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DNA Packaging by the *Bacillus subtilis* Defective Bacteriophage PBSX

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Defective bacteriophage PBSX, a resident of all *Bacillus subtilis* 168 chromosomes, packages fragments of DNA from all portions of the host chromosome when induced by mitomycin C. In this study, the physical process for DNA packaging of both chromosomal and plasmid DNAs was examined. Discrete 13-kilobase (kb) lengths of DNA were packaged by wild-type phage, and the process was DNase I resistant and probably occurred by a head-filling mechanism. Genetically engineered isogenic host strains having a chloramphenicol resistance determinant integrated as a genetic flag at two different regions of the chromosome were used to monitor the packaging of specific chromosomal regions. No dramatic selectivity for these regions could be documented. If the wild-type strain 168 contains autonomously replicating plasmids, especially pC194, the mitomycin C induces an increase in size of resident plasmid DNA, which is then packaged as 13-kb pieces into phage heads. In strain RB1144, which lacks substantial portions of the PBSX resident phage region, mitomycin C treatment did not affect the structure of resident plasmids. Induction of PBSX started rolling circle replication on plasmids, which then became packaged as 13-kb fragments. This alteration or cannibalization of plasmid replication resulting from mitomycin C treatment requires for its function some DNA within the prophage deletion of strain RB1144.

Bacteriophage PBSX, a normal resident of the *Bacillus subtilis* chromosome, is defective and does not exclusively package a unique genome. Several investigators have examined the DNA packaged by PBSX. It has been shown by sucrose density gradients to be homogeneous in size (16, 17, 18, 33, 34). Genetic transformation with phage-derived DNA has shown that this DNA can transform recipient cells of strain 168 for virtually any genetic marker (17, 33). There have also been several attempts to determine whether the DNA from the host chromosome is randomly inserted into the phage or whether there is some portion of the genome that is more highly represented in the phage-derived DNA. Unfortunately, two of the studies measured only the relative transformation frequencies of DNA from the phage compared with that of the unperturbed host cell (17, 33). No measurement of the transforming activity of DNA in the cell just before packaging was made.

Thurm and Garro (39) used marker frequency analysis to suggest that, after induction of PBSX, DNA in the host cell near both the origin of DNA replication (*purA*) and the site of prophage PBSX (*metC*) was amplified as much as 10-fold over DNA found just before induction. In another attempt to examine the identity of DNA packaged by PBSX, Okamoto et al. (33) used liquid-phase DNA hybridization to determine whether there was a subpopulation of molecules present in the phage at an increased frequency. They found none.

The results of these two studies seem to conflict. If random fragments of DNA were packaged and if the cell has amplified two distinct regions of DNA during the induction process, then why did the experiments of Okamoto et al. not show the presence of homologous DNA fragments (ostensibly *metC* and *purA* proximal) in the population of DNA isolated from PBSX? This study sought to answer this question by using the more sensitive and accurate recombinant DNA technology not available when either of the previous studies was undertaken.

MATERIALS AND METHODS

Strains. The *B. subtilis* strains used in this study are described in Table 1. Strains containing integrated plasmids were constructed by competent cell transformation, verified for insertion by hybridization, and checked for proper site of integration by PBS1 transduction mapping. These strains were shown to be devoid of an autonomously replicating plasmid by hybridization. They are designated as strain Ω plasmids, with Ω denoting plasmid integration.

Plasmids. Plasmid pJ-1 contains a 5.4-kilobase (kb) *Eco*RI fragment of *B. subtilis* DNA from the prophage PBSX region of the chromosome (2) recloned into the vector pDH5109. Plasmid pGS322 contains a 1.4-kb *Eco*RI fragment of *B. subtilis* DNA adjacent to operon *rrnB*. This fragment was originally isolated from phage J25 (38) by G. C. Stewart and then subcloned into pDH5109. Mapping studies have shown that this fragment resides approximately 60 map units from *thr-5* between *thr-5* and *aroG* (3a). Plasmid vector pDH5109 was kindly supplied by S. Chang. It is a chimera consisting of pC194 plus pBR322 joined at the *Hpa*II and *Cla*I sites, respectively. This ligation inactivates the pC194 replicon.

Plasmids pC194, pE194, and pUB110 are *Staphylococcus aureus* plasmids, all capable of replicating in *B. subtilis* (12). They express resistance to chloramphenicol, erythromycin, and kanamycin (neomycin), respectively. The complete nucleotide sequence is available for pC194 (23) and pE194 (22).

Media. TBAB (tryptose blood agar base) plates (Difco Laboratories, Detroit, Mich.) supplemented with 5 μg of the appropriate drug per ml (final concentration) (filter steri-

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TABLE 1. *B. subtilis* strains

Designation	Genotype or phenotype	Source (reference)
168	*trpC2*	University of North Carolina
1A78	*xtl-1 metC3 pyrA*	*Bacillus* Genetic Stock Center (4)
168ΩpJ-1	Integrant of plasmid pJ-1, *trpC2* Cm^r	This paper
168ΩpGS322	Integrant of plasmid pGS322, *trp*C2 Cm^r	This paper
1A12	*purA metB leuA*	*Bacillus* Genetic Stock Center
168(pC194)	Autonomous plasmid, *trpC2* Cm^r	This paper
168(pE194)	Autonomous plasmid, *trpC* Erm^r	This paper
168(pUB110)	Autonomous plasmid, *trpC2* Kan^r	This paper
RB1144	*pyrD ilvA1 thyA thyB* Δ(PBSX-*metC-proA*)	R. Buxton (5)
RB1144(pC194)	Autonomous plasmid *pyrD ilvA1 thyA thyB* Δ(PBSX-*metC-proA*)	This paper

lized) were used to maintain plasmid-bearing strains. Selection of prototrophic recombinants in the genetic analysis used Spizizen minimal medium (1) supplemented with 100 μg of the appropriate amino acid or nucleotide per ml (final concentration).

Genetic manipulations. Transformation of competent cells and protoplasts has been described previously (7, 14). PBS1 transductional mapping has also been described before (19).

Isolation of phage and phage-derived DNAs. Strain 168 was grown through at least four doublings in Penassay broth (Difco antibiotic assay medium no. 3) with shaking at 37°C. When an optical density of 0.25 (measured at 570 nm) was reached, freshly reconstituted mitomycin C (Sigma) was added to a final concentration of 0.5 μg/ml. Cells lysed in 90 to 120 min with continued shaking at 37°C. Debris was removed by a low-speed centrifugation step (10,000 rpm, 10 min, SS-34 rotor; Ivan Sorvall, Inc., Norwalk, Conn.), and the phage was pelleted by high-speed centrifugation (8,000 rpm, 18 h) (GSA rotor; Sorvall). The phage pellets were overlaid with phage buffer (32) and allowed to resuspend overnight on ice. Resuspended pellets were gently collected, layered onto cesium chloride step gradients, using layers with densities of 1.4, 1.3, and 1.2 by the method described by Miller (32), and centrifuged (23,000 rpm, 18 to 24 h, SW27 rotor; Spinco). Bands were collected and dialyzed against phage buffer to remove cesium chloride.

These purified phage particles were extracted with washed, buffer-saturated phenol to isolate phage-packaged DNA. After two extractions with phenol, two extractions with chloroform, and precipitation with ethanol, DNA could routinely be digested to completion with restriction endonucleases.

DNA manipulations. DNA preparations of whole cells, cells induced with mitomycin C, and cells lysed by phage were performed with care not to discriminate against any DNA species. Timed samples (to follow the fate of DNA during phage induction) were harvested by ethanol precipitation of the entire culture as described below to ensure that both cell-bound and free DNA species were represented in the final preparation.

To determine the order of packaging and DNA scission, we used the following protocol. Cells were grown in VY liquid medium (33) to an optical density of 0.25 (570 nm) (usually a 1L culture in a low-form flask in rapid agitation). A sample of 100 ml was drawn and chilled on ice (pretreatment sample), and chloramphenicol was added to a final concentration of 10 μg/ml. Mitomycin C (0.5-μg/ml final concentration) was added to the remainder of the growing culture, with five successive 100-ml samples being removed at 20-min intervals, chilled, and inhibited with chloramphenicol as above. Lysozyme (to 1 mg/ml) was added and allowed to digest for 15 min. Sodium dodecyl sulfate (SDS) (to a concentration of 1%) and phenylmethylsulfonyl fluoride (to 17.5 μg/ml) were added to the lysed, dilute preparation of cells. Each sample was then divided in half; to one-half, DNase I (to 20 μg/ml) and magnesium chloride (to 5 mM) were added and mixed. Both portions were incubated at 37°C for 60 min, and then 2.5 volumes of ice cold ethanol was added to each sample. After precipitation at 4°C overnight, the samples were centrifuged at 5,000 rpm for 60 min, dried in vacuo, and suspended in 10 ml of phage buffer. As before, the DNA was extracted with phenol and chloroform and reprecipitated with ethanol.

Restriction endonuclease digestions of DNA were performed according to the instructions of the manufacturer. Separation of DNA fragments by agarose gel electrophoresis, transfer of DNA to nitrocellulose, labeling of DNA by nick translation, DNA hybridization, and autoradiography have all been described by Maniatis et al. (27).

RESULTS

Physical examination of PBSX-derived DNA. Discrete 13-kb fragments of DNA were packaged by the phage (Fig. 1). When converted to molecular weight (13, 40), this estimate agrees well with the sedimentation coefficient value of 22S (34) and contour length measurements of electron micrographs, e.g., 12×10^6 (16) and 8.1×10^6, (18) as observed by others.

When phage-derived DNA was digested with restriction endonucleases, a discrete banding pattern became superimposed over the normal background seen when chromosomal DNA was digested (Fig. 2). If a unique population is

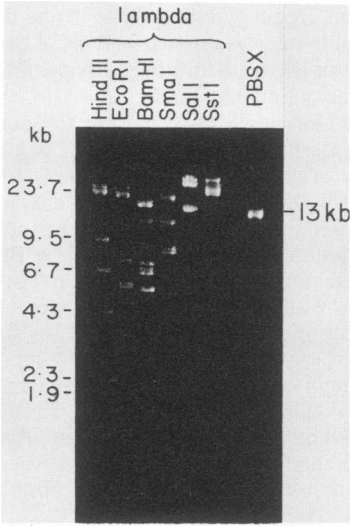

FIG. 1. Sizing of PBSX-derived DNA. The six lambda DNA lanes are restriction digests providing size standards.

preferentially packaged by the phage, then a precise and less complex pattern of bands would be observed. Hybridization analysis of these digests with fragments of DNA from the PBSX prophage region of the chromosome did not demonstrate any difference in either the size or intensity of hybridization to homologous fragments of phage-packaged DNA relative to chromosomal DNA (data not shown); i.e., none of the bands which were substantially enhanced by staining hybridized at all with any probe from the PBSX region. This is particularly relevant when the restriction map of this region of the chromosome is considered (L. M. Anderson, Ph.D. thesis, University of North Carolina, Chapel Hill, 1984). There are relatively few *Bam*HI sites in this region, so if a precise population of DNA fragments for this region is packaged by the phage, we would have expected the sizes of the homologous bands in *Bam*HI-digested DNA to change. Although it appears from the altered staining of some molecular weight species that there is preferential packaging (and this is supported by the inability of *Bam*HI-digested DNA to show a difference upon hybridization), the altered staining did not affect the hybridization to fragments from the PBSX region for which we have probes.

Resistance of 13-kb DNA to DNase I digestion. At 40 min postinduction with mitomycin C, 13-kb DNA became detectable in whole-cell DNA preparations (Fig. 3). This size class

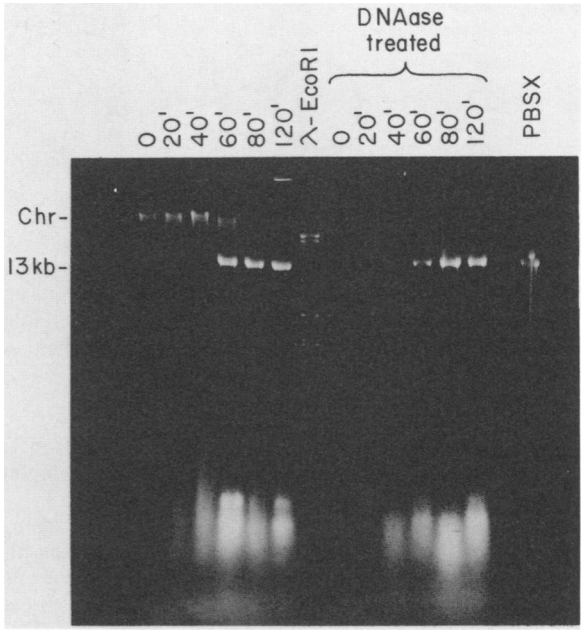

FIG. 3. Effect of DNase I on 13-kb DNA. At various times after mitomycin C treatment, DNA was purified by lysozyme and sodium dodecyl sulfate extraction and then divided in half. Samples on the left were electrophoresed directly, and those on the right were treated with DNase and then phenol extracted and electrophoresed. Chr, Chromosomal DNA.

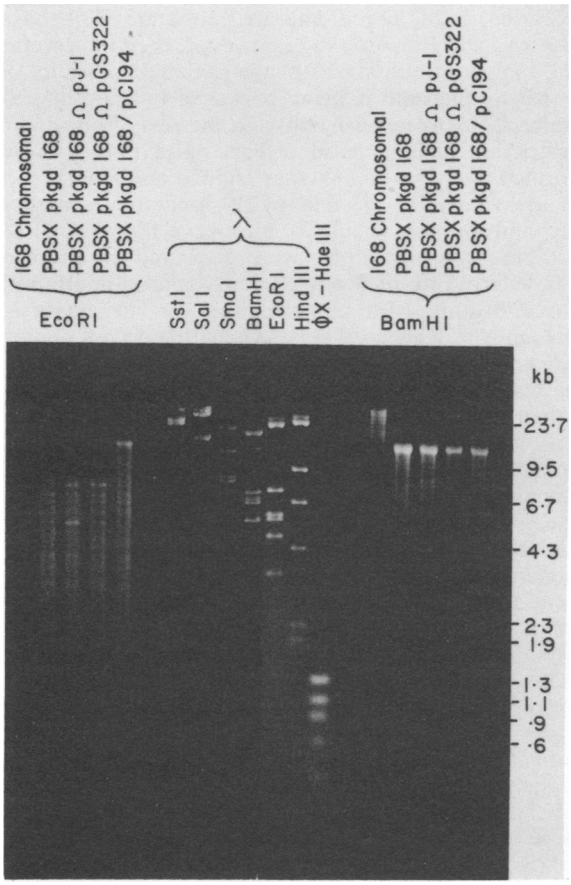

FIG. 2. Restriction endonuclease digests of DNA from CsCl-purified phage and chromosomal DNA with or without various plasmids integrated. Phage λ digested with various enzymes as explained in the text provides size standards.

became dominant at later times. When lysed cell preparations from these samples were treated with DNase I, the 13-kb DNA remained undegraded, whereas chromosomal DNA disappeared (Fig. 3). We interpreted this to mean that the majority of 13-kb DNA is not present in a pool before packaging by the phage, and therefore that the DNA is first packaged into the phage head and then cut. This observation is entirely consistent with the head-filling mechanism described for double-stranded DNA phages (11).

Genetic analysis of PBSX-packaged DNA. To determine whether any portion of DNA in the phage genome region was preferentially packaged, we used a novel genetic approach based on transformation assay. We have previously characterized a fragment of DNA from the *metA*-PBSX-*metC* region of the *B. subtilis* chromosome that contains the origin of replication of the defective phage PBSX (2; Anderson, Ph.D. thesis). In this study, we found that that same fragment is contained in plasmid pJ-1. We used this fragment to direct integration of the vector marker into the chromosome at its specific chromosomal site. We then used the chloramphenicol marker as a genetic flag to follow the fate of DNA from this region of the chromosome during the production of defective phage particles.

To provide a reference for the current studies, we also used a strain in which plasmid pGS322 was integrated into the chromosome at *rrnB* (Fig. 4). This positioned the same vector at a different site on the chromosome. As a control for consistency of results, we also made the transformation measurements relative to a chromosomal auxotrophic marker *leuA* positioned at 210° on the chromosome, which is well outside both of the regions under study.

We used the same preparation of competent cells as a recipient for DNA isolated from cells at three different stages of PBSX maturation: log-phase (zero-time) DNA,

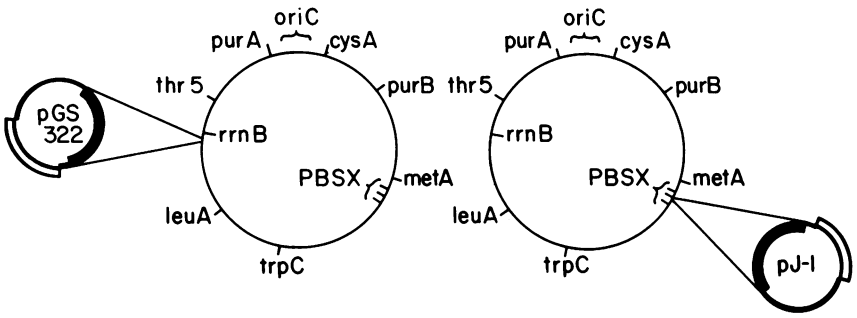

FIG. 4. Integration sites of plasmids pJ-1 and pGS322 into the *B. subtilis* 168 chromosome. (Drawing is not to scale.) Plasmid parts are: ■, cloned *B. subtilis* insert; ▭, pBR322 vector fragment; ▬, pC194 portion of vector pDH5109.

total cellular DNA isolated 80 min after induction, and phage-derived DNA. Each is described more completely below.

Log-phase (zero-time) DNA. DNA was isolated from the two strains having integrated plasmids during log-phase growth before phage induction. Several concentrations of DNA were used for transformation of strain 1A12 to establish a value of the transformants per microgram of DNA for each marker (Fig. 5A). Selection for chloramphenicol-resistant, adenine$^+$, or leucine$^+$ transformants generated the values shown. The saturation level of DNA for each of the three genetic markers was different, conceivably because of the difference in effective marker size. This indicates that no single DNA concentration can give the true proportion of chloramphenicol transformants in the population.

Postinduction (80-min) DNA. The proportion of markers present in the total cellular pool should reflect the positional effect due to replication of the chromosome both from the real origin of replication and from the phage origin of replication. Both of these regions have been reported to be enriched after phage induction (17, 39).

Figure 5B shows that the proportion of chloramphenicol-resistant transformants (relative to leucine) was higher in the pooled cellular DNA from the strain containing the plasmid integrated at the phage locus than in the strain with the integrant at the *rrnB* locus (*leu*/Cm = 10/1 versus 2/1). This was as expected from earlier genetic studies (39).

Phage-derived DNA. Figure 5C shows that when plasmid pJ-1 was integrated into the PBSX region of the chromosome, its chloramphenicol resistance marker was not preferentially packaged. The number of chloramphenicol-resistant transformants obtained relative to leucine was not greater than that seen when the same vector was integrated into an alternate site on the chromosome, i.e., pGS322 at *rrnB*. In fact, the plasmid marker integrated at *rrnB* appears to be packaged more frequently than the same marker when it is integrated at the PBSX locus.

The data from this study do not show a striking increase in the proportion of *purA* transformants relative to *leuA* transformants when compared over several DNA concentrations as might have been expected (39).

Fate of plasmid pC194 during PBSX induction. When plasmid pC194 was introduced into strains of *B. subtilis* carrying PBSX, it remained autonomous. Figure 6 shows the change in DNA size and form that occurred when strain 168 carrying plasmid pC194 was treated with mitomycin C to induce PBSX. The preinduction sample showed that large chromosomal DNA and several topological or multimeric forms of pC194 are present. At 80 min postinduction, 13-kb fragments of DNA appeared, and only two forms of the plasmid molecules were detectable. In phage-packaged DNA, 13-kb fragments were found with only a single plasmid form. The 13-kb band of DNA had homology to pC194.

We also found that DNA isolated from mature phage could efficiently transform competent cells and introduce monomeric pC194 into them (Table 2), whereas the zero-time sample of DNA and the 80-min postinduction sample could not. This result suggests that the plasmid was undergoing a transition into an unusual form, since plasmid transformation of competent cells is a very controlled event and does not normally occur without a purified plasmid preparation (6, 8). These data are consistent with those of Poluektova and Prozorov (35); however, their interpretation of the biological activity of phage-packaged plasmid DNA was that the plasmid is being packaged by the phage as a monomer. This is inconsistent with the rest of our findings.

Restriction endonuclease digests of pC194 DNA were performed (Fig. 7) with enzymes *Hin*dIII and *Hae*III, which each cleave within plasmid pC194 only once, and with *Eco*RI and *Bam*HI, which do not cleave the plasmid at all (23). These digest products were displayed on an agarose gel, transferred to nitrocellulose, and appropriately probed by hybridization with ^{32}P-labeled pC194 to identify the homologous sequences. The enzymes that do not digest the plasmid caused no change in the migration of the 13-kb band. If the plasmid had been integrated into the chromosome (2) and then packaged, these digestions would have caused the 13-kb band to be altered in size. This suggests that the plasmid DNA present in the 13-kb band is present as a multimer of only itself.

The enzymes that cut once within the plasmid caused all of the pC194 homologous material to migrate as linear monomers. From this result, we deduced that the plasmid is present in head-to-tail multimers in the 13-kb fragment. Since no other specific fragments were recovered from these digests, no particular permutation of the multimer was packaged by the phage.

We repeated the same type of experiment with strain 168 carrying plasmids pE194 or pUB110, which are *S. aureus* in origin yet are completely distinct plasmids already believed to replicate in normal cells through theta-structure intermediates (20, 37). Analogous 13-kb head-to-tail linear multimers of these two plasmids were also packaged by the phage (data not shown).

The effect of mitomycin C treatment on pC194 replication in host RB1144 that is deleted for a portion of PBSX (5) was also examined. Although no phage were produced from mitomycin C treatment of this strain, there was also no

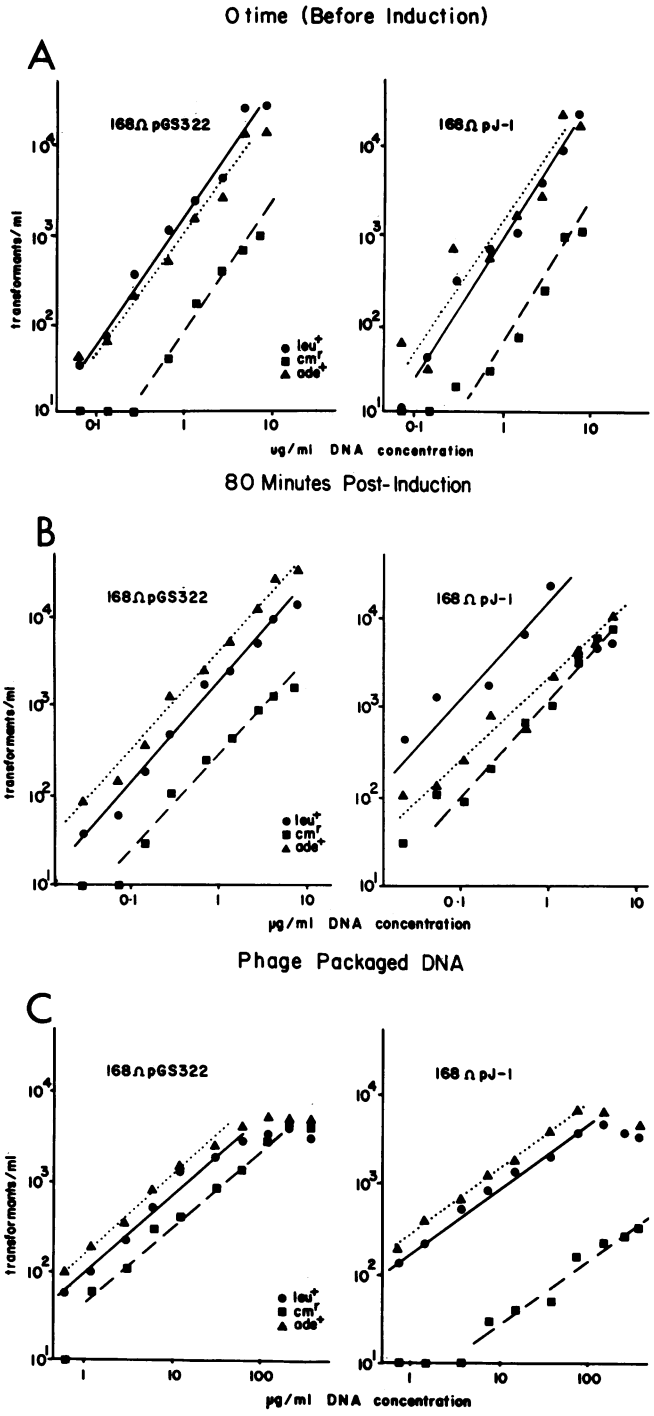

FIG. 5. Relative transforming activity at several DNA concentrations. (A) DNA isolated from cells during log-phase growth (zero time). (B) Total DNA from cells 80 min after induction with mitomycin C. (C) DNA from CsCl-purified DNA. In all cases the transformation used the same batch of recipient cells of strain IA12.

detectable alteration in the migration of pC194 homologous DNA which occurred when this strain was treated with mitomycin C (Fig. 8). This implies that the multimeric forms of plasmid are dependent on the presence of genes within the deleted region.

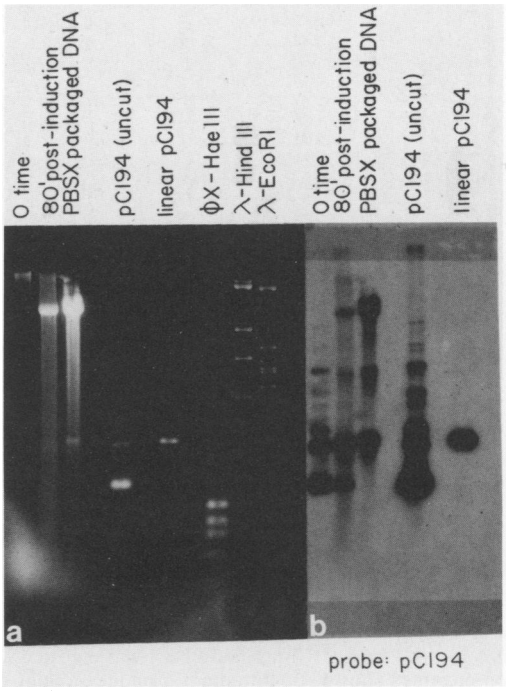

FIG. 6. Fate of plasmid pC194 during the induction of phage and packaging of DNA. (a) Stained gel with ethidium bromide. (b) Hybridization of the same gel with ^{32}P-labeled pC194 as a probe. Several multimeric forms of plasmid, which are not visible by stain because of low abundance, are revealed by hybridization.

DISCUSSION

We confirmed that PBSX packages discrete 13-kb lengths of DNA. The nature of the DNA varied with what was available when phage induction occurred. It was neither a unique homogeneous population (except in size) nor a population resulting from the packaging of random fragments within the cell, since an enhancement of some bands was detectable by ethidium bromide staining. The genetic data suggest that DNA from the chromosomal region of prophage PBSX is packaged less frequently (and not in direct proportion to its abundance at the time of induction) than DNA from a different region of the chromosome when measured by a neighboring nonphage marker, the integrated chloramphenicol resistance determinant. Additionally, unusual DNA replication intermediates of the plasmid seem to be packaged within the phage capsids if those plasmids are present at the time of induction. Since these plasmids carry no obvious viral packaging sequences, it must also be argued that the PBSX packaging system can recognize, at least with some

TABLE 2. Transformation of competent cells by plasmid pC194-containing samples[a]

Sample	No. of transformants per ml	
	Cm^r	*leuA*$^+$
Zero time (noninduced)	<10	1.8×10^2
80 min postinduction	10	6.0×10^2
Phage-derived DNA	2.2×10^2	2.28×10^3

[a] DNA was not saturating; hence, very few Cm^r transformants would be expected from noninduced samples since only multimeric forms of the plasmid transform. Cm^r transformants were verified to contain pC194 by small lysate analysis (37; data not shown).

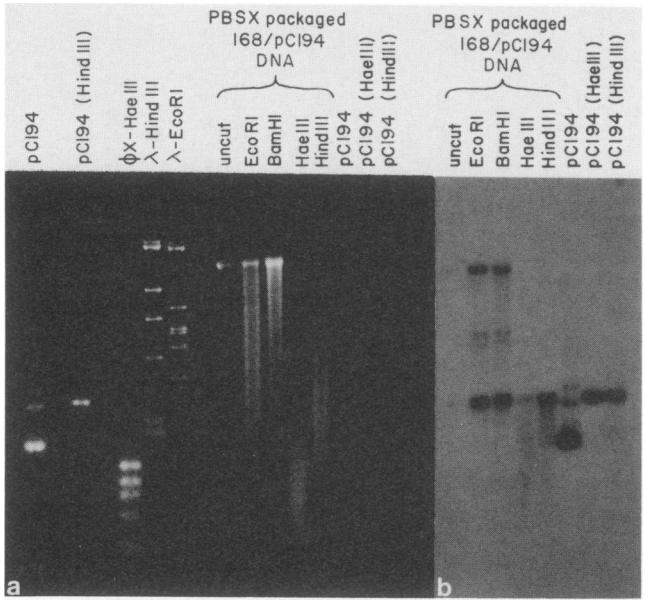

FIG. 7. Restriction endonuclease digests of phage-packaged DNA from strain 168 containing pC194 as an autonomous plasmid. Total DNA isolated from CsCl-purified PBSX was digested with the restriction enzymes shown. (a) Ethidium bromide staining reveals multiple bands typical of the heterogeneous contents of phage. The three right lanes containing pC194 purposely contained very little DNA to prevent obliteration of the autoradiogram shown (b).

efficiency, a feature of the plasmid DNA. Induction causes the formation of plasmid concatemers, and at least some significant effect of the phenomenon requires DNA from the region deleted in strain RB1144.

The mode of replication of plasmid pC194 in these perturbed cells that is suggested by these linear, multimeric forms is a rolling circle mechanism which generates head-to-tail multimers of the plasmid. The mode of replication of pC194 in *B. subtilis* has not been previously reported. However, Scheer-Abramowitz et al. (37) have analyzed the replication of plasmids pE194 and pUB110 by electron microscopy. These plasmids both form theta structures and replicate unidirectionally from a fixed origin. Unfortunately, the method of isolating molecules used by those authors for observation is biased toward molecules that would travel between covalently closed circular DNA and linear DNA in an ethidium bromide-cesium chloride density gradient. If these plasmids replicated by a rolling circle model in vivo, then this method of isolating the plasmid DNA would have discriminated against a long linear precursor.

The replication of plasmid molecules by a rolling circle mechanism is not totally inconsistent with a theta structure since other circular molecules (e.g., lambda) are known to replicate via both of these mechanisms (3). Furthermore, Khan et al. (25) have postulated the replication of a gram-positive plasmid by a rolling circle mechanism. Marrero et al. (28) have observed the same type of plasmid molecules packaged into phage SPO2 heads. Phages SPO2 and ϕ105 cannot normally transduce either plasmid pC194 or pUB110 without any phage-specific DNA (29), although phage were not examined after a UV or mitomycin C treatment analogous to that used here. Other phage (e.g., CP-51 and PBS1) can transduce and therefore package some form of plasmids without inserts (30, 36).

To hypothesize that two replication mechanisms are operating in the same system means that either there is a PBSX-associated shift from theta mechanism to rolling circle that is accompanied by a nicking step or that before PBSX induction, the proportion of rolling circle forms is extremely small and only becomes detectable after selection by the packaging process. For strain RB1144, this minor fraction would escape detection because phage packaging is faulty. The hybridization data tend to argue against the minor constituent hypothesis since the high-molecular-weight species were not detected even with highly radioactive plasmid probes. Hence, it appears most likely that the rolling circle structures are entirely dependent on some function of DNA in the deleted region.

The packaging recognition system for PBSX cannot be too stringent since so many types of DNA molecules are packaged. Therefore, the phage may be deficient in its ability to preferentially recognize any *cos*-type sequence (21). Nevertheless, DNA is packaged into the phage to become DNase resistant. Okamoto and Mudd (15) observed the tendency for PBSX to discriminate against some bacteriophage DNA. It is therefore possible to speculate that there must be some recognition signal functioning in this process, whether just a free end, as in the case of phage SPPI (10), secondary DNA structure, or the original cellular location of the replicating

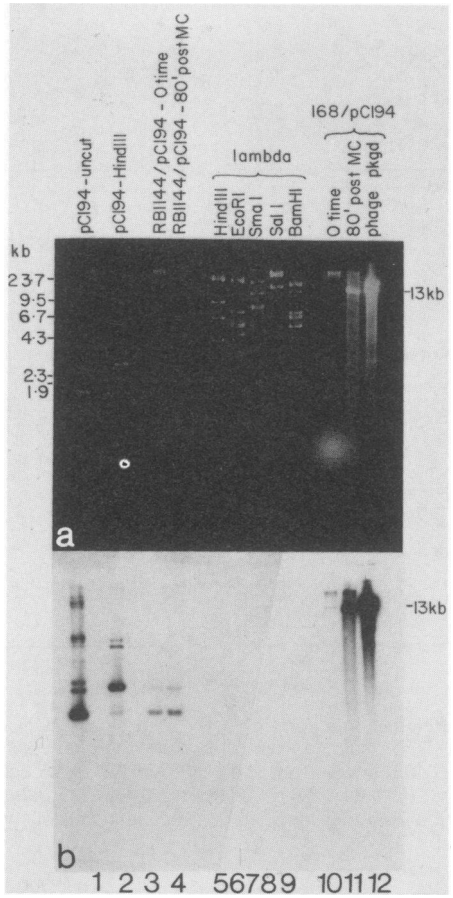

FIG. 8. Effect of mitomycin C (MC) treatment on pC194-homologous DNA in strain RB1144 containing pC194 as an autonomous plasmid (lanes 3 and 4) as compared with pC194 in strain 168 (lanes 10 to 12). Other lanes are as indicated. (b) Autoradiogram of panel a after probing with ^{32}P-labeled pC194 DNA.

molecule. Replicating DNA in *B. subtilis* is associated with the membrane (26), and this could be a method of selection used by PBSX (35) although it would logically have to follow from this that the PBSX *oriP* as well as phage SPO2 would not be membrane bound during replication (15).

The scission of the DNA required for packaging is also of interest. The phage must have a nuclease associated with its capsid at least during assembly to clip the DNA after packaging occurs. Evidence that this enzyme recognizes some specific sequence pattern has been presented by Huang and Marmur (24). The 5′ ends of PBSX DNA are not single stranded. Additionally, DNA packaged by PBSX and PBSY has dGMP and dTMP at its 5′ ends; PBSZ-packaged DNA ends in dAMP and dTMP. These results suggest that the scission of the DNA occurs with some specificity. It might be possible to identify the enzyme responsible for this cleavage by looking at the proteins found in complete PBSX particles (9).

We confirmed that PBSX was defective but not deficient in packaging. It could indeed package very precisely cleaved fragments of DNA that were not totally representative of the pool from which it may select. Additionally, the induction of phage either allowed a novel plasmid replication mechanism to be invoked (31) or cannibalized plasmid DNA by adopting it into the phage DNA replication mechanism that was induced.

ACKNOWLEDGMENTS

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