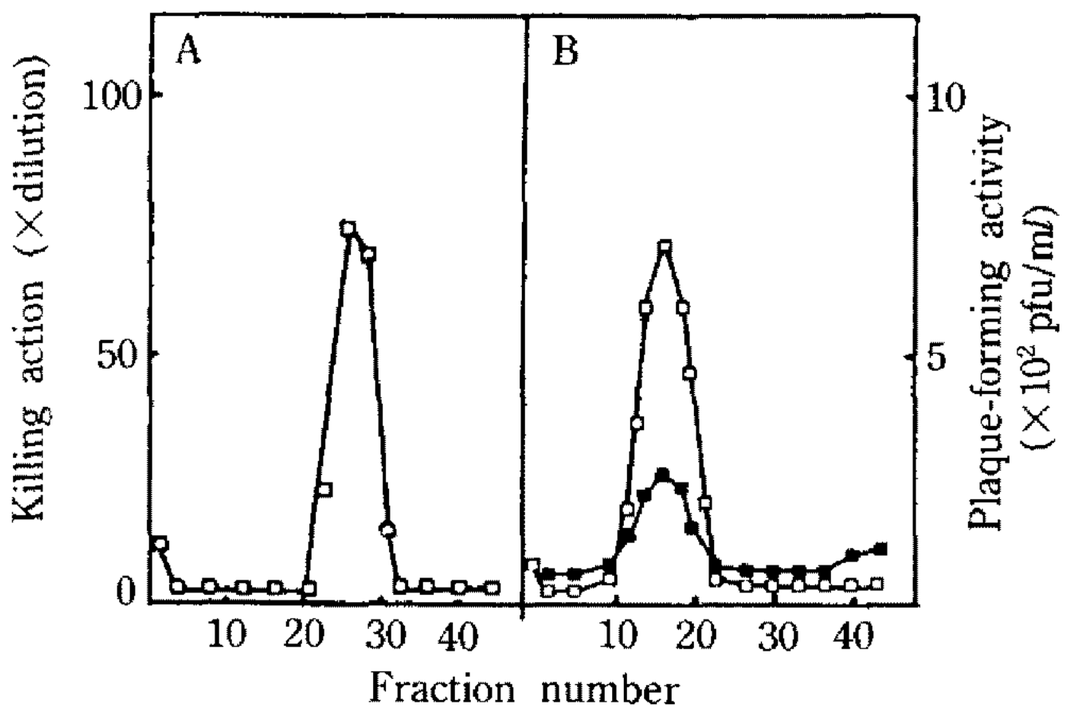

Fig. 1. Ultracentrifugation profile of the killing agents and phage in a 5 to 20% linear sucrose gradient. Fraction No. 1 represents the bottom of the tube. A) killing activity of FA23 lysate, assayed on FA10; B) Plaque-forming activity of F-2 lysate, assayed on FA23. □, Killing activity; ■, Plaque-forming activity.

ques, typical of temperate phage, on strains FA10 and FA23. Plates on FA1 were very turbid, small, and hard to count. FA23 gave easily recognizable turbid plaques with a similar efficiency of plating. Thus, FA23 was used as the host for growing and assaying Sigma FA1. Lysogenic cells from the center of a turbid plaque on FA23 were inducible and released Sigma FA1 into medium after treatment with mitomycin C.

Two activities of Sigma FA1

Sigma FA1 was further purified by CsCl equilibrium density gradient centrifugation. Plaque-forming (against FA23) and killing (against FA10) activities were examined for each fraction. Again, the peak fractions for these two activities coincined with each other (data not shown).

To rule out the possibility that the killing activity was due to some agent other than the Sigma FA1 particle, the homogeneity of the purified Sigma FA1 preparation was checked with immunological methods. The results of simple immunodiffusion showed the uniformity of the Sigma FA1 particles (Fig. 2). Sigma FA1 particles grown in FA23 cells were serologically identical to the original virion observed in mitomycin C-induced lysates of FA23 (Fig. 2).

To confirm the two activities of Sigma FA1, neutralization tests were performed. Thus, purified Sigma FA1 first mixed with strain FA10 or FA23, and the titer on the supernatant of centrifugation at 4,000×g was measured for the plaque-forming or killing activity, respectively. It was found that FA10 and FA23 strains neutralized phage-forming and killing activities, respectively (data not shown). The two Sigma FA1 activities were stable at pH values between 5.0 and 9.4, and showed the same spectra of sensitivity to proteinases examined (Sigma FA1 was inactivated by proteinase K and resistant against trypsin and subtilisin under the conditions tested). These results reasonably suggest that the activites could be attributed to the same particle, Sigma FA1.

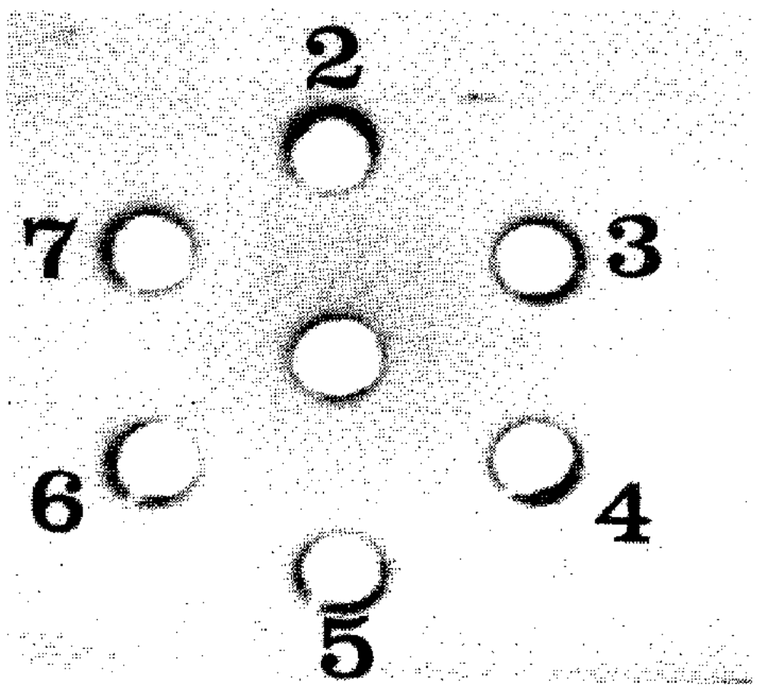

Fig. 2. Ouchterlony immunodiffusion tests.
The center well contained mouse antiserum against purified Sigma FA1. The peripheral wells contained mitomycin C-induced lysate of FA23 (well 4), Sigma FA1 (3×10^8 particles) (well 3), Sigma FA1 (10^8 particles) (well 2), cleared culture fluid of uninfected FA23 (well 5), crude Sigma FA1 (Scraped plaque on FA23) (well 6), and Sigma FA1 (3×10^8 particles) (well 7).

Molecular basis of Sigma FA1

The number and approximate molecular size of viral proteins were analyzed by SDS-PAGE. The

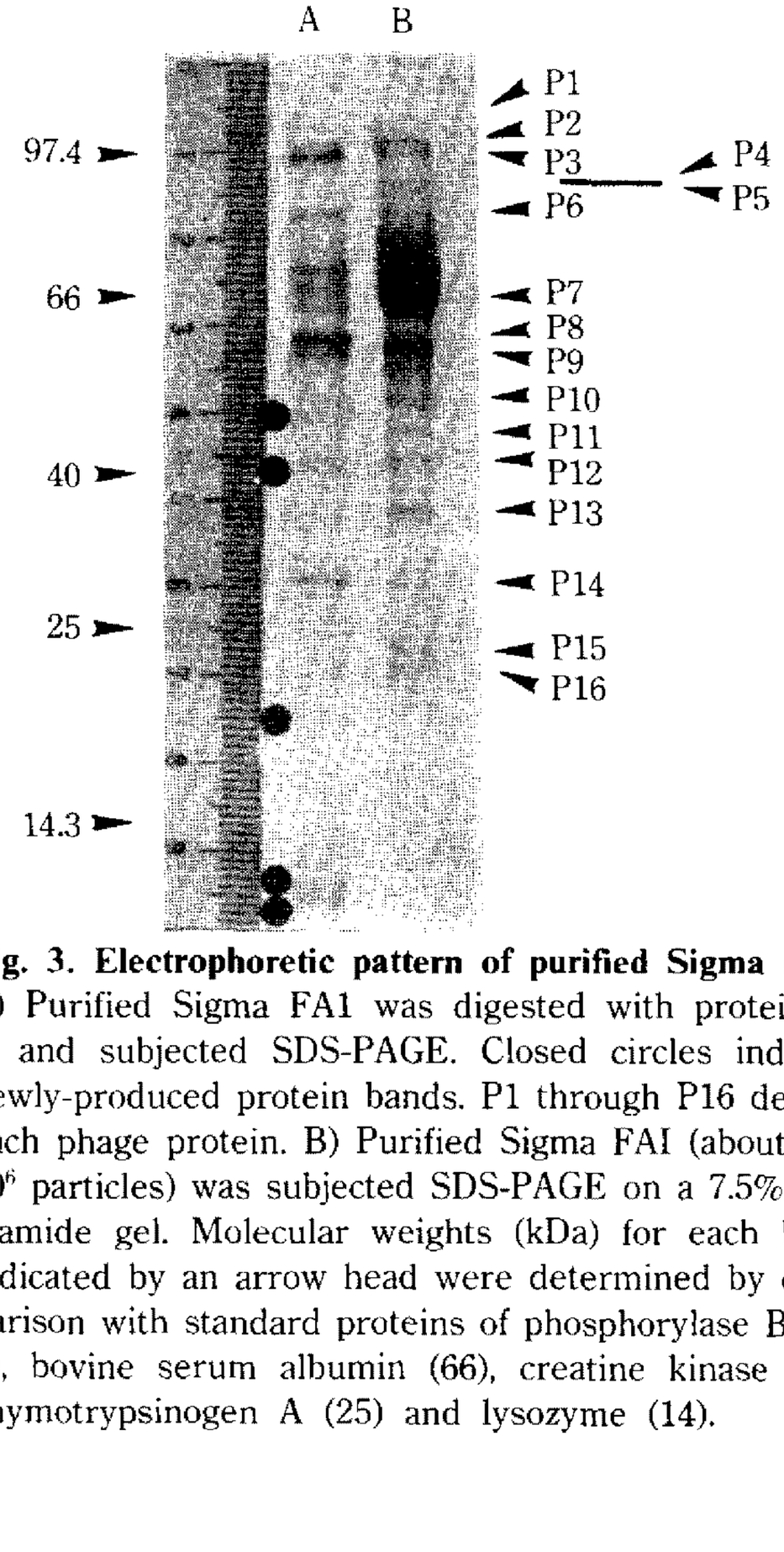

Fig. 3. Electrophoretic pattern of purified Sigma FA1.
A) Purified Sigma FA1 was digested with proteinase K and subjected SDS-PAGE. Closed circles indicate newly-produced protein bands. P1 through P16 denote each phage protein. B) Purified Sigma FAI (about 5×10^6 particles) was subjected SDS-PAGE on a 7.5% acrylamide gel. Molecular weights (kDa) for each band indicated by an arrow head were determined by comparison with standard proteins of phosphorylase B (97.4), bovine serum albumin (66), creatine kinase (40), chymotrypsinogen A (25) and lysozyme (14).

structural proteins were resolved into 15 bands, and the electrophoretogram revealed that the protein P7 (molecular weight 66,000) accounted for 79 % of the total protein (Fig. 3); it is presumed to be the major constituent of the band. The molecular sizes of the polypeptides were estimated to be 110, 100, 95, 84, 82, 74, 66, 59, 57, 50, 44, 42, 38, 30, 34, and 22 kilodaltons.

Kinetics of killing

Sigma FA1 was mixed with a logarithmic phase culture of FA10 at an MOI of 20. After appropriate incubation, surviving cells were counted (Fig. 4a). The extent of killing with various amounts of Sigma FA1 was also investigated. A linear correlation was obtained between the logarithms of the number of surviving cells and the Sigma FA1 dose (Fig. 4b) suggesting the killing action of Sigma FA1 to be a single-hit process (28). To corfirm the killing by a single particle, the number of FA10 cells was varied against a constant number of Sigma FA1 partiles. When the MOI value was sufficiently low, the number of FA10 cells killed was equal to the number of Sigma FA1 particles.

Physiology of infected cells and phage DNA, and killing action

Physiological change of host cells have been reported in the course of killing by bacteriocin (29) or by phage which showed abortive infection (30). To clarify the events occuring in the course of killing in the FA10-Sigma FA1 system, we examined the physiological changes in FA10 cells after Sigma FA1 infection, using FA23 as the control. To determine whether macro-molecular synthesis is inhibited in FA10, cumulative incorporation of precursors of DNA, RNA, and protein into the acid-insoluble fraction was measured at various times after infection. Incorporation of labeled thymidine, uridine, and leucine into macromolecules began to decline about 5 min after infection (Fig. 5). To study the relationship between killing by Sigma FA1 and the

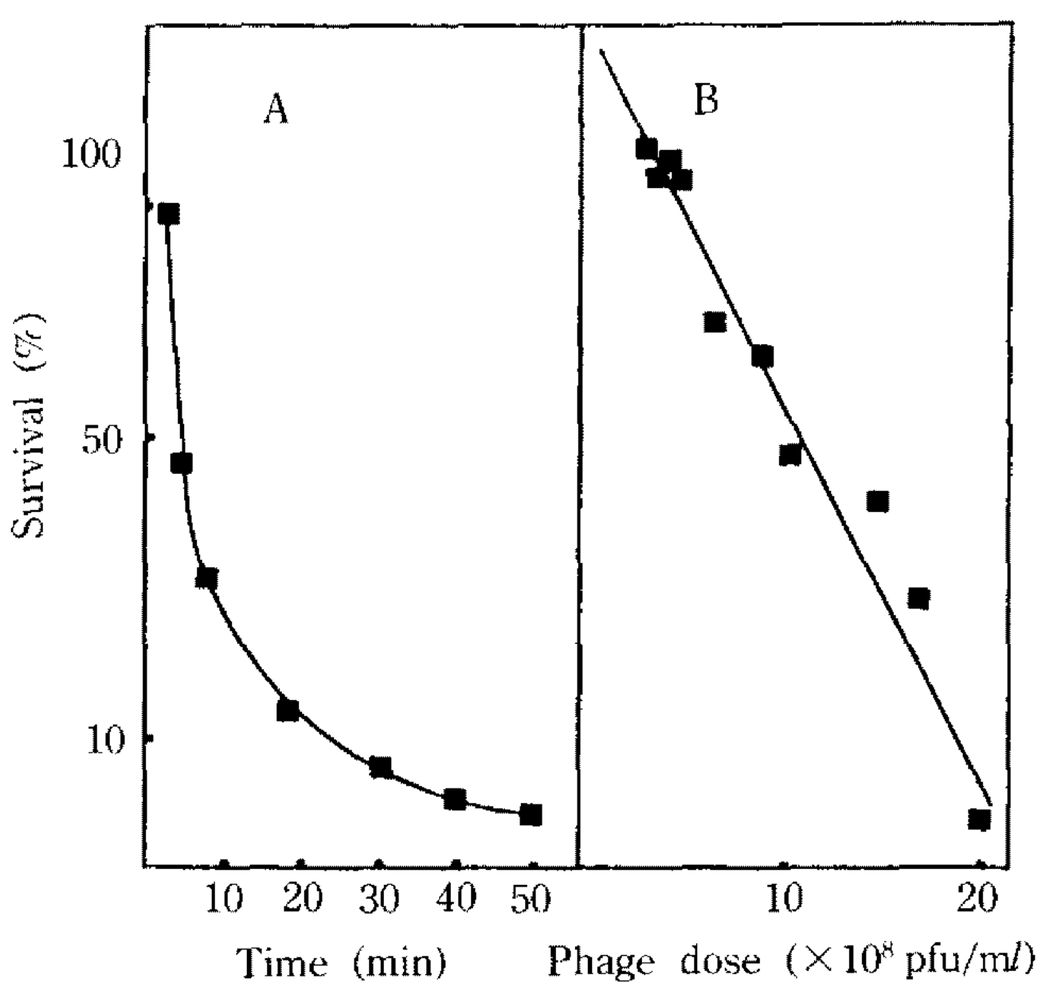

Fig. 4. Kinetics of killing action of Sigma FA1.
A) Time courses. Suspensions of FA10 (3×10^6 cells/m*l*) in kimura C were infected at time zero with Sigma FA1 at an MOI of 20 and incubated at 37℃. At several time intervals, the reaction mixtures were diluted with kimura C, and the surviving cells were counted by spreading on an NBY plate. B) Relationship of the survival ratio to the Sigma FA1. Suspensions of FA10 (3×10^6 cells/m*l*) in kimura C were mixed with various amounts of Sigma FA1 (10^9 pfu/m*l*) to give the final phage concentration indicated and then incubted at 37 ℃ for 20 min.

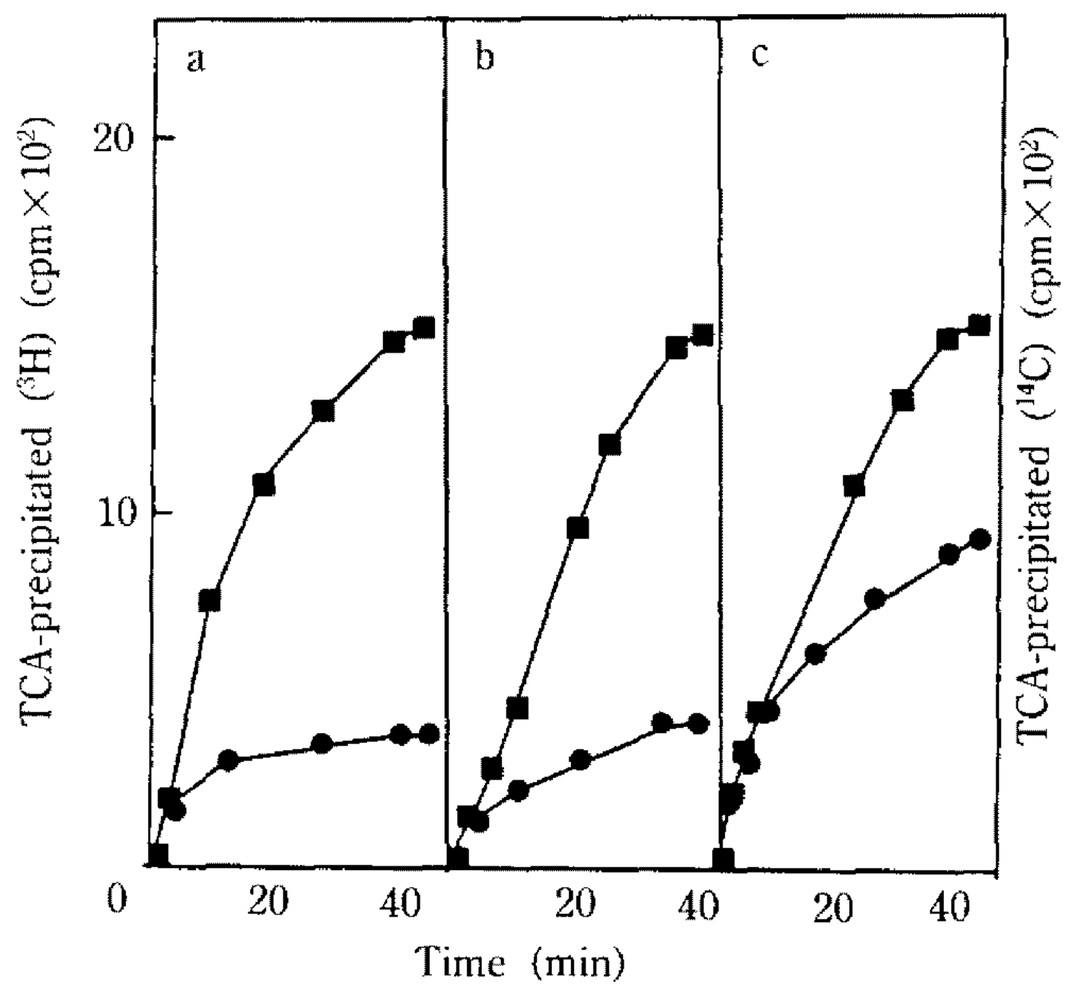

Fig. 5. Biosynthesis of DNA, mRNA and protein.
Cells were grown and infected with Sigma FAI. Radiolabeled tracers were added at time zero. Samples (10 μ*l*) were withdrawn at the indicated times and preincubated in 1 m*l* of cold 10% TCA. The TCA-precipitate was collected on a GFC (Whatman Co.), and the radioactivity (^{3}H for nucleic acid, ^{14}C for protein was determined. (a) DNA: (b) RNA: (c) protein. ■, Sigma FA 1-infected FA cells. ●, uninfected cells.

membrane transport activity of FA10 cells, we measured the uptake of leucine after infection. However, no difference was observed between the degrees of inhibition of the activity in FA10 and FA23 cells. No degradation of the chromosomal DNA of FA10 cells was observed (as measured by the TCA-precipitable radioactive DNA) up to 120 min after infection, at which time 99% of the cells had been killed.

We wished to determine whether infected Sigma FA1 DNA was degraded by a host restriction system. The extent of degradation was estimated by the liberation of radioactive fragments of phage DNA into the acid-soluble fraction of the infected culture, with the restricted fragments detected by agarose gel electrophoresis. It was found that when Sigma FA1 DNA was infected into FA10 cells, it was rapidly degraded, with a half-life of less than 1 min (agarose gel electrophoresis experiment) or with a half-life of about 7 min (TCA precipitation experiments). These results indicate that Sigma FA 1 DNA could not be expressed at all after its infection into FA10 cells. This was confirmed through the observation that early proteins synthesized in FA23 for Sigma FA1 were not detectable in FA10 cells (data not shown). From these results, it was inferred that the expression of the phage genome was not necessary for the killing activity. This notion was supported by the observation that killing (60% of that in the nonirradiated control) was still caused by heavily UV-irradiated Sigma FA1, whose plaque-forming activity was reduced to 10^{-4} (data not shown). In the cases of known abortive infection (16, 29, 30), killing-sensitive bacteria carry a phage or harbor a plasmid. It has been reported that non-permissive *B. subtilis* cells lysogenized with bacteriocin PBSX become sensitive to phage SP-10 under conditions in which PBSX is induced by treatment with mitomycin C, and it has been suggested that the resistance of the host cell to SP-10 is controlled by the lysogenized PBSX (31). Even though we ran experiments to determine whether Sigma FA1 could multiply in FA10 cells which had been pretreated with mitomycin C, no multiplication of Sigma FA1 in such cells could be detected, and the host cells were killed with a reduced production of bacteriocin. This suggests that the inability of Sigma FA1 to multiply in FA10 was not caused by the structured bacteriocin. We also tried to detect bacteriocin activity of Sigma FA1-infected FA10 cells without mitomycin C treatment, but no induction of bacteriocin could be observed.

It has been known that some of the inducible particles structurally related to bacteriophage components could act bacteriocidally. Therefore, the killing activities of the lysates obtained above were examined by cross streak methods. 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 strains were detected in other *Bacillus* species tested. By the detection method used, killing activity was found not in the supernatants, but in the precipitates after ultracentrifugation, indicating the high molecular weight nature of the killing agent.

Discussion

It has been known that a temperature phage either grows lytically or lysogenizes when it infects a bacterial cell. Sigma FA1, the temperate phage described in this report, behaved as a temperate phage on two strains (FA23 and FA24) of *B. circulans*. However, Sigma FA1 also showed bacteriocin-like killing activity against three other strains (FA 10, FA13 and FA24). Expression of the phage genome was confirmed to be absent in at least one of these latter strains.

In some bacteria-virus interactions, an abortive infection has been reported as a phenomenon, in which no progeny phage are produced and the host cells are killed (12, 16, 29, 30). In these cases, phage DNA is infected normally into the host cell and the normal infectious process starts, but the presence of a prophage or a plasmid dictates that the normal events cease before the production of progeny.

Membrane depolarization is suggested to be a primary cause of some of the abortive infectios (30). Although we initially thought that Sigma FA1 might have abortively infected FA10 cells and caused cell death, this view is unlikely to be the case for seve-

ral reasons. In the course of killing of FA10 cells by Sigma FA1, (i) the injected phage DNA was rapidly degraded in the host cells, (ii) the phage genome was not expressed, (iii) killing occured even when heavily UV-irradiated phage was used, (iv) no lysogenization of Sigma FA1 was observed in rare surviving colonies, and (v) no prophage or plasmid was detectable in FA10 cells. These festures are clearly different from those of known abortive infections and seem to suggest that the killing of FA10 is caused by some proteinaceous component of Sigma FA1.

Proteinaceous killing agents with a narrow host range are known as bacteriocin (32). The modes of action of some low-molecular-weight bacteriocins have been determined. They can be ionphores, DNAses, RNAses (34), or phospholipases (35). If ion-permeable channels were formed by Sigma FA1 and were the primary cause of cell death, a drastic leakage of K^+ would be observed only in the killing sensitive host (FA10) relative to the phage-productive host (FA23). The results indicate, however, that the extent of leakage of K^+ in both strains was the same for at least the first 10 min of infection, during which time the killing was nearly completed (data not shown). This result indicates that while membrane depolarization occured as reported for T1 (36) when Sigma FA1 adsorbed host bacterial cells, it did not appear to be a primry cause of cell death. In addition, Sigma FA1 reduced leucine transport in both strains by about 50%. This also indicates that membrane disfunction as not specific for killing-sensitive cells. Although it can not be ruled out, the possibility that Sigma FA1 induced killing is caused by DNAse activity is less likely, since no obvious destruction of host chromosomal DNA was observed and also, specific inhibition of protein synthesis in FA10 was not observed in the course of killing. Genes which might be concerned with the shifting of host cells into a stable phage factory would not be expressed as a result of the restriction of Sigma FA1 DNA (data not shown). However, the result indicated that the accummulated leucine did not dissipate completely. Moreover, the process of killing FA10 (Fig. 4a) seemed to be an event proceeding the restoration of the membrane in FA23. On the other hand, the frequency with which the killing-resistant mutants were obtained suggests that the killing-sensitive host became resistant by a one-step mutation, and it is inferred that the putative target for the killing action of Sigma FA1 would be some reactant for the phage enzyme injected or some regulatory protein in the host cell.

The infection of FA10 cells with Sigma FA1 resulted in cell death without the production of any bacteriophage at an MOI of 0.5, or without any cell lysis at an MOI of 50 or higher. These results indicate that Sigma FA1 action on FA10 cells is different from the mode of action of some bacteriophages in lysis from without (33). As mentioned above, Sigma FA1 may be regarded as a defective phage, such as PBSX (12). In phage PS17 of *Pseudomonas aeroginosa*, only the tail part of the phage showed killing activity similar to R1 (34). In this case, killing-sensitive strains were the same as phage-producing strains. These observations suggest a continuity in evolution between bacteriophage and defective phage. We think Sigma FA1 may be an example of the evolution of a bacteriophage with a defective phage activity. In our case, however, Sigma FA1 as a bacteriocin is not a defective phage but a complete phage, and we use the term structured bacteriocin instead of defective phage.

요 약

*Bacillus circulans*로부터 mitomycin C처리에 의해 유발되는 Bacteriophage Sigma FA1을 서당밀도균배원심법 (sucrose gradient centrifugation)에 의해 분리, 정제하였다. Sigma FA1의 감수성숙주에 대한 치사작용은 single-hit 방식이었다. 한편, 세포에 감염된 후 phage DNA는 천천히 분해되기 시작했으며, 본 연구의 결과는 Sigma FA1의 세포에 대한 치사작용이 기존의 것과는 다른 것임을 입증시켰으며, 이는 단백질분해기능에서 유래되는 듯 했다.

References

1. Taniguchi, H., F. Odashima, M. Igarashi, Y. Maruyama and M. Nakamura: *Agric. Biol. Chem.*, **46**, 2107 (1982)

2. Kim, C.H.: *Nihonseikagakukaishi*, **61**, 34 (1986)
3. Kim, C.H.: *Agric. Biol. Chem.*, **54**, 2767 (1990)
4. Sata, H., M. Umeda, C.H. Kim, H. Taniguchi and Y. Maruyama: *Biochim. Biophys. Acta*, **991**, 338 (1989)
5. Kim, C.H., D.-S. Kim, H. Taniguchi and Y. Maruyama: *J. Chromatography*, **512**, 131 (1990)
6. Kim, C.H., H. Sata, H. Taniguchi and Y. Maruyama: *Biochim. Biophys. Acta*, **1048**, 223 (1990)
7. Kim, C.H., S.-T. Kwon, D.-S. Lee, H. Taniguchi and Y. Maruyama: *Kor. J. Appl. Microbiol. Biotechnol.*, **18**, 309 (1990)
8. Kim, C.H., H. Taniguchi and Y. Maruyama: *Arch. Biochem. Biophys.*, Submitted (1991)
9. Kim, C.H., H. Taniguchi and Y. Maruyama: *Nihonnougeikagakukaishi*, **64**, 379 (1989)
10. Kim, C.-H., S.-T. Kown, D.-S. Lee, H. Taniguchi and Y. Maruyama: *Kor. J. Appl. Microbiol. Biotechnol.*, **18**, 221 (1990)
11. Bredley, D.E.: *Bacterial Rev.*, **31**, 230 (1967)
12. Ghisottic, D., S. Zangrossi and G. Siromi: *J. Virol.*, **48**, 616 (1983)
13. Glenn, J. and D.H. Duckworth: *J. Virol.*, **30**, 421 (1979)
14. Susskind, M.M., A. Wright and D. Botstein: *Virology*, **62**, 367 (1974)
15. Rettenmier, C.W. and H.E. Hemphill: *J. Virol.*, **13**, 870 (1974)
16. Yasbin, R.E., A.T. Genesan and F.E. Young: *J. Virol.*, **13**, 916 (1974)
17. Marmur, J.: *J. Mol. Biol.*, **3**, 208 (1961)
18. Malamy, M.H., M. Fiandt and W. Szybalski: *Mol. Gen. Genet.*, **119**, 207 (1972)
19. Kimura, R.: *Jpn. J. Med. Pro.*, **48**, 685 (1961)
20. Kageyama, M. and F. Egami: *Life Sci.*, **9**, 471 (1962)
21. Adams, M.H.: *Bacteriophages*, Interscince Publishers, Inc., New York, 27 (1959)
22. Maniatis, T.E., E.F. Fritsch and J. Sambrook: *Molecular cloning: a laboratory manual*, Cold Spring Harbor, New York (1982)
23. Birdsell, D.C., G.M. Mathaway and L. Ruitberg: *J. Virol.*, **4**, 264 (1969)
24. Ouchterlony, O.: *Prog. Allergy* **5**, 1 (1958)
25. Jurd, R.D.: Gel Electrophoresis of Protein, (B.D. Hames and D. Rickwood eds.) IRL Press, Oxford, England, 236 (1981)
26. Gonzalez, J.M., Jr., H.T. Dulamge and B.C. Carton: *Plasmid*, **5**, 351 (1981)
27. De Lay, J.: *J. Bacteriol.*, **101**, 738 (1970)
28. Jacob, F., L. Siminovitch and E. Wollman: *Ann. Inst. Pasteur* (Paris), **83**, 295 (1952)
29. Susskind, M.M., A. Wright and D. Botstein: *Virology*, **62**, 367 (1974)
30. Goldberg, I.D. and T. Bryan: *J. Virol.*, **2**, 805 (1968)
31. Tagg, J.R., A.s. Dajani and L.W. Wannamaker: *Bact. Rev.*, **401**, 722 (1976)
32. Kao, S.H. and E.P. Bakker: *J. Virol.*, **34**, 95 (1980)
33. Shinomiya, T. and S. Shiga: *J. Virol.*, **32**, 958 (1979)
34. Konisky, J.: *Annu. Rev. Microbiol.*, **36**, 125 (1982)
35. Ozaki, M.Y. Higashi, H. Saito, T. An, and T. Amano: *Biken J.*, **9**, 201 (1966)
36. Keweloh, H. and E.P. Bakker: *J. Bacteriol.*, **60**, 347 (1984)
37. Thomas, C.A., Jr. and L.A. MacHattie: *Ann. Rev. Biochem.*, **36**, 487 (1967)

(Received September 4, 1991)