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Bacteriocinlike Killing Action of a Temperate Bacteriophage φBA1 of *Bacillus aneurinolyticus*

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A new temperate phage, φBA1, was isolated from *Bacillus aneurinolyticus*. φBA1 had an icosahedral head with a diameter of about 70 nm and a tail about 20 nm long and contained a circularly permuted, linear duplex DNA of about 38 × 10^6 daltons. This phage showed two activities: bacteriocin-like killing activity against five strains of *B. aneurinolyticus* and normal temperate phage activity against three other strains. φBA1 killed sensitive cells by a single-hit process. After adsorption of φBA1 to cells sensitive to killing, the content of intracellular ATP increased for the first 5 min and then gradually decreased. Phage DNA injected into the cell immediately after infection was degraded rapidly. Killing was also caused by heavily UV-irradiated φBA1. Killing-resistant mutants showed normal adsorption of φBA1 and normal injection of the DNA with its instantaneous restriction. Our results indicate that the killing action of φBA1 is different from the phenomenon of abortive infection and suggest that the killing might be caused by a proteinaceous component of φBA1.

Bacillus aneurinolyticus is a species closely related to *Bacillus brevis* and also shares many properties with *Bacillus laterosporus* and some with *Bacillus circulans*. The most specific biochemical trait of *B. aneurinolyticus* is the production of thiaminase (EC 3.5.99.2) (9), but this group of bacteria has so far been left as an unassigned group taxonomically by some authors (31). In the course of a search for a prophage of *B. aneurinolyticus*, a temperate phage was isolated together with structured bacteriocins of various morphologies. This phage, called φBA1, 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, see reference 4), little is known about bacteriocinlike killing action of a complete phage which does not accompany the expression of the phage genome. The limited cases of killing action of a complete phage include the abortive infection of *Escherichia coli* phage (8, 10, 27, 33), *Salmonella* phage (38), and *Bacillus subtilis* phage (32, 42) onto sensitive hosts which are lysogenic for another prophage or carry a plasmid. We are not aware of any example of killing activity of a complete phage which is independent of the expression of phage DNA or which is active on an apparently prophage-free host.

In this report, we describe an inducible prophage of *B. aneurinolyticus*, characterization of its bacteriocin-like killing activity, and evidence which differentiates the killing action of φBA1 from abortive infections like those listed above.

MATERIALS AND METHODS

Microorganism, media, and buffers. The origins of 20 strains of *B. aneurinolyticus* used in this study were described elsewhere (1). All bacterial strains grown either in NBY, which contained 1% polypeptone (Daigo Eiyo), 0.5% meat extract (Wako Pure Chemicals), and 0.1% yeast extract (BBL Microbiology Systems), or in HIBY, which contained 2.5% heart infusion broth (Nissui) and 0.1% yeast extract. SM buffer, which was used to dilute the phage and to prepare sucrose or CsCl gradients, contained 0.05 M Tris hydrochloride (pH 7.6), 0.1 M NaCl, 0.008 M $MgSO_4$, and 0.01% gelatin. TMS buffer contained 0.01 M NaCl, 0.01 M $MgCl_2$, and 0.05 M Tris hydrochloride (pH 8.0). TES contained 0.01 M Tris hydrochloride (pH 8.0), 0.15 M NaCl, and 0.002 M EDTA. SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [26]), φ80 buffer (24), and Kimura C medium (20) were prepared as described.

Screening of killing agents and phages. Exponentially growing *B. aneurinolyticus* was supplemented with 0.2 μg of mitomycin C per ml and incubated further at 37°C for 6 h with shaking. The resulting lysates were treated with DNase and RNase (1 μg/ml each at 37°C for 1 h), and the supernatant of low-speed centrifugation (5,000 × *g*) was tested for killing by the cross-streak test and assayed semiquantitatively by the serial dilution method (15); 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 (2) with strain KA23 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°C, suspended in 0.2 ml of φ80 buffer, and purified through a linear 5 to 20% (wt/vol) sucrose gradient at 37,000 × *g* for 30 min at 4°C. Killing activity of the fractions was examined as above.

Purification of φBA1. After repeated single-plaque isolation, a phage stock was prepared by plating on KA23. The crude preparation was treated with DNase and RNase (10 μg/ml each, 37°C, 1 h), 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 CsCl (ρ = 1.3, 1.5, and 1.7 g/cm^3) at 50,000 × *g* for 90 min. The opalescent band obtained was centrifuged for 24 h at 150,000 × *g* in SM buffer containing CsCl (0.8 g/ml) and then

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dialyzed against TMS buffer. Phage DNA was isolated from the CsCl-purified ϕBA1 by phenol extractions (25). T_m and the buoyant density of ϕBA1 DNA were determined by using ϕ105 DNA as a standard (3).

Mapping of recognition sites of restriction endonuclease. Conditions of cleavage for each endonuclease (Nippon Gene) were those recommended by suppliers. Fragments were analyzed by electrophoresis on 0.5 to 1.0% agarose and 3.5 to 12% polyacrylamide gels. The molecular sizes were estimated against fragments of λ DNA cut by *Hin*dIII or of pBR322 cut by *Hin*fI.

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS) electrophoretic analysis was performed by the method of Laemmli (23). The concentration of acrylamide was 3.75% in the stacking gel and 10% in the separating gel.

Uptake of radioactive precursors by ϕBA1-infected cells. Cells were grown in Kimura C to a concentration of 5×10^7 cells per ml, supplemented with a radiolabeled precursor, and infected with ϕBA1 at a multiplicity of infection (MOI) of 25. At the indicated time, 50-μl samples were transferred into 1 ml 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/ml) and thymine (2 μg/ml). Radiolabeled precursors were added immediately before infection.

Preparation of radiolabeled phage. KA23 cells (4×10^8 cells per ml) were infected with ϕBA1 at an MOI of 5×10^{-5}. After adsorption for 20 min, deoxyadenosine (1 mg/ml) and thymine (2 μg/ml) were added along with [^{3}H]thymidine (450 μCi/ml) and incubated for confluent soft agar plaques on NBY agar plate. Phage particles were purified as described above.

ATP determination. Cells were grown in NBY to a concentration of 10^8 cells per ml, washed, suspended in 10 ml of Kimura C, and infected with ϕBA1 at an MOI of 20. At the indicated times, portions (1 ml) were filtered through a membrane filter (pore size, 0.45 μm) and extracted in 5 ml of boiling water for 20 min (5). ATP was determined with firefly extracts (FLE-50; Sigma) (37).

K^+ content of cells. Cells were grown in NBY to 8×10^7 cells per ml, centrifuged, and suspended in 10 ml of 0.05 M Tris hydrochloride (pH 8.0)–0.1 M NaCl–0.013 M sodium glutamate–7×10^{-4} M $MgSO_4$ at 4×10^8 cells per ml. ϕBA1 (5×10^{11}/ml) was then infected at an MOI of 25. Samples (1 ml) were withdrawn onto the filter, heated in a HNO_3-$HClO_4$ mixture at 75°C, and analyzed with an atomic absorption spectrophotometer (model 207; Hitachi).

Immunological methods. Anti-ϕBA1 serum was raised by injecting purified ϕBA1 ($\times 10^{11}$ in 0.5 ml of gelatin-free SM buffer) into rabbits (male, 2.5 kg) eight times at 4-day intervals. Immunodiffusion in 0.6% agar was done by the Ouchterlony technique (29). Immunoelectrophoresis (14) was performed in 0.6% agar containing 0.19 M Tris hydrochloride (pH 8.6) at 50 V for 4 h.

Analysis of phage DNA after infection. KA10 cells (10^8 cells per ml) growing in HIBY were infected with DNA-labeled ϕBA1 at an MOI of 13. At various times, a 1-ml sample was chilled and spun down, and the precipitate was analyzed through slot-lysis electrophoresis by the method of González et al. (12) with 0.7% agarose. To measure the conversion of radiolabeled phage DNA into acid-soluble material, KA10 cells (10^9 cells per ml) were infected with ϕBA1 at an MOI of 0.1. At various times, 0.1 ml was taken into 1 ml of cold 10% TCA and kept at 0°C for 1 h. The precipitate was collected on a membrane filter (pore size, 0.45 μm), washed with 5% TCA, and counted.

Analysis of phage-coded protein synthesis. Strain KA10 or KA23 was grown to 1.5×10^8 cells per ml in Kimura C, infected with ϕBA1 at an MOI of 20, and incubated at 37°C. At the indicated times, a 5-ml portion was transferred into 0.5 ml of Kimura C containing 1 μCi of U-^{14}C-labeled amino acid mixture and incubated for 5 min at 37°C. The reaction was terminated by the addition of 1 ml of ice-cold 0.02 M NaN_3–0.01 M Tris hydrochloride (pH 8.0). Cells were spun down and suspended in 0.1 ml of a solution containing lysozyme (5 mg/ml), 0.02 M NaN_3, and 0.01 M Tris hydrochloride (pH 8.0), incubated at 37°C for 30 min, and then combined with 0.1 ml of a solution containing 5% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.005% bromphenol blue, and 0.25 M Tris hydrochloride (pH 6.8). Proteins of the lysed cells were analyzed by 10% polyacrylamide gel electrophoresis as above and fluorography with Amplify (Amersham Corp.) by exposing X-ray film (Fuji RX) for 3 to 5 days.

Isolation of killing-resistant mutants. Exponentially growing KA10 was suspended in 0.05 M Tris malate buffer (pH 6.0) at 10^8 cells per ml and treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NMNG) (100 μg/ml) at 37°C for 30 min. Cells were suspended in NBY at 10^7 cells per ml, incubated at 37°C to a concentration of 4×10^8 cells per ml, and spread on a lawn of ϕBA1. Surviving colonies were confirmed for resistance to ϕBA1 by repeated cross-streaks.

Denaturation and renaturation experiment of ϕBA1 DNA. ϕBA1 DNA prepared as above was processed as described (40).

RESULTS

Production of killing agents and phage. All the strains of *B. aneurinolyticus* tested were lysed within about 5 h at 37°C 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. Of 20 lysates examined, 17 were able to kill two or more of the other strains of *B. aneurinolyticus* but not the producer strain itself. Killing was observed only among strains of *B. aneurinolyticus*, and no sensitive strain was detected in the other *Bacillus* species tested, including *Bacillus thiaminolyticus*, *B. brevis*, *B. circulans*, *B. laterosporus*, and *B. subtilis*.

When killing agents were purified by sucrose gradient centrifugation, they were found in fractions of different sucrose concentration specific for each particle (Fig. 1). Electron microscopic observation of almost entire fractions revealed that the peak fraction of killing activity was enriched in phage or phage-tail like particles in all cases (Fig. 2). They looked morphologically like phage (KA23 and KAS232 fractions), phage tails (KA1 and KA6 fractions), or phage tails with fibers (KA10 and KA17 fractions). Besides the active particles observed in the peak fraction, crude lysates of various strains contained minor inactive particles, e.g., empty heads in KA1 and KA17 lysates (Fig. 2D) and tail-like structures in KA23 and KAS232 lysates (data not shown). Plaque-forming activity could not be detected in any of the lysates except for that of KAS232. Interestingly, it was found that the peak fraction for both the killing (against KA10, KA13, KA14, KA17, and KA22) and the plaque-forming (against KA1, KA23, and KA24) activities coincided with each other in the KAS232 lysate (Fig. 1C). Electron microscopy showed morphologically singular and homogeneous particles in the peak (Fig. 2F) and the neighboring

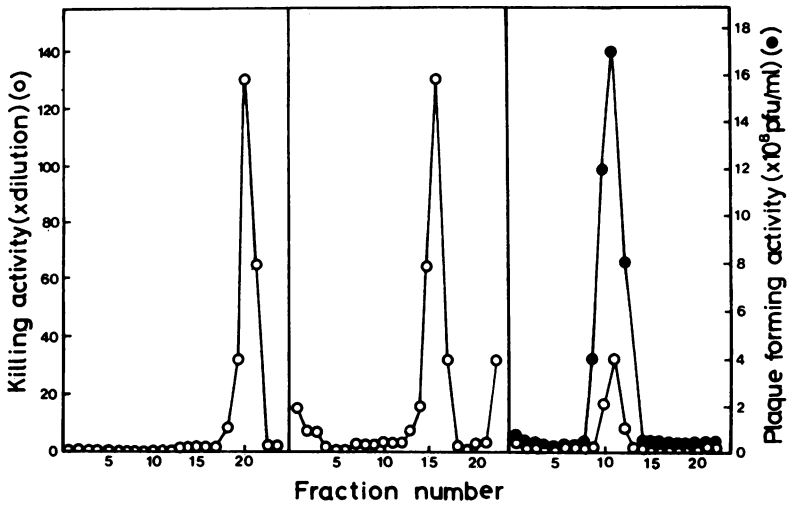

FIG. 1. Centrifugation profile of the killing agents and phage in a 5 to 20% linear sucrose gradient. Fraction no. 1 represents the bottom of the centrifuge tube. (Left) killing activity of KA1 lysate, assayed on KA23; (center) killing activity of KA23 lysate, assayed on KA10; (right) killing activity (○) and plaque-forming activity (●) of KAS232 lysate, assayed on KA22 and KA23, respectively.

fractions. The phage particle was called φBA1. φBA1 made turbid plaques, typical of temperate phage, on strains KA1, KA23, and KA24. KA23 and KA24 gave easily recognizable turbid plaques with similar efficiencies of plating. Plaques on KA1 were very turbid, small, and hard to count. Thus, KA23 was used as the host for growing and assaying φBA1. Lysogenic cells from the center of a turbid plaque on KA23 was inducible and released φBA1 into medium after treatment with mitomycin C.

Two activities of φBA1. φBA1 was further purified by CsCl equilibrium density gradient centrifugation. Plaque-forming (against KA23) and killing (against KA10) activities were examined for each fraction. Again, these two activities coincided with each other (data not shown). To rule out the

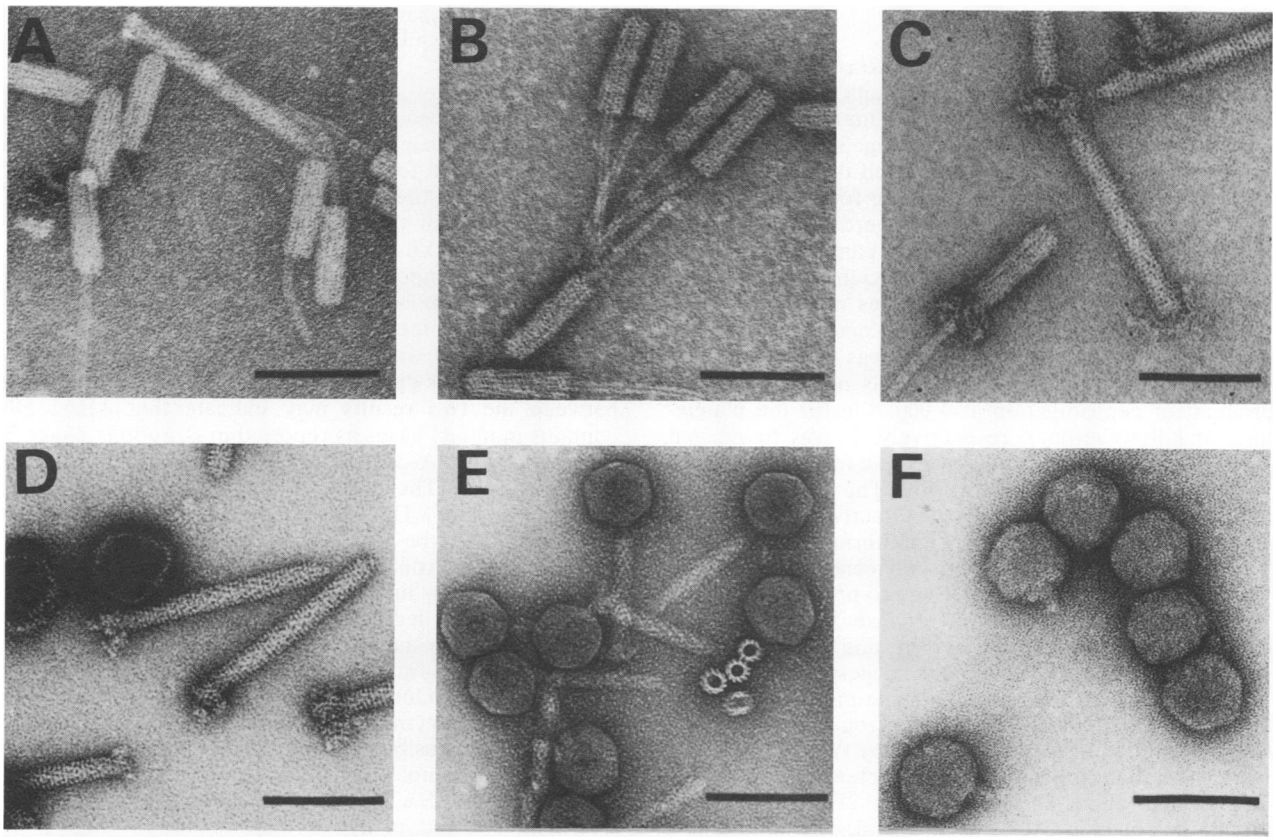

FIG. 2. Electron micrographs of samples taken from the peak fraction of sucrose gradient centrifugation. Samples were negatively stained with 1% uranyl acetate. Shown are lysates of KA1 (A), KA6 (B), KA10 (C), KA17 (D), KA23 (E) and KAS232 (F). Bars, 100 nm.

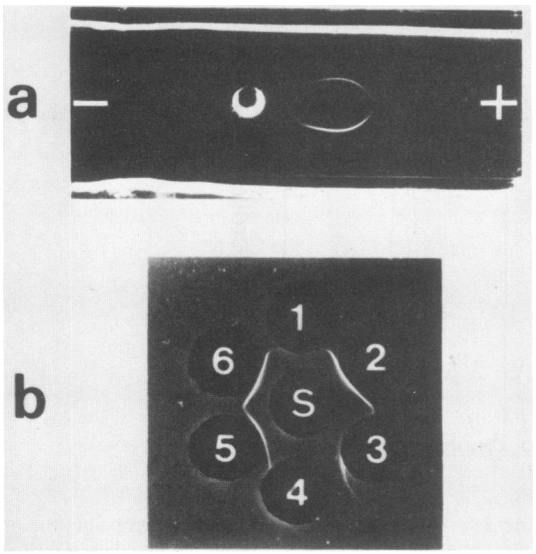

FIG. 3. Serological test of the homogeneity of purified ϕBA1. (a) Immunoelectrophoresis of ϕBA1. Particles (5×10^9) of ϕBA1 suspended in TMS buffer were subjected to electrophoresis as described in Methods. Diluted (2×10^{-1}) anti-ϕBA1 rabbit serum was then added in two troughs (upper and lower) and incubated overnight. (b) Ouchterlony immunodiffusion patterns. The center well (S) contained rabbit antiserum against purified ϕBA1. The peripheral wells contained mitomycin C-induced lysate of KAS232 (well 1), ϕBA1 (5×10^9 particles) (well 2), ϕBA1 (10^9 particles) (well 3), cleared culture fluid of uninfected KA23 (well 4), crude ϕBA1 (scraped plaque on KA23) (well 5), and ϕBA1 (5×10^9 particles) (well 6).

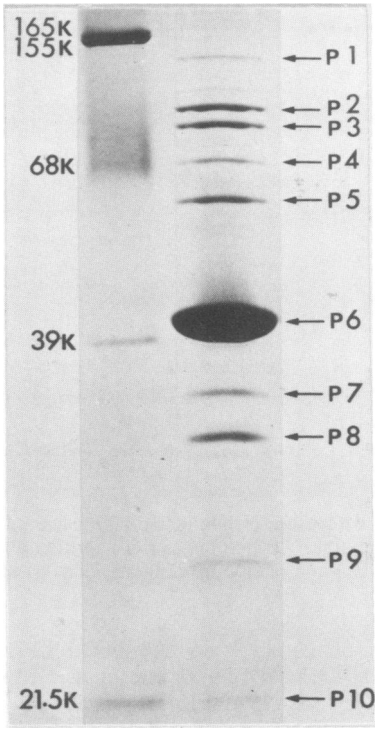

FIG. 4. SDS-polyacrylamide gel electrophoresis of purified ϕBA1. ϕBA1 (about 8×10^9 particles) was subjected to SDS electrophoresis on a 10% acrylamide gel. Molecular weights (in thousands) for each band indicated by an arrow were determined by comparison with standard proteins (Boehringer Mannheim Biochemicals) of known molecular weight that were coelectrophoresed. P1 through P10 denote each phage protein.

possibility that killing activity was due to some agent other than the ϕBA1 particle, the homogeneity of the purified ϕBA1 preparation was checked with immunological methods. The immunoelectrophoretogram (Fig. 3a) and results of simple immunodiffusion (Fig. 3b) showed the uniformity of the ϕBA1 particle. ϕBA1 particles were found to be negatively charged at pH 8.6 and moved towards the anode as a serologically singular species. ϕBA1 particles grown in KA23 cells were serologically identical to the original virion observed in mitomycin C-induced lysates of KAS232 (Fig. 3b). To confirm two activities of ϕBA1, neutralization tests were performed. Thus, purified ϕBA1 was first mixed with strain KA10 or KA23, and the titer was measured on the supernatant of centrifugation at $4,000 \times g$ for the plaque-forming or killing activity, respectively. It was found that KA10 or KA23 strains neutralized plaque-forming or killing activity, respectively (data not shown). The pH stability and proteinase sensitivity of the two ϕBA1 activities were compared with each other. Both plaque-forming and killing activities were stable at pH values between 6.2 and 9.0 and showed the same spectra of sensitivity to proteinases examined (ϕBA1 was inactivated by pronase E and resistant against trypsin, papain, chymotrypsin, and subtilisin under the conditions tested). These results suggested that the two activities could be attributed to the same particle, ϕBA1.

Characteristics of ϕBA1. Electron micrographs of purified ϕBA1 showed the morphology of Bradley type C phage (4), having an icosahedral head with a diameter of about 70 nm and a short tail about 20 nm long (Fig. 2F). The buoyant density of the phage in CsCl was 1.51 g/ml. The number and approximate molecular size of viral proteins were analyzed by SDS-polyacrylamide gel electrophoresis. The structural proteins were resolved into 10 bands, and the electrophoretogram revealed that the protein P6 (molecular weight, 43,000) accounted for 81% of the total protein (Fig. 4); it is presumed to be the major constituent of the head. The molecular sizes of the polypeptides were estimated to be 130, 102, 88, 68, 57, 43, 37, 34, 26, and 21 kilodaltons.

The melting temperature of ϕBA1 DNA in SSC was 87°C. This value corresponds to a guanine-plus-cytosine content of 43% (6). On the other hand, the buoyant density of DNA in the CsCl gradient was 1.6904 g/cm^3, which corresponded to 31% guanine-plus-cytosine content (34). The discrepancy between the two results may indicate that ϕBA1 DNA contains modified bases as reported in *B. subtilis* phage SP8 (16). ϕBA1 DNA was digested with various restriction endonucleases. The sizes of fragments were estimated against digests of λ DNA. The molecular size of ϕBA1 DNA was estimated to be about 38×10^6 daltons.

Although electron microscopy of intact ϕBA1 DNA revealed exclusively linear molecules (Fig. 5a), physical mapping experiments led us to the result that ϕBA1 DNA was circular. This was shown typically in the fact that an enzyme which gave two fragments from ϕBA1 DNA in a single digestion experiment exhibited two cutting sites when used as the second enzyme in double digestion trials. Hence, we examined the possibility that ϕBA1 DNA was a circularly permuted linear molecule like T2 or T4 DNA (41). Electron microscopy of ϕBA1 DNA after denaturation-renaturation treatment showed many circular molecules (Fig. 5b). In addition, the electrophoretic pattern after single digestion with *Xba*I or *Bal*I (which cleaved ϕBA1 DNA at a single site

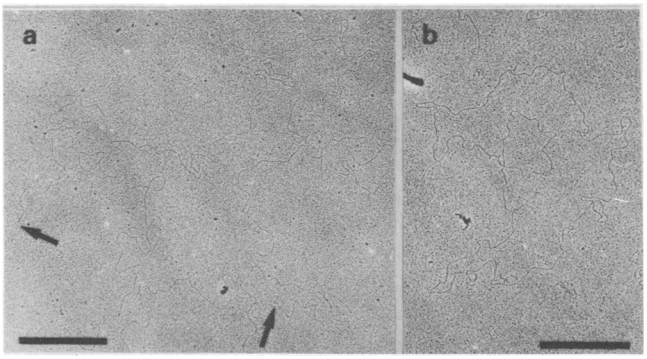

FIG. 5. Electron micrographs of φBA1 DNA. Samples for electron microscopy were prepared by the method of Kleinschmidt (21). The grids were stained with alcoholic uranyl acetate and then shadowed. Bars, 1 μm each. (a) Intact φBA1 DNA. Arrows show the end of the molecule. (b) Homoduplex molecule of φBA1 DNA after denaturation-renaturation treatment. φBA1 DNA was denaturated in 0.2 M NaOH and dialyzed for 10 h against 0.3 M NaCl–0.03 M sodium citrate. The solution was then heated at 65°C for 40 min and cooled to 4°C.

when used in a second digestion) was ambiguous, whereas the patterns after the second digestion with other enzymes were clear and discrete (Fig. 6). From these results, φBA1 DNA was indicated to be a linear, circularly permuted molecule. Further, φBA1 DNA was digested completely by BAL 31 under conditions in which circular DNA of a plasmid of comparable size (RP4) was not digested (data not shown).

The physical map of φBA1 DNA is shown in Fig. 7. φBA1 DNA was resistant to the restriction endonucleases *Ava*I, *Bam*HI, *Bcl*I, *Eco*RI, *Kpn*I, *Sac*I, *Sma*I, and *Xho*I. The mean length of the linear molecule was 15.1 ± 1.2 μm,

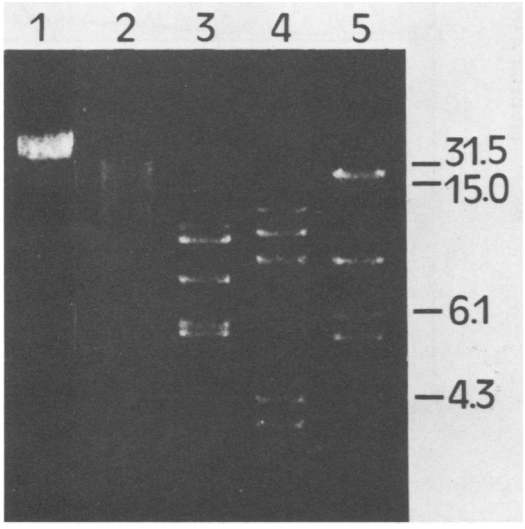

FIG. 6. Agarose-gel electrophoresis after double digestion. φBA1 DNA was digested first with *Xba*I, ethanol precipitated (25), and then digested with *Pst*I, *Pvu*II, or *Mlu*I. Samples were subjected to 1% agarose gel electrophoresis. Lane 1, intact φBA1-DNA. Products are shown for digestion with *Xba*I (lane 2), *Xba*I plus *Pst*I (lane 3), *Xba*I plus *Pvu*II (lane 4), and *Xba*I plus *Mlu*I (lane 5). Positions for the molecular weight standards (in millions) are shown on the right.

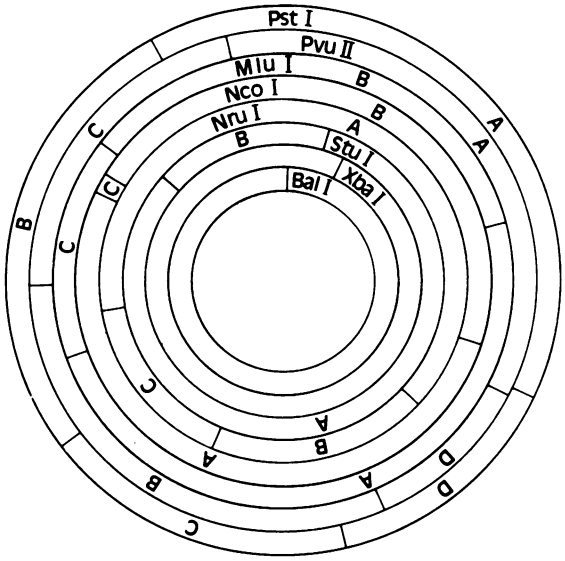

FIG. 7. Restriction endonuclease map of φBA1 DNA. The circularly-permuted linear molecule is drawn as a circle. The sites of cleavage by endonucleases were based on the analyses of electrophoretic patterns of single and double digestions of φBA1 DNA. A, B, C, and D designate each restriction fragment.

corresponding to a molecular size of 38.1 × 10^6 daltons against the internal standard.

The latent period for φBA1 was about 90 min, and the burst size was 70 after one-step growth in NBY. The frequency of lysogenization was 1 to 3% at an MOI of 20 on NBY agar.

Kinetics of killing. φBA1 was mixed with a logarithmic-phase culture of KA10 at an MOI of 25. After appropriate

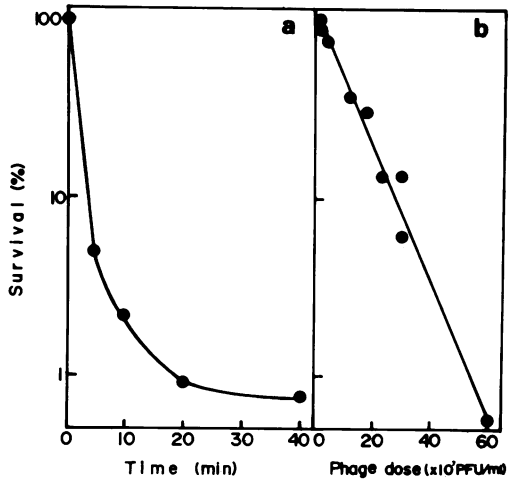

FIG. 8. Kinetics of killing action of φBA1. (a) Time course of killing. Suspensions of KA10 (3 × 10^7 cells per ml) in Kimura C were infected at time zero with φBA1 at an MOI of 25 and incubated at 37°C. At time intervals, the reaction mixture was diluted with Kimura C, and the surviving cells were counted by spreading on an NBY plate. (b) Relationship of the survival ratio to the concentration of φBA1. Suspensions of KA10 (3 × 10^7 cells per ml) in Kimura C were mixed with various amounts of φBA1 (10^{10} PFU/ml) to give the final phage concentrations indicated and then incubated at 37°C for 30 min. The surviving cells were counted as in panel a.

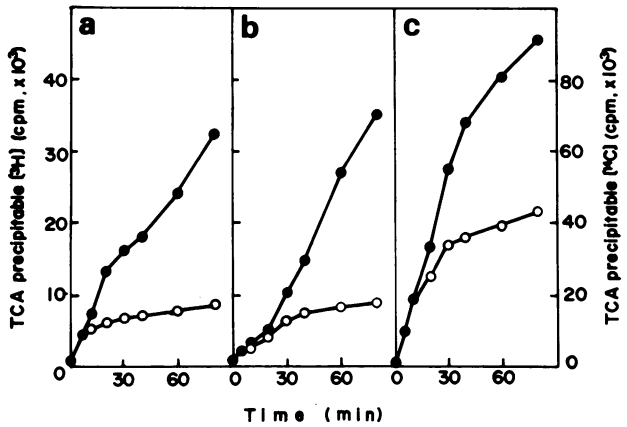

FIG. 9. Macromolecular synthesis in uninfected (●) and ϕBA1-infected (○) KA10 cells. Cells were grown and infected with ϕBA1 as described in Methods. Radiolabeled precursor was added at time zero. Samples (50 μl) were withdrawn at the indicated times and precipitated in 1 ml of cold 10% TCA. The TCA-precipitable material was collected on a membrane filter, and the radioactivity (^3H for DNA and RNA, ^{14}C for protein) was determined. (a) DNA synthesis; (b) RNA synthesis; (c) protein synthesis.

incubation, surviving cells were counted (Fig. 8a). The extent of killing with various amounts of ϕBA1 was also investigated. A linear correlation was obtained between the logarithms of the number of surviving cells and the ϕBA1 dose (Fig. 8b), suggesting the killing action of ϕBA1 to be a single-hit process (13). To confirm the killing by a single particle, the number of KA10 cells was varied against a constant number of ϕBA1 particles. When the MOI value was sufficiently low, the number of KA10 cells killed was equal to the number of ϕBA1 particles (data not shown).

Physiology of infected cells. Physiological changes of host cells have been reported in the course of killing by bacteriocin (38) or by phage which showed abortive infection (8, 10, 27, 32, 33, 38, 42). To clarify the events occurring in the course of killing in the KA10-ϕBA1 system, we examined the physiological changes in KA10 cells after ϕBA1 infection, using KA23 as the control.

To determine whether macromolecular synthesis is inhibited in KA10, 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 7 min after infection (Fig. 9). We then investigated the effect of ϕBA1 on the cellular ATP content of KA10 (which was killed) and KA23 (which made phage until it lysed) (Fig. 10). The content of intracellular ATP temporarily increased for the first 5 min after infection and gradually decreased in KA10 cells. Only a very slight leakage of ATP into the medium was observed at 30 min and later. In KA23 cells, the content of intracellular ATP temporarily decreased and then recovered to the same level of uninfected control.

To study the relation between killing by ϕBA1 and the membrane transport activity of KA10 cells, we measured the uptake of leucine after infection. However, no difference was observed between the degrees of inhibition of the activity in KA10 and KA23 cells (data not shown). The K^+ content of infected cells was also measured to determine the change of membrane potential after infection. ϕBA1 induced a rapid loss of K^+ from both KA10 and KA23 cells (Fig. 11). No degradation of the chromosomal DNA of KA10 cells was observed (as measured by the TCA-precipitable radioactive DNA) up to 80 min after infection, at which time 97% of the cells had been killed (data not shown).

Fate of ϕBA1 DNA. We wished to determine whether injected ϕBA1 DNA was degraded by a host restriction system. The extent of degradation was estimated by the liberation of radioactivity into the acid-soluble fraction of the infected culture, and the restricted fragments were detected by agarose gel electrophoresis. It was found that ϕBA1 DNA was injected into KA10 cells, where it rapidly degraded, with a half-life of less than 1 min (agarose gel electrophoresis experiments) or with a half-life of about 5 min (TCA precipitation experiments; data not shown). These results indicate that ϕBA1 DNA could not be expressed at all after its injection into KA10 cells. This was confirmed through the observation that early proteins synthesized in KA23 for ϕBA1 were not detectable in KA10 cells (Fig. 12). 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 (70% of that in the nonirradiated control) was still caused by heavily UV-irradiated ϕBA1, whose plaque-forming activity was reduced to 10^{-4} (data not shown). In the cases of known

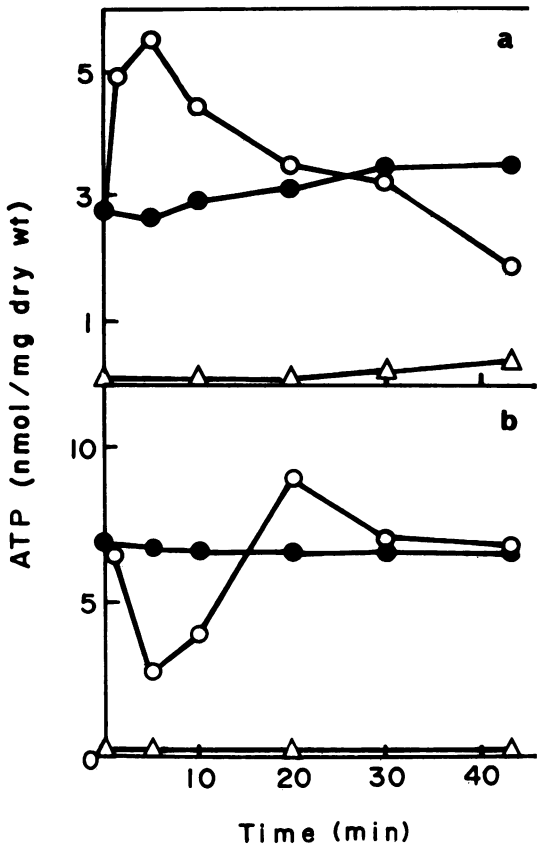

FIG. 10. Effect of ϕBA1 on cellular ATP content. Exponentially growing cells suspended in Kimura C were infected with ϕBA1, and cellular ATP was measured as described in Methods. Extracellular ATP was determined on the membrane (Millipore, Sartrius) filtrate of the infected cell suspension. A supernatant of the reaction mixture was also measured to confirm the adsorption on those membranes negligible. (a) KA10; (b) KA23. Symbols: ○, intracellular ATP content of ϕBA1-infected cells; ●, intracellular ATP content of uninfected control cells; △, extra-cellular ATP.

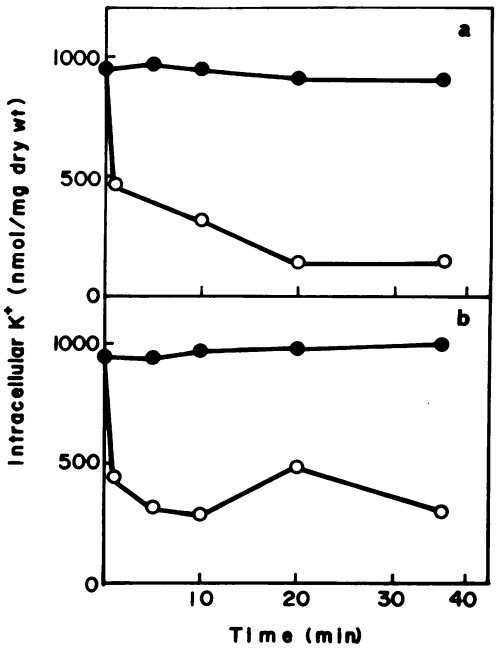

FIG. 11. Effect of φBA1 on cellular K^+ content. KA10 (a) and KA23 (b) cells were incubated as described in Methods. At the indicated times, 1-ml samples were filtered through a membrane filter. K^+ contents of cells on the filter was measured as described in Methods. Symbols: ●, uninfected control cells; ○, φBA1-infected cells.

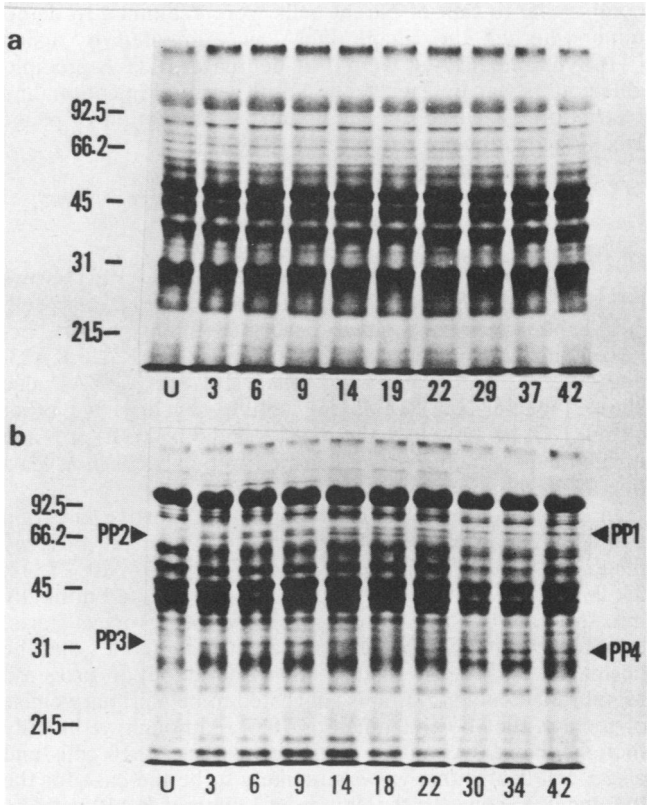

FIG. 12. Expression of early genes in the two kinds of host cell. Pulse-labeling of phage-specific proteins is described in Methods. At each time (indicated at the bottom of each lane), infection was terminated, the mixture was transferred into the labeling mixture, and labeling was continued for 5 min. Radiolabeled polypeptides in KA10 (a) or KA23 (b) are shown. Lane U represents the proteins synthesized through the 5-min labeling by the cell before phage infection. The proteins were separated and fluorographed as described in Methods. Phage-specific proteins are indicated as pp1 through pp4. pp1 (68 kilodaltons) was detected at 3 min and later, pp2 (66 kilodaltons) was detected at 3 to 22 min, pp3 (36 kilodaltons) was detected at 3 to 14 min, and pp4 (34 kilodaltons) was detected at 22 min and later. The molecular size standards used were soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, and phosphorylase B (21.5, 31, 45, 66.2 and 92.5 kilodaltons, respectively).

abortive infection (8, 10, 27, 32, 33, 38, 42), killing-sensitive bacteria carry a prophage or harbor a plasmid. Screening for plasmid DNA in *B. aneurinolyticus* strains revealed the existence of three or more covalently closed circular DNA of different molecular sizes in some of the killing-sensitive strains (e.g., KA17; approximate molecular sizes of 2, 7, and 20 megadaltons). However, no comparable DNA band could be detected in the killing-sensitive strain KA10 (19). Further, no complete phage were detected in the lysate of KA10 induced by mitomycin C. Phage-tail-like bacteriocin had been lysogenized in KA10 (Fig. 2). This particle was concluded to be a bacteriocin because of the existence of two killing-sensitive host strains (KA2 and KAS232).

It has been reported that nonpermissive *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 (11). We suspected that φBA1 and the structured bacteriocin in KA10 (Fig. 2C) had a common immunity repressor, and we ran experiments to determine whether φBA1 could multiply in KA10 cells which had been pretreated with mitomycin C. No multiplication of φBA1 in such cells could be detected, and the host cells were killed with a reduced production of bacteriocin (data not shown). This suggests that the inability of φBA1 to multiply in KA10 was not caused by the structured bacteriocin. We also tried to detect bacteriocin activity of φBA1-infected KA10 cells without mitomycin C treatment, but no induction of bacteriocin could be observed.

Isolation of mutants resistant to killing. Killing-resistant mutants of KA10 were isolated by NMNG mutagenesis from surviving colonies on the φBA1 lawn at an approximate frequency of 8×10^{-7} (55 isolates). To determine the phage-adsorbing ability of these mutants, exponentially growing resistant cells were infected with φBA1 at an MOI of 0.01, incubated for 40 min at 25°C, and centrifuged, and the unadsorbed phage in the supernatant fluid were counted. Killing-resistant mutants could be divided into three groups on the basis of their phage adsorption potency (Table 1). Further, 14 mutants which showed the adsorption potency

TABLE 1. Classification of φBA1-resistant mutants of strain KA10

Group	No. of mutants	Adsorption of φBA1[a]
A	14	>70
B	13	30–70
C	28	0–30

[a] Each value represents the percentage of the adsorbability against that of the wild-type KA10. Injection of φBA1 DNA occurred in 12 of the 14 group A mutants and was not determined for groups B and C.

comparable to that of parent cells were examined to determine whether the phage DNA was injected by using [^{3}H]thymidine-labeled ϕBA1. A decrease in TCA-precipitable counts was interpreted to be the result of injection (and restriction) of DNA. In 12 mutants, the injection of phage DNA occurred as in parent cells (Table 1).

DISCUSSION

It has been known that a temperate phage either grows lytically or lysogenizes when it infects a bacterial cell. ϕBA1, the temperate phage described in this report, behaved as a temperate phage on three strains (KA1, KA23, and KA24) of *B. aneurinolyticus*. However, ϕBA1 also showed bacteriocin-like killing activity against five other strains (KA10, KA13, KA14, KA17, and KA22). 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 cell is killed (8, 10, 27, 32, 33, 38, 42). In these cases, phage DNA is injected 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 infections (10). Although we initially thought that ϕBA1 might abortively infect KA10 cells and cause cell death, this view is unlikely to be the case for the following reasons. In the course of killing of KA10 cells by ϕBA1, (i) the injected phage DNA was rapidly degraded in the host cell, (ii) the phage genome was not expressed, (iii) killing occurred even when heavily UV-irradiated phage was used, (iv) no lysogenization of ϕBA1 was observed in rare surviving colonies, and (v) no prophage or plasmid was detectable in KA10 cells. These features are clearly different from those of known abortive infections and seem to suggest that the killing of KA10 is caused by some proteinaceous component of ϕBA1.

Proteinaceous killing agents with a narrow host range are known as bacteriocins (39). The modes of action of some of these low-molecular-weight bacteriocins have been determined. They can be ionophores, DNases, RNases (22), or phospholipases (30). If ion-permeable channels were formed by ϕBA1 and were the primary cause of cell death, a drastic leakage of K^+ would be observed only in the killing-sensitive host (KA10) relative to the phage-productive host (KA23). The results indicate, however, that the extent of leakage of K^+ in both strains was the same at least for the first 10 min of infection (Fig. 11), during which time the killing was nearly completed (Fig. 8a). This result indicates that membrane depolarization occurred as reported for T1 (18) when ϕBA1 adsorbed host bacterial cells, but it did not appear to be a primary cause of cell death. In addition, ϕBA1 reduced leucine transport in both strains by about 50%. This also indicates that membrane disfunction was not specific for killing-sensitive cells. The possibility that ϕBA1-induced killing is caused by DNase or RNase activity is less likely, although it could not be ruled out, since no obvious destruction of host chromosomal DNA was observed and, also, specific inhibition of protein synthesis in KA10 was not observed in the course of killing.

On the other hand, there was a marked difference in cellular ATP content between KA10 and KA23 after ϕBA1 infection. The rapid increase of intracellular ATP content in the initial 5 min in KA10 (Fig. 10) suggests that a large part of the ATP consumption ceased immediately after infection. The subsequent decrease of intracellular ATP is considered to result from a reduction in ATP synthesis (e.g., because of membrane depolarization [Fig. 11] or a decreased rate of respiration [data not shown]), since attempts to recover ATP from extracellular fluid were in vain and since ATP-consuming activities such as membrane transport or protein synthesis (Fig. 9c) seemed to continue partly. In KA23, cellular ATP transiently decreased (Fig. 10), suggesting the depolarization of membrane (Fig. 11). Subsequent restoration may be due to the expression of early gene(s) of ϕBA1 to give membrane proteins (Fig. 12). From this, it might be inferred that killing is a result of the failure of infected cells to recover from the labile to the stable state. Genes which might be concerned with the shifting of host cells to a stable phage factory would not be expressed as a result of the restriction of ϕBA1 DNA (Fig. 12). However, the results indicating that the accumulated leucine did not dissipate completely and that intracellular ATP of KA10 did not leak outside of cells suggest that the failure of membrane restoration did not exert such a critical effect on the intracellular ATP level. Moreover, the process of killing KA10 (Fig. 8a and Fig. 10a) seemed to be a foregoing event compared with the restoration of the membrane in KA23. 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 ϕBA1 would be some reactant for the phage enzyme injected or some regulatory protein in the host cell.

The infection of KA10 cells with ϕBA1 resulted in cell death without production of any bacteriophage at an MOI of 0.5 or without any cell lysis at an MOI of 50 or higher (data not shown). These results indicate that ϕBA1 action on KA10 cells is different from the mode of action of some bacteriophages in lysis from without (17).

As mentioned above, ϕBA1 may be regarded as a defective phage such as PBSX (7, 28, 36). In phage PS17 of *Pseudomonas aeruginosa*, only the tail part of the phage showed killing activity similar to pyocin R1 (35). 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 ϕBA1 may be an example of the evolution of a bacteriophage with a defective phage activity. In our case, however, ϕBA1 as a bacteriocin is not a defective phage but a complete phage, and we use the term structured bacteriocin instead of defective phage.

In the characterization of ϕBA1 DNA, it was deduced to be circularly permuted from the results of electrophoresis of endonuclease fragments and electron microscopic observation of linear molecules. There have been methods accepted for the identification of a permuted DNA molecule such as shear-break or denaturation-renaturation experiments (41). Our method could be a convenient alternative for them.

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