JOURNAL OF VIROLOGY, Feb. 1970, p. 237-246

Vol. 5, No. 2
Printed in U.S.A.

Characterization of Inducible Bacteriophages in *Bacillus licheniformis*

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Received for publication 29 September 1969

Two morphologically distinct and physically separable defective phages have been found in *Bacillus licheniformis* NRS 243 after induction by mitomycin C. One of them (PBLB) is similar to the defective phage PBSX of *B. subtilis*, which has a density of 1.373 g/cm^3 in CsCl and a sedimentation coefficient of 160*S*. PBLB incorporates into its head mainly bacterial deoxyribonucleic acid (DNA) which has a sedimentation coefficient of 22*S* and a buoyant density in CsCl of 1.706 g/cm^3. The other phage (PBLA) has a morphology similar to the temperate phage ϕ105 of *B. subtilis*; the head diameter is about 66 nm, and it possesses a long and noncontractile tail. PBLA has a density of 1.484 g/cm^3 in CsCl and the phage-specific DNA, which is exclusively synthesized after induction by mitomycin C, has a density of 1.701 g/cm^3. PBLA DNA is double-stranded and has a sedimentation coefficient of 36*S*, corresponding to a molecular weight of 34 $\times$ 10^6 to 35 $\times$ 10^6 daltons. The phage DNA has one interruption per single strand, giving single-stranded segments with molecular weights of 13 $\times$ 10^6 and 4 $\times$ 10^6 daltons. Common sequences between the two phage DNA species and with their host DNA have been demonstrated by DNA-DNA hybridization studies. Both phage particles kill sensitive bacteria. However, all attempts thus far to find an indicator strain to support plaque formation have been unsuccessful.

It now appears that many species of the genus *Bacillus*, as well as other genera, can be induced by a variety of agents to produce defective, nonplaque-forming phage particles (17). One such defective phage, PBSX of *Bacillus subtilis*, has been characterized (10, 11). The phage is produced upon induction by mitomycin C and apparently contains exclusively host deoxyribonucleic acid (DNA) of molecular weight 8.4 $\times$ 10^6 daltons packaged into the phage heads.

B. licheniformis, treated with mitomycin C, produces two morphologically distinct and physically separable defective phage particles. One of them is PBSX-like in that the particle contains mostly host DNA and the other phage particle contains phage-specific DNA which is synthesized after induction. The characterization of these phages and their DNA is the subject of this report.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. The *B. licheniformis* strain used almost exclusively in this study was wild-type NRS 243. It is naturally resistant to the antibiotics streptomycin and erythromycin and, unlike most other *B. licheniformis* strains, does not produce much capsular material. A thymine-requiring mutant of NRS 243 was isolated by aminopterin treatment by the method described by Okada et al. (9). The aminopterin concentration used was 200 μg/ml. The mutant, designated NRS 243 *thy*$^-$, has a high thymine requirement (approximately 5 μg/ml), can utilize either thymine or thymidine, and has a generation time in minimal medium (*see* below) with appropriate supplements of 50 min. Other *B. licheniformis* strains used were ATCC 9945A M17 (7) and IRC-1 (provided by D. Dubnau). The *B. subtilis* strains used were 168 *thyA thyB trp-2* (18), W23 *str-2 ery-2 mic-2*, and S31 (17). Phage T7 was prepared by infecting *Escherichia coli* B-3 *thy*$^-$ and purified as described by Richardson (13). Ribonucleic acid (RNA) phage Qβ and phage λ were used as markers in CsCl buoyant density determinations. The method of purification of PBSX has been described previously (10).

Media and solutions. VY broth contained 25 g of Veal Infusion (Difco) and 5 g of Yeast Extract (Difco) in 1 liter of water. Minimal medium contained Spizizen salt solution (15) supplemented with 0.5% glucose, 0.1% acid-hydrolyzed Casamino Acids (Difco, vitamin-free), and 50 μg/ml of L-sodium glutamate. A 5- to 20-μg amount of thymine or thymidine was added per ml to both liquid media when they were used to support the growth of NRS 243 *thy*$^-$. L agar contained 10 g of Tryptone (Difco), 10 g of Yeast

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Extract (Difco), 5 g of NaCl, and 1.25 mg of $MnCl_2$ in 1 liter of medium solidified with 1.5% agar. L soft agar contained the same constituents except that 0.7% agar was used.

SSC was a solution of 0.15 M NaCl with 0.015 M sodium citrate (*p*H 7); 2 × SSC, 6 × SSC, and 10 × SSC were solutions with the appropriate multiple salt concentrations. TMK contained 0.01 M tris(hydroxymethyl)aminomethane (Tris) (*p*H 7.5), 0.005 M $MgCl_2$, and 0.3 M KCl. Redistilled phenol, which was saturated with 0.2 M phosphate or Tris buffer and adjusted to *p*H 7.5, was used to extract phage DNA. Phenol was removed from the DNA solution by extensive dialysis against either SSC or 0.01 M Tris (*p*H 7.5) containing 0.15 M NaCl. Phage DNA was also prepared by a 4 to 8 hr deproteinization at 37 C with 0.5% Sarkosyl (Geigy Industrial Chemicals, Ardsley, New York) and 500 μg/ml of Pronase, which was preincubated at 37 C for 30 min before use. Bacterial DNA was isolated by Marmur's method (8).

Preparation and assay of phages. Phage lysates were generally prepared by induction either with mitomycin C (Kyowa Hakko Kogyo Co., Tokyo, Japan) or by thymine starvation. A culture of the bacteria which was grown at 37 C to a turbidity of about 40 units (Klett-Summerson colorimeter equipped with a red filter) was treated with mitomycin C for about 5 min. The concentration of mitomycin C used in each experiment is specified. The antibiotic was then removed by centrifugation, and the culture was suspended in the same volume of fresh medium. Lysis, which was usually completed in 3 hr, was monitored by changes in turbidity. Induction by thymine starvation was achieved by first allowing the bacteria to grow to a turbidity of about 40 units in minimal medium containing 20 to 50 μg/ml of thymine. The culture was then centrifuged in the cold, the pellet was washed once with Spizizen salt solution, and the cells were finally resuspended in the same volume of minimal medium without added thymine. Lysis of the culture occurred gradually and was completed after 18 hr of shaking at 37 C. The cell debris was removed by centrifugation prior to concentration of the lysate.

When the volume of the phage lysate was small, it was concentrated by placing the lysate inside dialysis tubing and embedding it in solid polyethylene glycol 4000 (PEG, Union Carbide Chemicals, New York, N.Y.). After standing at room temperature for a few hours, the concentrated lysate was dialyzed against TMK to remove PEG. Large-scale preparations of the phage lysate were concentrated by the method described by B. Alberts (*personal communication*). NaCl (0.5 M) and PEG 6000 (0.9%) were added to the lysate, and the mixture was allowed to stand in the cold overnight. The sediment thus formed was collected by low-speed centrifugation (Sorvall, 5,000 rev/min for 5 min) and resuspended in 0.01 of the original volume of TMK. Chloroform was added dropwise to precipitate PEG, which could then be removed by another low-speed centrifugation. The concentrated lysate was dialyzed against TMK and subjected to subsequent purification steps. Radioactively labeled phages were prepared by adding radioactive thymine or thymidine (New England Nuclear Corp., Boston, Mass.) to the growth medium. Such preparations were purified by layering them on the top of a glycerol gradient and centrifuging in a Spinco model L ultracentrifuge. Fractions from the peak regions were pooled and dialyzed against TMK. Purification of phages was also achieved by CsCl-density gradient centrifugation. The phage lysate was layered on the top of a preformed step gradient which consisted of three layers of CsCl solution of different densities, 1.7, 1.45 and 1.25 g/cm³, and centrifuged for 2 hr at 35,000 rev/min at 5 C in an SW 39 rotor of the Spinco model L ultracentrifuge. Alternatively, the phage lysate could be adjusted to an initial density of 1.42 g/cm³ with CsCl and similarly centrifuged for 36 hr until equilibrium is reached. Two phage bands at densities of 1.48 and 1.37 g/cm³ were collected, and CsCl was removed by dialysis against TMK.

The assay and identification of the *B. licheniformis* phages were carried out by using the following criteria. (i) The size of the phages was determined by sedimentation in a glycerol gradient. (ii) The density of the phages was measured by equilibrium sedimentation in a CsCl-density gradient. (iii) The phage concentration was assayed by estimating the radioactively labeled DNA incorporated into phage particles and thus rendered resistant to hydrolysis by nucleases. In this assay, the phage lysate which contained labeled DNA was treated with a mixture of pancreatic deoxyribonuclease (500 μg/ml) and snake venom phosphodiesterase (300 μg/ml) at 37 C for 45 min. The mixture of the two nucleases is subsequently referred to as DNase. The amount of phage produced was then estimated as trichloroacetic acid (5%)-insoluble radioactivity. (iv) The phages could also be assayed by killing activity. This procedure involved mixing 0.1 ml of a culture of a sensitive strain with 3 ml of soft L agar and overlaying the mixture on an L agar plate. The phage lysate (about 0.02 ml) was spotted on the seeded plate and incubated at room temperature for about 24 hr. Clear spots on the seeded plate were scored qualitatively as positive killing. (v) Phage particles were also characterized by electron microscopy (10).

Gradient sedimentation. For the separation of phage particles, a linear gradient of glycerol (10 to 25%) containing TMK was used. When a Spinco SW 25.3 rotor was used, the gradient was centrifuged at 15 C and 23,000 rev/min for 100 min, or it was centrifuged at 25,000 rev/min for 50 min with an SW 39 rotor. For the sedimentation of DNA, a linear sucrose gradient (5 to 20%) was used. Neutral gradients consisted of 1.0 M NaCl, 0.01 M Tris (*p*H 7.5), and 0.01 M ethylenediaminetetraacetic acid (EDTA); alkaline gradients were prepared in 0.9 M NaCl, 0.1 M NaOH, and 0.01 M EDTA. Centrifugation was carried out in a Spinco SW 25.3 rotor at 15 C and 23,000 rev/min for 15 hr.

Analytical centrifugation. The Spinco model E analytical ultracentrifuge equipped with ultraviolet optics was used to determine the density of phage particles and their DNA species. Centrifugation was performed at 25 C at 44,770 rev/min for 18 hr. The markers used in the respective determinations were phage Qβ, 1.445 g/cm³; phage λ, 1.508 g/cm³; and *B. subtilis* phage PBS2 DNA, 1.722 g/cm³. The latter

density is based on that of *E. coli* DNA, taken to be 1.710 g/cm³.

DNA-DNA hybridization. The method described by Denhardt (4) was followed. DNA was denatured by heating at 100 C for 5 min in 2 × SSC and diluted to give a DNA concentration of 10 μg/ml in 6 × SSC. Unlabeled denatured DNA at various concentrations was immobilized on membrane filters (HAWP 25 mm, Millipore Corp., Bedford, Mass.) and hybridized with a constant amount of labeled, denatured DNA. The results represented the average of duplicate experiments.

RESULTS

Identification of B. licheniformis phages. Mitomycin C treatment, which is the most efficient method, was adopted as the method of choice for the induction of phage. When a culture of *B. licheniformis* NRS 243 *thy*⁻ was treated for a short time with mitomycin C (0.5 to 3 μg/ml for 5 min), the turbidity continued to increase until a peak was reached in about 80 min, followed by a decrease until lysis was completed at the end of about 3 hr. When ³H-thymidine was added to the culture, radioactively labeled particles of *B. licheniformis*, prepared as outlined above, could be analyzed on a glycerol gradient. A typical profile is shown in Fig. 1C. The radioactively labeled phages sedimented in two distinct peaks, corresponding to sedimentation coefficients of 220 to 250*S* and 160*S*. These values were estimated by using *B. subtilis* phage PBSX (160*S*) as the standard. The larger phage was termed PBLA; the smaller, PBSX-like *B. licheniformis* phage is referred to as PBLB. The phages were relatively unstable in the presence of sucrose; hence, glycerol gradients were routinely used in their preparation and characterization.

The buoyant densities of *B. licheniformis* phages in CsCl were also determined; the values were found to be 1.48 g/cm³ for PBLA, using *E. coli* phage λ as marker (1.508 g/cm³), whereas the buoyant density of PBLB was 1.373 g/cm³, with the RNA phage Qβ (1.445 g/cm³) as a density marker. Because of the large difference in buoyant densities, the phages were also separable by preparative CsCl-density gradient centrifugation. The fractions obtained by either size or density separation showed two distinct types of intact phages as observed under the electron microscope (Fig. 2). The head of PBLB had a hexagonal outline with a diameter of 41 nm and a long, contractile tail. PBLA had a head 66 nm in diameter and a thin, long and noncontractile tail with a knoblike structure at the end. This resembles the temperate phage φ105 of *B. subtilis* (2) in morphology, although PBLA appeared to have a slightly larger head.

The specificity of killing activity of the NRS 243 phage lysate is shown in Table 1, where "+" indicates killing and "−" indicates immunity. The crude lysate showed the same range of killing activity as the purified PBLA and PBLB. Thus far we have not been able to find a bacterial strain which serves as an indicator for killing activity of either one but not the other phage. Both phages exhibited killing activity similar to PBSZ, which is the defective phage produced by *B. subtilis* W23. All three defective phages kill the *B. subtilis* strains 168 and S31. PBLA and PBLB were different from PBSZ in that the latter could also kill *B. licheniformis* NRS 243 from which the two

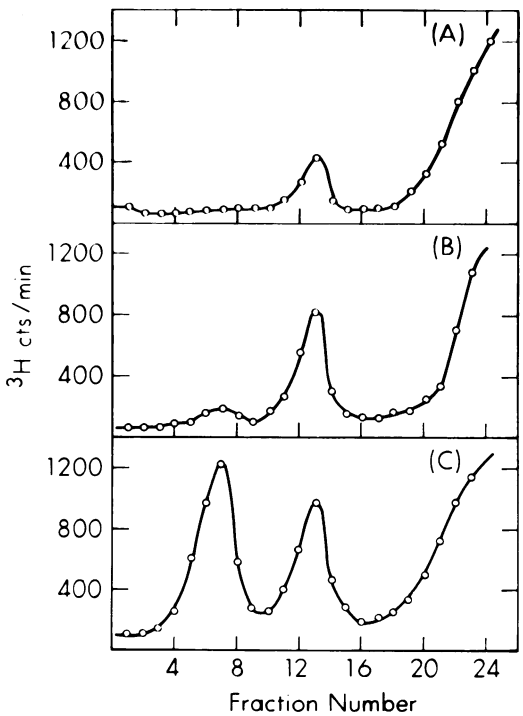

FIG. 1. *Glycerol gradient sedimentation of phage lysates obtained by different methods of induction. The culture of NRS 243 thy⁻ was grown in minimal medium containing 20 μg/ml thymine and ³H-thymidine (10 μc/ml) to a turbidity of about 40 units. It was then divided into three portions: (A) induction by thymine deprivation, (B) induction by mitomycin C (1 μg/ml) treatment and resuspension of the culture in the same minimal medium without thymine and thymidine, and (C) induction by mitomycin C (1 μg/ml) and resuspension in the identical medium as before induction but supplemented with 20 μg/ml of thymine and ³H-thymidine (10 μc/ml). After completion of lysis, the lysate was treated with DNase, and then sedimented at 35,000 rev/min in the Spinco SW 39 rotor for 50 min. Fractions were collected, and radioactivity was determined as trichloroacetic acid-precipitable material.*

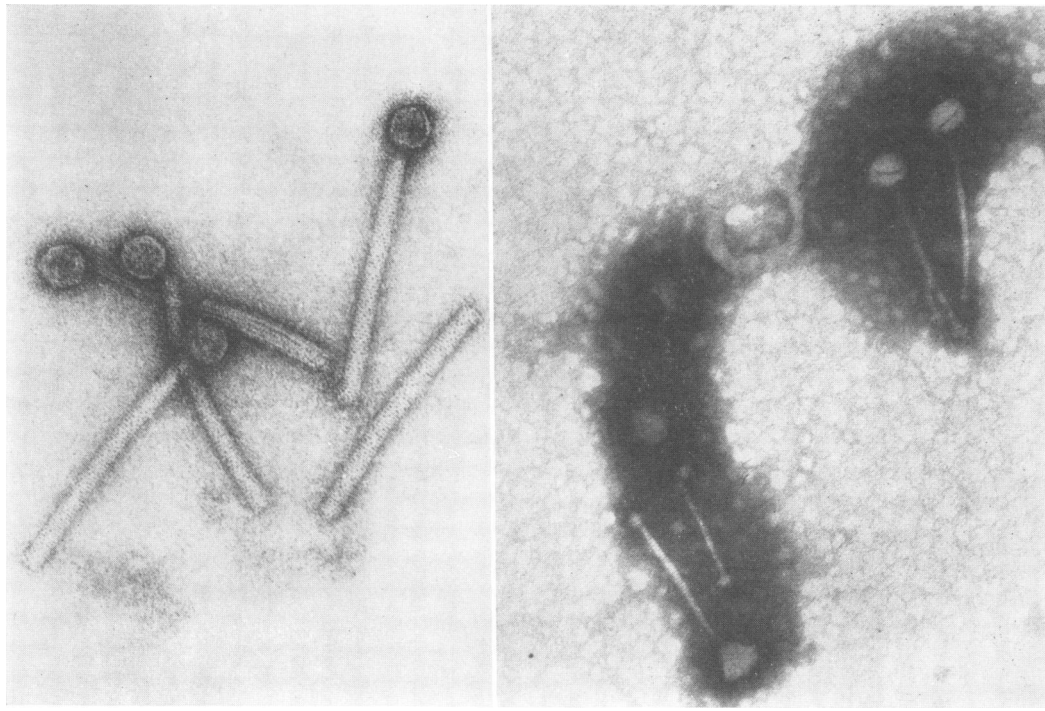

FIG. 2. *Electron micrographs of phage PBLA (right) negatively stained with silicotungstic acid stain, and PBLB (left) negatively stained with methanolic uranyl acetate. PBLB magnified × 129,700.*

TABLE 1. *Specificity in killing activity of defective phages*

Phage	Bacterial strains					
	B. subtilis			*B. licheniformis*		
	168	W23	S31	NRS 243	9945A	IRC-1
Crude lysate of NRS 243.....	+	−	+	−	+	+
PBLA.....	+	−	+	−	+	+
PBLB.....	+	−	+	−	+	+
PBSZ.....	+	−	+	+	+	+

phages were induced, whereas *B. subtilis* W23, the host of PBSZ, was resistant to the *B. licheniformis* phages. As with other defective phages, the parental *B. licheniformis* strain was immune to the killing activity of both phages it produced. Thus far, all attempts to find a host in which PBLA and PBLB might be supported to replicate and produce plaques have not been successful. This limits the conditions for preparing PBLA and PBLB phage lysates to induction.

Induction process. Mitomycin C induced the simultaneous production of both PBLA and PBLB phages. However, the relative amounts of the two phages produced were dependent on mitomycin C concentration. Under identical growth conditions, the optimal concentration of mitomycin C for the induction of PBLA was 2 μg/ml or greater, whereas that of PBLB was about 0.5 μg/ml. Concentrations of mitomycin C higher than 0.5 μg/ml actually caused a decrease in the yield of PBLB in favor of more PBLA production. Induction by thymine deprivation produced only PBLB. This was demonstrated by the exclusive presence of PBLB when a lysate obtained by thymineless induction was examined under the electron microscope. It was further confirmed by equilibrium density gradient studies, as well as by glycerol gradient analysis. A phage lysate was prepared by thymineless induction from a culture of NRS 243 *thy*$^-$, the DNA of which was labeled with ^{3}H-thymidine. When the lysate was analyzed on a glycerol gradient, the sedimentation profile (Fig. 1A) showed only one peak of 160*S* material, corresponding to the sedimentation coefficient of PBLB. When an intermediate amount of mitomycin C (1 μg/ml) was also used to induce the culture in addition to

thymine deprivation, the production of PBLB was almost doubled, as shown in Fig. 1B. Fig. 1C shows the result of phage production when thymine, at the same concentration before and after mitomycin C treatment, was added to the medium.

To determine the origin of the DNA that was incorporated into the phage particles, a culture of *B. licheniformis* NRS 243 *thy*$^-$ was grown in the presence of ^{3}H-thymidine of high specific activity. After induction by mitomycin C, the culture was resuspended in a medium containing ^{14}C-thymidine. To increase the sensitivity of this experiment, the specific activity of the ^{3}H-labeled thymidine was ten times higher than that of ^{14}C-labeled thymidine. After treatment with DNase, the phage lysate was analyzed on a glycerol gradient, and the profile is shown in Fig. 3. It can be seen that PBLA incorporated exclusively ^{14}C-thymidine which was added after induction, whereas PBLB contained DNA which was synthesized both before and after induction.

Since the DNA synthesized after induction could be incorporated into both types of phage, an experiment was designed to estimate the relative rate of de novo DNA synthesis after induction by mitomycin C. The rate was studied by adding ^{3}H-thymidine to the culture at various times after induction and measuring the radioactivity that was eventually incorporated into each phage (Fig. 4). The rate of PBLB DNA synthesis decreased with time after induction, ceasing when the culture began to lyse. On the other hand, the optimal rate of PBLA DNA synthesis occurred about 45 min after induction, and decreased sharply. The PBLB results are consistent with those of *B. subtilis* phage PBSX, where the induction of the defective phage can take place in the apparent absence of DNA synthesis (10). It is conceivable that a small amount of host DNA synthesized late in induction might have been prevented from being packaged into PBLB phage particles and thus escaped detection by this assay method.

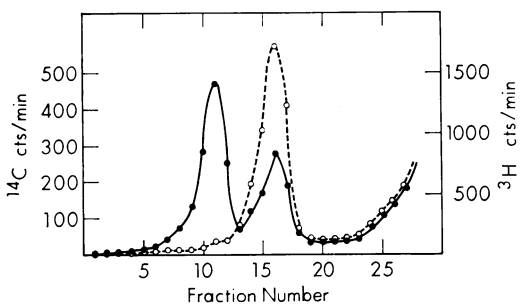

FIG. 3. *Glycerol gradient sedimentation of Bacillus licheniformis phage lysate. NRS 243 thy*$^-$ *was grown in minimal medium supplemented with 20 μg/ml of thymine and* ^{3}*H-thymidine (40 μc/ml). Induction was performed by treating the culture with 2 μg/ml of mitomycin C for 5 min, and resuspending the culture in the same medium containing 20 μg/ml of thymine and* ^{14}*C-thymidine (4 μc/ml). The lysate was treated with DNase before it was layered on the glycerol gradient. Radioactivity in each fraction was determined as trichloroacetic acid-precipitable material. Symbols: ●,* ^{14}*C counts; ○,* ^{3}*H counts.*

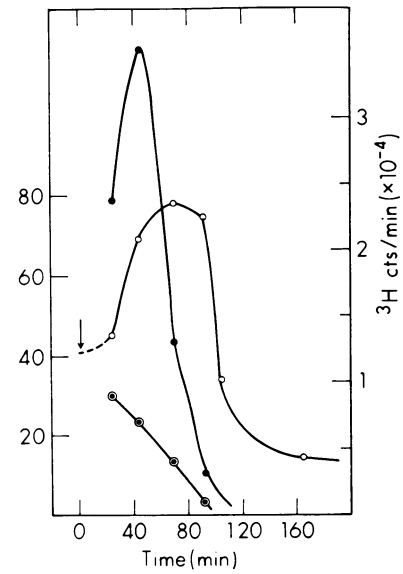

FIG. 4. *Rate of incorporation of precursor into phage DNA. The culture was grown in minimal medium supplemented with 20 μg/ml of thymine and was induced by mitomycin C at a concentration of 1 μg/ml for 5 min. At designated times after induction a sample of 0.5 ml was removed,* ^{3}*H-thymidine (10 μc/ml) was added to the fraction, and the incubation was continued until lysis was complete. Each sample was treated first with DNase and then analyzed on a glycerol gradient. The arrow indicates the time of addition of mitomycin C, and the dotted line shows the time required to resuspend the culture. Symbols: ○, turbidity; ●,* ^{3}*H-thymidine incorporated into PBLA; ◉,* ^{3}*H-thymidine incorporated into PBLB.*

Finally, we also attempted to lyse the cells at various times after induction, but before phage maturation, to try to determine whether both phages were produced simultaneously. Immediately after induction by mitomycin C, ^{3}H-thymidine was added to the culture of NRS 243 *thy*$^-$. At various times thereafter, samples were removed and lysed with lysozyme (200 μg/ml) and then analyzed on glycerol gradients. The results showed that both types of phages were formed at the same time in a one-step burst fashion when the turbidity of the culture began to

decrease. These results do not distinguish whether both phages arise from the same or different induced cells.

Physical properties of the phage DNA molecules. DNA molecules from phages PBLA and PBLB were extracted by phenol and their buoyant densities were determined by using PBS2 DNA (1.722 g/cm^3) as the density marker. The densities were found to be different. PBLA DNA had a density of 1.701 g/cm^3, and PBLB DNA had a density 1.706 g/cm^3 (Fig. 5). The similarity of the density of *B. licheniformis* (1.707 g/cm^3) is consistent with the postulated incorporation of bacterial DNA into PBLB.

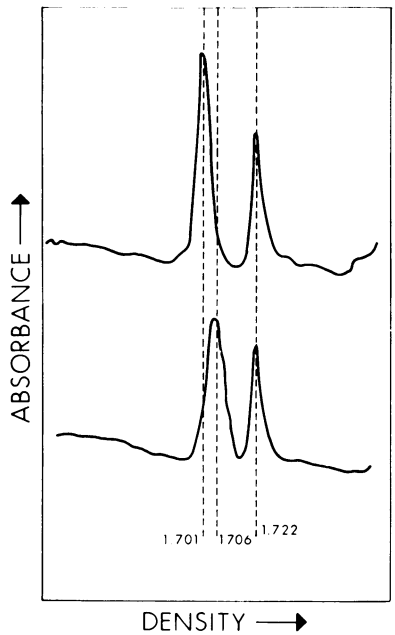

FIG. 5. *Buoyant density profile of defective phage DNA species. The upper pattern is the PBLA DNA, and the lower pattern is the PBLB DNA. B. subtilis phage PBS2 DNA (ρ = 1.722 g/cm^3) was used as a density marker.*

PBLB DNA sedimented as a symmetrical band in either neutral or alkaline sucrose gradients as shown in Fig. 6A and B. The sedimentation coefficients were estimated to be 22*S* under neutral conditions and 25*S* in the alkaline form. T7 DNA (taken as 32*S* for the native form) and PBSX DNA (22*S* for native form) were used as standards. Under neutral conditions, PBLA DNA also gave rise to a sharp and symmetrical band when it was sedimented in a sucrose gradient (Fig. 7A). This apparently homogeneous PBLA DNA was found to have a sedimentation coefficient of 36*S* corresponding to a native molecular weight of 34 $\times$ 10^6 to 35 $\times$ 10^6 daltons, as calculated from Studier's equation (16). However, when the same material was analyzed in an alkaline sucrose gradient, the profile (Fig. 7B) showed a major component of 38*S*, which coincided with the denatured T7 DNA standard and a 25*S* minor component comprising about 20 to 25% of the radioactivity. The molecular weights of the single-stranded forms were about 13 $\times$ 10^6 daltons and 4 $\times$ 10^6 daltons, respectively. The sum of these two components agrees with the expected single-stranded molecular weight of 17 $\times$ 10^6 to 18 $\times$ 10^6 daltons, calculated as one-half the molecular weight determined under native conditions. The same sedimentation profiles were obtained irrespective of the method of phage DNA extraction, i.e., the phenol method or the Sarkosyl and Pronase treatment. Furthermore, to assure that the appearance of the smaller component

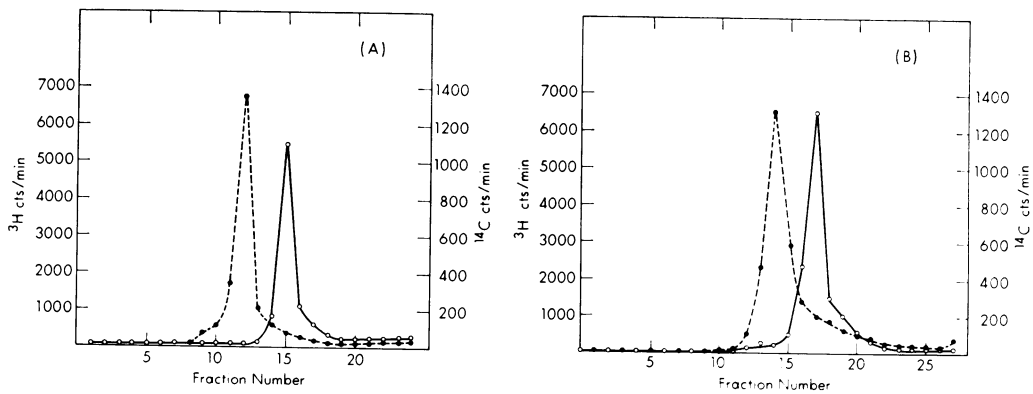

FIG. 6. *Sucrose gradient sedimentation of phage PBLB DNA. Purified and ^{3}H-thymidine-labeled phage preparation was first mixed with ^{14}C-thymidine-labeled T7 phage and the DNA species were isolated with Pronase and Sarkosyl treatment. PBLB DNA sedimented in (A) neutral pH and (B) alkaline pH. Symbols:* ○, *^{3}H counts;* ●, *^{14}C counts.*

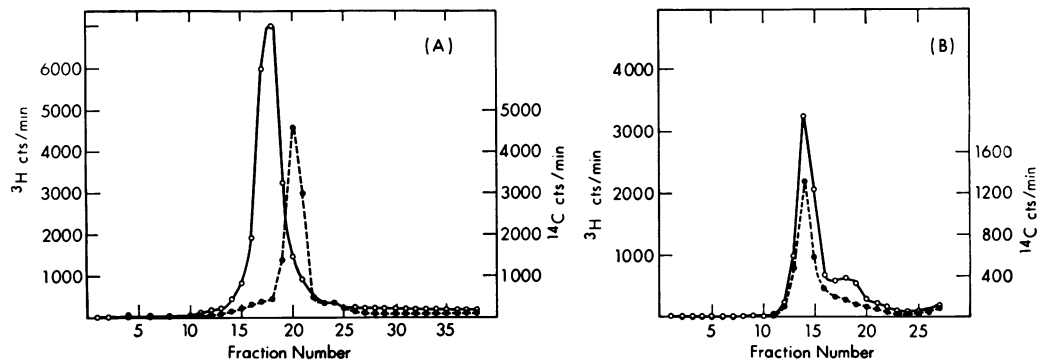

FIG. 7. *Sucrose gradient sedimentation of phage PBLA DNA. ^{3}H-labeled PBLA and ^{14}C-labeled T7 phages were mixed, and the DNA species were isolated with Pronase and Sarkosyl treatment. DNA sedimented in (A) neutral pH and (B) alkaline pH. Symbols: ○, ^{3}H; ●, ^{14}C counts.*

was not an artifact of the DNA extraction procedure, T7 phage was routinely mixed with the purified phage preparation and the mixture was then deproteinized. The properties of phages PBLA and PBLB and their DNA are summarized in Table 2.

DNA-DNA hybridization. Sequence homology between the DNA molecules of the two defective phages of *B. licheniformis*, as well as their relationship to host bacterial DNA, was studied by hybridization on membrane filters by using the technique described by Denhardt (4). A constant amount of radioactive, denatured phage DNA was used to hybridize with various amounts of unlabeled, denatured DNA samples that were immobilized on the membrane filters. The relationships between DNA of PBLB and PBLA and between that of PBLB and host bacterial DNA as well were compared with the homologous PBLB-PBLB DNA hybridization (Fig. 8A). When an excess amount of DNA is immobilized on the membrane filter, more than 50% of the ^{3}H-labeled PBLB DNA will hybridize with either PBLA DNA or *B. licheniformis* DNA. This level of hybridizability was the same as that for homologous PBLB-PBLB DNA annealed under identical conditions. A parallel experiment using radioactive, denatured PBLA DNA was also performed (Fig. 8B). With sufficient unlabeled DNA immobilized on the filter, radioactive, denatured PBLA DNA annealed with PBLB DNA and bacterial host DNA with similar efficiency, amounting to about 45% of the input counts. This level was lower than the corresponding homologous PBLA-PBLA DNA hybridization, which reached a level of more than 60% of the input. These results demonstrated that there was extensive sequence homology between the DNA of the defective phages and *B. licheniformis* host DNA, as well as between the two defective phage DNA species themselves. In addition, DNA isolated from *B. subtilis* phage ϕ105 was also used to hybridize with radioactive, denatured PBLA DNA under the conditions described by Fig. 8B. A lower level of hybridizability, amounting to about 30% of the input counts, was obtained.

DISCUSSION

Several bacterial species have now been demonstrated to harbor defective phagelike particles which can be detected when induced by chemical or physical means (3, 12). Some of these particles, even though they possess a full complement of phage components and contain DNA, cannot form plaques on sensitive bacteria. Thus, the defective phages are obtainable only by induction. Two closely related *Bacillus* species, *B. subtilis* and *B. licheniformis*, have been carefully examined with regard to their ability to produce such defective phage particles. The properties of the *B. subtilis* defective phage, PBSX, have been reported earlier (10, 11), and this communication deals with the characterization of the inducible phages of *B. licheniformis*, PBLA and PBLB.

By all the criteria studied, PBSX-like phages are produced by both *Bacillus* species. These phages are readily identifiable by their characteristic size, buoyant density, and morphology. This type of defective phage is unique in that it incorporates into its phage head preexisting bacterial DNA of homogeneous molecular size with a sedimentation coefficient of 22*S*, equivalent to a molecular weight of 8.4×10^6 daltons in double-stranded form. The DNA molecules isolated from PBSX-like defective phage particles can further be identified with bacterial DNA by their similar buoyant density in CsCl and by DNA-DNA hybridization studies. In the case of PBSX DNA, transforming activity is also demonstrable. On the other hand, the defective phages of *B. subtilis* and of *B. licheniformis* are distinguishable from each other mainly by their killing activity, which is

listed in Table 1. The range of killing activity of the *Bacillus* defective phage is not limited by species specificity.

Even though *B. subtilis* and *B. licheniformis* have DNA species which differ in their overall base composition and the two organisms displayed limited genetic homology (5), the ability to produce this defective, PBSX-like defective particle has been conserved. Surprisingly, mapping studies on the location of PBSX mutants on the *B. subtilis* chromosome do not place the genetic markers within the major conserved genetic regions of the *B. subtilis* chromosome (E. Siegel and A. Garro, *unpublished data*).

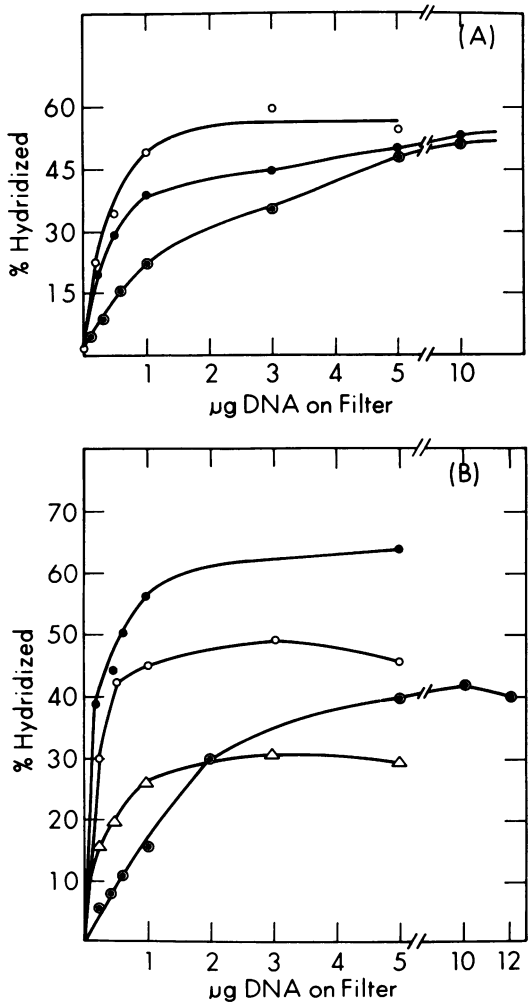

FIG. 8. *DNA-DNA hybridization studies. (A) Hybridization of ^{3}H-labeled PBLB DNA with unlabeled DNA that was immobilized on the membrane filter. ^{3}H-PBLB DNA input was 7,000 counts/min to each filter. (B) Hybridization of ^{3}H-labeled PBLA DNA with unlabeled DNA on the filter. ^{3}H-PBLA DNA input was 18,000 counts/min to each filter. Unlabeled DNA: ○, PBLB DNA; ●, PBLA DNA; ◉, B. licheniformis NRS 243 DNA; △, B. subtilis phage φ105 DNA.*

TABLE 2. *Physical properties of defective phages of Bacillus licheniformis and their DNA*

B. licheniformis phage	Phage particles		DNA		
	Density (g/cm^3)	Size	Density (neutral, g/cm^3)	Size	
				Neutral	Alkaline
PBLA	1.484	220–250*S*	1.701	36*S*	38*S* + 25*S*
PBLB	1.372	160*S*	1.706	22*S*	25*S*

Upon induction by mitomycin C, *B. licheniformis* produces a second type of defective phage, PBLA, in addition to the PBSX-like particle. The two defective phages of *B. licheniformis* are quite different in structure under the electron microscope as well as in other physical properties such as size and buoyant density, hence making possible their physical separation. Preliminary immunological experiments indicated that PBLA and PBLB were also unrelated serologically, since antiserum against PBSX was capable of neutralizing PBSZ (the defective phage produced by *B. subtilis* strain W23) and PBLB, whereas it had no effect on PBLA.

After mitomycin C treatment, DNA continues to be synthesized in the induced bacteria. PBLA incorporated exclusively DNA that was synthesized de novo after induction, whereas PBLB packaged both preexisting and newly synthesized host DNA. The rate of synthesis of the newly synthesized DNA molecules that were eventually incorporated into the two phage particles followed different time courses. The PBLB-specific DNA was synthesized at the maximum rate 45 min after mitomycin C induction. In the case of PBLA DNA, there was a lag period prior to optimum phage-specific DNA synthesis, suggesting the possibility that a different DNA synthesizing machinery, other than that of the host, was induced as a result of mitomycin C treatment.

PBLA DNA, when sedimented in a neutral sucrose gradient, gives a sharp and symmetrical band. In view of its sedimentation profile, it seemed unlikely that this DNA contained cohesive ends similar to that of *E. coli* phage λ. The same DNA preparation of PBLA, which was intact under neutral conditions, resolved into two components in alkaline gradients of 13×10^6 and 4×10^6 daltons with a mass ratio of about 4:1. This ratio is roughly that of the molecular weights.

The sum of the two components is also in good agreement with 50% of the molecular weight of the double-stranded native form. Furthermore, no single-stranded material larger than 13×10^6 daltons was observed. These results imply that both strands of the native DNA molecule are not intact, each strand possessing a single interruption. The above results can best be interpreted by assuming that each PBLA phage DNA molecule contains two long segments and two short segments. Two models for the structure of PBLA DNA may be entertained. The short segment of each strand may be situated at opposite ends of the native DNA molecule. In this case, the interruption in each single strand is separated by some distance, thus rendering the DNA molecule intact under neutral conditions. As a second model, it may be proposed that the two short segments are opposite each other. In this case the two interruptions are slightly displaced in the native DNA molecule to maintain its intactness. Phage particles possessing intrinsic single-stranded breaks in their DNA have been demonstrated to be biologically infectious as in the case of *E. coli* phage T5 (1). Thus far, we have not been able to obtain a *B. subtilis* or a *B. licheniformis* strain which can support the propagation of PBLA. One of the possibilities is that PBLA is defective. The single-strand breaks in PBLA DNA molecules might represent errors in the excision process after mitomycin C induction.

Despite the difference in buoyant density between PBLA DNA (1.701 g/cm^3) and host bacterial DNA (1.707 g/cm^3), some sequence homology has been demonstrated between them by DNA-DNA hybridization. PBLA DNA also displays some base sequence homology with the DNA of the *B. subtilis* phage ϕ105. These two phages appear to be morphologically related by electron microscopy. Since PBLB DNA originates from the host chromosome, it is to be expected that there would be extensive base sequence homology between the phage and *B. licheniformis* DNA (Fig. 8).

Attempts were made to analyze by CsCl-density gradient centrifugation the separated fragments of denatured PBLA DNA, fractionated by alkaline sedimentation (Fig. 7B), to renature by themselves under neutral conditions. The densities of the renatured fragments of PBLA DNA indicated they were heterogeneous in base composition, since the longer fragments (38*S* in alkali) had a renatured buoyant density of 1.701 g/cm^3, whereas the smaller fragments (25*S* in alkali) had a renatured density of 1.706 g/cm^3, similar to that of host DNA (W. M. Huang, *unpublished data*). This preliminary finding and the observation that PBLA DNA hybridizes efficiently with *B. licheniformis* DNA (Fig. 8) suggest that PBLA DNA contains representative host sequences. This conclusion would have to be confirmed by additional experiments.

The above studies have been mainly concerned with the physical properties of the two *B. licheniformis* phages. Phage assays were carried out by determining the extent of incorporation of radioactive precursors into their DNA molecules. This method is laborious and much less sensitive than plaque assays. One question which remains to be answered is whether both phages are produced by a single bacterium or are derived from separate cells. However, in the absence of the more sensitive techniques, this question remains unanswered for the present.

One of the most interesting aspects of the production of the defective phages of *Bacillus* species which incorporate host DNA is the manner by which the host chromosome is packaged by the phage particle. In the case of *B. licheniformis*, phage PBLB contains exclusively host DNA and no PBLA DNA. This observation emphasizes that the packaging mechanism is selective with respect to the DNA that is eventually incorporated into the phage head. The packaging mechanism and its selectivity should prove an interesting area for future research. It would, for instance, give us a better understanding of the uptake of transducing fragments by generalized transducing phages (6).

ACKNOWLEDGMENTS

We thank J. Mangan for carrying out the electron microscopy. This investigation was supported by a Public Health Service grant (GM-11946-06) from the National Institute of General Medical Sciences. J. Marmur was supported by a Career Award from the Health Research Council of the City of New York (I-322).

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