Defective Bacteriophage PBSH in *Bacillus subtilis*

II. Intracellular Development of the Induced Prophage

MARTIN HAAS AND HIROSHI YOSHIKAWA

Space Sciences Laboratory, University of California, Berkeley, California 94720

Received for publication 27 September 1968

Treatment of *Bacillus subtilis* strain 168 with mitomycin C caused induction of a defective prophage, PBSH. During induction, extensive deoxyribonucleic acid (DNA) synthesis took place. Concurrently, a change in marker frequency of the bacterial DNA was noticed. The frequency of only one marker, *ade-16*, the marker closest to the origin of the bacterial chromosome, was enhanced manyfold. DNA from whole phage particles transformed all bacterial markers at a frequency equal to that of DNA in the noninduced culture, except *ade-16*, the frequency of which was enhanced 30 to 100 times. Analysis of a double isotope experiment demonstrated that 14% of the phage DNA was derived from preinduction bacterial DNA. The other 86% of DNA in phage particles was DNA replicated after induction. Density label experiments with 5-bromodeoxyuridine showed that postinduction DNA synthesis took place preferentially at the origin region of the bacterial chromosome. Measurement of the molecular weight of DNA replicated after induction clearly showed that postinduction DNA replication is chromosomal. No evidence for prophage detachment and autonomous phage DNA replication was found. The data indicated that, after mitomycin C action, the bacterial chromosome underwent multiple reinitiation at the origin, while normal sequential DNA replication was stopped. The pool of replicated bacterial DNA was fragmented randomly. This DNA was packaged into PBSH particles which were released after cell lysis.

Phages have been useful as a source of perturbations in the bacterial cell. Defective phages enable one to apply smaller perturbations to a bacterial system and, in so doing, to study phenomena which are normally masked behind the biochemical and genetic activity of a replicating phage. In particular, the careful choice of defective phages, whether intentionally induced or fortuitously detected, may allow a highly specific probe into some aspect of bacterial physiology.

Defective phages which are not variants of known temperate phages have often been found in the last few years (7, 8, 19). Such phages are generally found by induction and lysis of supposedly nonlysogenic bacterial strains. Since they are noninfective, only observation under the electron microscope or fractionation of such a phage lysate may show their existence. Production of defective phages may be due to spontaneous reversion of the defective genes.

In this paper, we describe a new type of defective phage, PBSH, which produced large numbers of phage particles after induction, without detectable replication of phage deoxyribonucleic acid (DNA). Some of the properties of PBSH to be shown are listed below. (i) Though seemingly a defective phage, it is present in low numbers in

noninduced bacterial cultures and can be efficiently induced by well-controlled induction conditions. (ii) The phage produced after induction does not form plaques on many supposedly nonlysogenic strains tested. (iii) The induced culture shows extensive DNA synthesis, most of which is cellular DNA replication. (iv) The liberated phage particles harbor bacterial genes. The distribution of bacterial markers in the phage particles is unusual. These properties suggest that PBSH is neither a defective phage in the usual sense, nor a normal temperate phage (3). As is shown in this paper, phage PBSH is defective due to the absence of any detectable phage DNA replication, but it is produced simultaneously with multiple reinitiation of the bacterial chromosome at the origin.

A possible mechanism of PBSH induction is discussed.

MATERIALS AND METHODS

Bacterial strains and media used in the experiments described in this paper, other than the transformation method, and induction and purification of PBSH and PBSH DNA have been described in the preceding paper (7).

Isolation of DNA from mitomycin C (MC)-treated

cells. Cells were killed in ice-cold 0.1 M ethylenediamine-tetraacetate (EDTA), pH 8.2, containing 0.02 M Na₂N₃ and then were incubated to make protoplasts (23). Protoplasts were incubated with 1 mg of Pronase (Calbiochem, Los Angeles, Calif.) per ml overnight, then mixed with CsCl to give a density of 1.70 g/ml. Fractions were collected after centrifugation in a swinging-bucket rotor for 48 to 60 hr at 35,000 rev/min. DNA was identified by determination of the optical density at 260 and 280 nm, by radioactivity after precipitation with 10% trichloroacetic acid on 0.45 μ m membrane filters (Millipore Corp., Bedford, Mass.) after heating to 80 C in 0.2 N NaOH for 30 min, and by transforming activity. It has been observed in this laboratory that isolation of DNA by centrifugation of whole protoplasts in CsCl yields double optical density peaks at the DNA region of the gradients. However, only one DNA peak is found as judged by radioactivity or transforming activity.

Assay of DNA synthesis in vivo. At intervals during the labeling procedure (see legend to Fig. 8), 1-ml samples in duplicate were taken to determine DNA synthesis kinetics. Samples were at once mixed into 1 ml of ice-cold 0.1 M EDTA (pH 8.2) containing 0.02 M sodium azide and 1 mg of lysozyme per ml. Samples were then lysed by 60 min of incubation at 37 C. NaOH was added to a concentration of 0.2 N, and samples were heated to 80 C for 30 min to hydrolyze ribonucleic acid (RNA). The samples were precipitated with 10% trichloroacetic acid, and radioactivity was counted on membrane (Millipore Corp.) filters in a liquid scintillation counter.

Transformation and transduction. Transformation was carried out by a modification of the method of Anagnostopoulos and Spizizen (1). Recipient cells were grown in medium C+G which is medium C (22) with the addition of 0.05% casein hydrolysate, 50 μ g of L-tryptophan per ml, 100 μ g of L-glutamic acid per ml, and 20 μ g of base requirement per ml until just before the stationary phase. Cells were then quickly mixed with 20% glycerol and frozen in liquid nitrogen until used. When used for transformation, the frozen cells were quickly thawed at 37 C and diluted 1:10 in medium CT. Medium CT is medium C to which we added 0.01% casein hydrolysate, 5 μ g of L-tryptophan per ml, 100 μ g of adenine per ml, and 5 μ g of L-histidine per ml. Cells were grown for 90 min in medium CT to competence, at which time they were at a concentration of about 10⁸/ml. An 0.1-ml (or 0.05 ml) amount of the DNA to be tested by transformation was added either to 1 ml or to 0.5 ml of competent cells and was incubated for 40 min. Transformed cells were diluted in medium CG (22) and were plated on selective media. Transduction with purified phage PBSH was carried out by the same method, except that whole phage was used instead of purified DNA, with 120 min of incubation. This does not change the marker frequency ratios as compared to 40 min of incubation with purified DNA; i.e., there is no evidence for selective transduction as a function of time. Transformation and transduction of all recipient cells were simultaneously carried out with DNA extracted from spores of strain 168TT as a control. DNA was extracted from a purified spore

preparation germinated for 2 hr in medium S (21) in the presence of 200 μ g of chloramphenicol per ml and without thymine. Germinated spores were treated for 20 min with 500 μ g of lysozyme per ml and 2% sodium dodecylsulfate, after which DNA extraction was completed by the method of Marmur (13). All transformations and transductions were repeated at least twice, and platings were in duplicate for each marker. DNA and phage used in transformation and transduction experiments were added at a concentration well below the saturation level.

Recipient cells used were 168 Leu⁻, Met⁻, *ade-6* (MU8U5U6); 168 Leu⁻, Met⁻, *ade-16* (MU8U5U16); 168 Leu⁻, Met⁻, Thr⁻ (MU8U5U5); 168 Leu⁻, Met⁻, His⁻ (MU8U5U2); 168 Leu⁻, Met⁻, Tyr⁻ (MU8U5U12) (26, 27); and 168 Leu⁻, Met⁻, *cys-14*. This last mutant was obtained by transformation of strain 168 LMA-16 (MU8U5U16) with a saturating concentration of DNA from strain C14 (a cysteine-requiring strain obtained from I. Takahashi) and by selecting for *ade-16* prototrophs. Since *cys-14* is linked to *ade-16* (4), some of the *ade-16* prototrophs were also cysteine auxotrophs. One of these cells picked up was used as a recipient and was designated *leu met cys-14*.

Other strains used were 168LTT (MY2Y1U5) and a transformant of this strain to leucine prototrophy, 168TT [MY2Y1(U5)1].

RESULTS

Marker frequency analysis of DNA during induction. Although no strain of *B. subtilis* has been found to produce plaques after PBSH infection, competent cells of various auxotrophic mutants of strain 168 were transformed either by the phage or by DNA extracted from phage (7). A linear relationship between the number of transformed cells and the multiplicity of the phage was observed (Fig. 1). It is not yet clear whether this represents true transducing activity of the phage or transformation by DNA released from the phage in the mixture with competent cells. Figure 2 shows that the number of transformants obtained with increasing time of contact between competent cells and the phage rose slowly, with a maximum between 120 and 200 min of contact. Both transformation and transduction of dominant auxotrophic traits are normally complete after 20 to 30 minutes of contact between the transforming (transducing) agent and the recipient cells (9). The atypical behavior observed in Fig. 2 suggested that PBSH transduction took place as a result of the release of phage DNA into the competent cell mixture, after which transformation took place. This suggestion was supported by the fact that PBSH transduction was deoxyribonuclease-sensitive (*unpublished data*). Therefore, we use the phrase transducing activity to refer to the transforming activity of the whole phage.

The transforming activity of any marker *x* relative to the activity of the *met* marker repre-

FIG. 1. Linearity of transduction with increasing concentrations of phage PBSH. A stock suspension of PBSH in AAM (1% ammonium acetate with 0.01 M $MgCl_2$) was diluted in AAM, and 0.1 ml of each dilution was used to transduce 1 ml of LMA-16 (MU8U5U16) recipient cells grown to competence. One of the samples, multiplicity of infection (MOI) = 200, was also plated at intervals on nonselective minimal plates at a dilution of 10^8 to determine killing of the recipient cell population by phage PBSH during transduction. No significant loss of cell viability in the recipient cell population was detected after 40 min of incubation with phage at an MOI of 200.

FIG. 2. Time course of transduction with PBSH. Recipient cell MU8U5U16 was transformed with whole-phage PBSH at a concentration in the transformation mixture of optical density (at 260 nm) = 0.030, corresponding to an MOI of 200. At the indicated times, the transformation mixture was diluted with medium CG and plated. The experimental conditions for obtaining competent cells are described in Materials and Methods. The decrease in the number of *ade-16* transformants beyond 200 min of incubation can be explained by the experiments of Kammen et al. (10), who showed that transformants may expel their DNA after long incubation times. It is not clear why the same decrease is not observed for the *met* transformants.

sents the frequency of marker *x* to *met* in a given DNA population when these values are normalized versus spore DNA (20). By this method, one can determine the relative frequency of chromosomal markers in PBSH DNA. All markers along the bacterial chromosome were transduced by the phage (Fig. 3). The remarkable finding was that, of all markers examined, only the *ade-16* marker, which is located near the chromosomal origin, was transformed 30- to several hundred-fold more frequently than the *met* marker. This indicated that a PBSH stock contained 30 to several hundred times more *ade-16* transducing particles than *met*-transducing particles. Since the DNA extracted from a bacterial culture just before induction transformed these markers at a normalized ratio of 4.7, the change in genetic composition of bacterial DNA in transducing phage PBSH took place during induction and outgrowth of the phage. Therefore, we decided to follow the change in marker frequency in induced cells during phage growth by extracting DNA after MC addition and assaying for transforming activity on a series of recipient cells. Figure 4 shows the growth curve obtained in this experiment and the times at which 25-ml samples were withdrawn and their DNA isolated.

In Fig. 5, the nutritional markers used in the frequency analysis of in vivo MC-treated DNA and their positions on the chromosome are given. The positions of these markers on the chromo-

FIG. 3. Normalized transduced marker frequencies of PBSH. The ratios of marker *x* to *met* are plotted as a function of marker position *X* along the *B. subtilis* chromosome (see Fig. 4). Symbols: ●, observed marker frequency in phage; ○, marker frequency $g_n(X)$ of cellular DNA in the bacterial culture at the time of induction. This is the expected marker frequency if preinduction DNA is randomly packaged into phage heads. The reason for the low value for *ade-6*, a marker not genetically linked to any other known marker (4), is unknown.

some are average values of several marker-frequency analyses (H. Yoshikawa and N. Sueoka, unpublished data; 16). The normalized ratio of *ade-16* to *met* was 4.7 for the DNA preparation from cells just before MC treatment (sample 0⁻). This indicated that each replicating chromosome had more than one replication point under the growth conditions used, assuming that

FIG. 4. Growth curve of strain 168TT in Penassay Broth. Samples (25 ml) were withdrawn at the indicated times and immediately filtered to remove MC. Cells were resuspended in buffer and DNA was extracted. Under these conditions, where MC (4 μ g/ml) was not removed after 15 min of contact, no phage were produced, though the culture lysed without delay, as compared to cultures which are resuspended after 15 min of MC action.

reinitiation had occurred bilaterally on both replicating branches. The value for n , the average number of replication positions per chromosome, was calculated from the formula $g_n(X) = 2^{n(1-X)}$ (reference 20) as 2.23. Using this value, the expected frequencies $g_n(X)$ of the other markers were then calculated.

Table 1 shows the change in the relative marker frequency of *ade-16*, *leu*, and *met* during MC induction. The complete data for the other six recipients used are not given, but the normalized marker frequency ratios, expressed as transformants of marker x /transformants for methionine, are given in Table 2. Only the genetic ratio for the *ade-16* marker increased with duration of MC action, up to a value of 70 or more in phage PBSH. The other marker frequencies were equal, within experimental error, to their frequencies in the preinduced culture.

The increase in the ratio of *ade-16* to *met* could be due to specific loss of methionine (terminus)-transforming activity as a function of duration of MC action. It is clear from Table 2 that this is not the case. If methionine-transforming activity were lost during MC action, then all of the other marker frequencies should also be observed to increase as a function of time of MC contact. This was verified by plating for all three transformants from each recipient cell: marker x , *leu*, and *met*. In so doing, we could also determine the ratios of marker x to *leu*. These data showed that only the *ade-16* to *leu* marker ratio increased during MC action, whereas all other marker ratios remained constant. The data given in Table 2 are summarized in Fig. 6. It is clear that only the *ade-16* marker ratio increased after MC treatment.

The genetic data presented above show that,

Recipient markers used in marker-frequency analysis of in vivo mitomycin-C treated DNA

	cys-14							
	ade-16	ade-6	thr	his	leu		tyr met	
Origin								Terminus
X	0	0.07 0.04	0.27	0.46	0.62		0.95	1.0
$g_n(X)$	4.7	4.2 4.5	3.20	2.30	1.79		1.06	1.0

X Position of marker on the bacterial chromosome

$g_n(X)$ Marker frequency under growth conditions giving an average of n replication positions per chromosome. $g_n(X) = 2^{n(1-X)}$; here $n = 2.23$

FIG. 5. Recipient markers used in marker frequency analysis of in vivo MC-treated DNA. The X positions of the marker on the bacterial chromosome are average estimates by marker-frequency analysis (16). The value $g_n(X)$ is the marker frequency under growth conditions giving an average of n replication positions per chromosome $g_n(X) = 2^{n(1-X)}$, (21). Here $n = 2.23$; this value was calculated from the normalized ratio of *ade-16*/*met* = 4.7, obtained in a Penassay Broth culture just before MC addition.

TABLE 1. Transforming activity of cellular DNA isolated after MC treatment^a

DNA	<i>ade-16</i>	<i>ade-16/leu</i> ^b	<i>leu</i>	<i>leu/met</i> ^b	<i>met</i>	<i>ade-16/met</i> ^b
0 ⁻	560 ^c	5.3 (2.7)	105	1.96 (1.8)	54	10.4 (4.7)
0 ⁺	552	7.4 (3.7)	75	1.39 (1.28)	54	10.4 (4.7)
10 min	372	7.6 (3.8)	49	1.32 (1.21)	37	10.0 (4.5)
20 min	292	12.6 (6.3)	23	.89 (.82)	26	12.3 (5.5)
30 min	556	13.6 (6.8)	41	1.0 (.92)	40	13.9 (6.3)
40 min	730	15.2 (7.6)	48	1.4 (1.28)	34	21.4 (9.7)
50 min	512	19.7 (9.8)	26	1.0 (0.92)	26	19.7 (8.9)
60 min	801	27.6 (13.8)	29	1.26 (1.15)	23	34.8 (15.7)
φ	655	31.8 (15.9)	20	1.81 (1.67)	11	60 (27)
Spore	619	2.0	305	1.09	280	2.21

^a Isolation and purification of the DNA samples used in this experiment are described in Materials and Methods. Recipient cell used was MU8U5U16.

^b Variations in efficiency for various markers of each recipient cell were normalized (values in parentheses) by use of spore DNA and by dividing the obtained marker frequency ratios by the ratio found for spore DNA. Spore DNA contains all markers at equal frequency (22).

^c Number of transformants counted, average of two experiments (four plates).

TABLE 2. Normalized marker ratios of *in vivo* MC-treated DNA^a

Marker	<i>X</i> ^b	<i>g_n(X)</i> ^c	Duration of MC contact (min)								PBSH
			0 ⁻	0 ⁺	10	20	30	40	50	60	
<i>ade-16</i> . . .	0	4.7	4.7	4.7	4.5	5.5	6.3	9.7	8.9	15.7	17-70
<i>cys-14</i> . . .	0.04	4.4									3.0
<i>ade-6</i> . . .	0.07	4.2	2.2	.65	.75	.65	1.1	1.6	1.35	1.6	1.75
<i>thr</i> . . .	0.27	3.2	2.9	2.0	1.9	1.9	2.0	2.3	2.8	3.0	2.9
<i>his</i> . . .	0.46	2.3	1.93	1.0	1.2	0.9	1.3	1.4	1.5	1.65	2.3
<i>leu</i> . . .	0.62	1.8	1.8	1.24	1.38	1.05	1.42	1.7	1.7	1.55	1.7
<i>tyr</i> . . .	0.95	1.06	1.04	1.09	.88	.88	1.13	1.26	1.2	1.37	1.44
<i>met</i> . . .	1.00	1.00									

^a All ratios refer to the methionine marker (marker *x/met*).

^b Position of marker on the bacterial chromosome.

^c Expected marker frequency of untreated bacterial DNA under conditions giving *n* replication points per chromosome; here *n* = 2.23. All markers have been normalized for recipient-cell variations with *B. subtilis* spore DNA as standard.

after MC induction of phage PBSH, there is an increase in the frequency of one marker in particular (*ade-16*) but no increase in the frequency of a marker situated only 0.04 map unit further along the chromosome. The value 0.04 was estimated from linkage data of Dubnau et al. (4); 0.04 map unit corresponds to about 60×10^6 daltons of DNA (24). The increase is relative to all other markers, as the specific activity of the *ade-16* marker did not increase. The specific activity of all markers decreased as a result of MC treatment. A decrease in transforming activity of DNA after treatment *in vivo* with MC has been shown (12). Therefore, the relative increase of *ade-16* frequency may be due to differential inactivation of the markers. However, this possibility seems to be ruled out by the finding that the *ade-16* region of the chromosome is preferentially replicated

after phage induction (see below). Therefore, it seems that at least two mechanisms are active simultaneously: a general decrease in DNA specific activity during MC action, and separate synthesis of DNA in the origin region of the bacterial chromosome.

Incorporation of bacterial DNA into PBSH. To determine the source of transducing DNA present in PBSH particles and to follow any phage DNA synthesis, a double-label experiment was performed. Figure 7 shows the growth curve in this experiment and the different labeling periods; the results are given in Table 3. From ³H- and ³²P-specific activities of the purified PBSH DNA and preinduction DNA, we calculated that (i) 14% of the PBSH DNA was of bacterial preinduction origin; (ii) a total of 40% of the preexisting bacterial DNA found its way into phage particles;

FIG. 6. Normalized marker frequencies of *in vivo* MC-treated bacterial DNA. In each case, recipient cells were plated for selection of transformants for three unlinked markers, marker *x*, *leu*, and *met*. The changes of marker *x* frequency versus *met* or versus *leu* are plotted as a function of the duration of MC action. Symbols (upper figure): ○, *ade-16/met*; ◇, *thr/met*; ●, other markers/*met*. Lower figure: ○, *ade-16/leu*; ●, other markers/*leu*.

(iii) after MC induction, total DNA in the culture increased some threefold; (iv) the purified phage tails (density 1.295 g/ml) contained 1% of either label. This is the level of contamination. This experiment gave a crude but essential picture of DNA synthesis after induction.

A considerable part of the preexisting (pre-induction) bacterial DNA was taken up into phage particles. Since DNA extracted from phage particles was comparatively homogeneous in molecular weight (7), it is inferred that, as a result of MC induction, a mechanism was activated which cut the DNA available in the cell into pieces of equal molecular weight which were packed into phage heads. Induction with MC in the absence of thymine resulted in production of phage particles, but most of the DNA in the phage particles was bacterial DNA of preinduction origin. This is described below.

Mode of DNA replication after MC treatment in synthetic medium. Figure 8A shows the growth curves of cultures grown in synthetic medium C+GB, induced with MC, and resuspended in the absence of thymidine (TdR), with 5-bromo-deoxyuridine (5-BuDR), and in the presence of

FIG. 7. Growth curve of double labeling experiment. Before induction, cells (strain MY2Y1U5) were pre-labeled with (per ml) 1 μ Ci (2 μ g) of ^3H -TdR in 200 ml of low-phosphate Penassay Broth (Difco). At a Klett reading of 18, the cells were centrifuged quickly at 37 C and were resuspended in an equal volume of prewarmed medium without TdR. At this point, 50 ml of culture was withdrawn and was poured over ice-cold 0.1 M EDTA, pH 8.2, containing 0.02 M sodium azide. To the remaining 150-ml culture, 4 μ g of MC per ml was added and then 2 mCi of ^{32}P was added. After 15 min of incubation, the cells were centrifuged at 37 C and resuspended in 150 ml of prewarmed medium containing 20 μ g of TdR per ml and 2 mCi of ^{32}P without MC until lysis. The lysate was treated as previously described (7), and the CsCl density gradient was completely fractionated, instead of only recovering the phage and tail bands. The ^{32}P control sample was induced similarly, only ^{32}P label was used throughout the growth period.

TdR. There was a 20-min delay in growth of the thymineless and 5-BuDR cultures as compared to the TdR sample, and a 20-min delay in their lysis. All three cultures lysed at the same rate once lysis started (Fig. 8A). Incorporation of ^{32}P into DNA (Fig. 8B) in the absence of TdR was expected to be small or zero, but it was found to be up to 25% of the ^{32}P incorporation in the presence of TdR. However, before cell lysis there was no significant breakdown of DNA to an acid-soluble level in the presence of TdR or 5-BuDR. During outgrowth with 5-BuDR, DNA synthesis, as measured by ^{32}P uptake, was only 40% of that in the TdR sample and 60% more than during thymineless outgrowth. It is clear, therefore, that in medium C+GB with 5-BuDR there was only limited DNA replication.

Figure 9 shows the CsCl density gradients of phage PBSH grown after thymineless, 5-BuDR, and TdR induction. The activities of the ^3H pre-label and ^{32}P postlabel in DNA extracted from

TABLE 3. Results from three CsCl gradients of double-label experiments^a

Determination	DNA (counts/ min)	Total counts/min in phage		DNA total yield (μ g)	Specific activity (counts per min per μ g)		Total counts/min in tails	
		³ H ^b	³² P		³ H	³² P	³ H	³² P
³² P-labeled control phage (150 ml)....			8,080,000	300		27,000		147,000
³ H- ³² P-labeled phage (150 ml).....		$(90.5 \pm 4.5) \times 10^3$	2,590,000	320	284 $\pm$ 14	8,000	1,000	50,000
³ H-DNA control (150 ml).....	228,900			114	2,000			

^a The experimental procedures are given in Fig. 7.

^b Tritium counts were performed twice, immediately after finishing the experiment and when 98% of the ³²P activity had decayed. The values given are the averages of the two measurements.

purified phages are given in Table 4, together with the activities in DNA from the whole lysates. The ratios of ³H to ³²P in the various types of phage DNA were 4.4 to 10 times greater than those of the various types of DNA in the whole lysates. This indicated that a large part of newly synthesized ³²P-DNA was not incorporated into phage DNA. Since approximately 40% of ³H-DNA was taken up into phage, only 4 to 9% of the ³²P-DNA synthesized was packaged into phage particles. This result suggested that newly synthesized ³²P-DNA might be preferentially expelled from the cells during the induction period. Similar excretion of DNA has been reported in *B. subtilis* (5, 25).

It was shown above that only the frequency of the *ade-16* marker increased after MC treatment. To show whether this increased marker frequency was caused by inactivation of all markers except *ade-16* or by replication of the *ade-16* region of the chromosome, 5-BUdR phage and 5-BUdR phage DNA synthesized during induction were analyzed.

Figure 9B shows a density shift between the 5-BUdR-grown phages transducing *ade-16* and those transducing *met*. It follows that, in spite of the poor phage production in MC-induced cells grown in the presence of 5-BUdR, the region containing *ade-16* transducing DNA was replicated preferentially to the region containing methionine transducing activity. Figure 10 shows the CsCl density (1.35 g/ml) gradient of 5-BUdR phage prepared in a large batch. A shift between the phages containing ³H-prelabeled DNA and those containing ³²P-labeled postinduction DNA was observed. DNA was extracted from the 5-BUdR phages. Figure 11 shows a CsCl density gradient of DNA extracted from the 5BUdR phages. The increased frequency of the *ade-16* marker in the

heavy-density peak (HH) and its low frequency in the light (LL) nonreplicated peak indicated that replication occurred preferentially at the origin region of the bacterial chromosome. This is consistent with the data of the marker-frequency analysis and strongly suggests that the *ade-16* region of the chromosome replicated many times during MC treatment in the presence of TdR.

The large amount of DNA synthesis after induction in the presence of TdR and the formation of discrete light, light-heavy, and heavy DNA bands of PBSh DNA, when grown in 5-BUdR after induction, showed conclusively that PBSh induction involves ordered DNA replication rather than a major repair-type replication at the lesions caused by MC binding (Table 5).

Fate of intracellular DNA after MC induction: molecular weight change in the DNA pool. Two possible mechanisms were considered to account for the preferential replication of the *ade-16* marker during MC induction. One is that the prophage detaches from the bacterial genome together with the *ade-16* region and is then replicated autonomously, resulting in a net increase of the *ade-16* marker in the DNA pool (15, 17). This would also lead to the appearance of heavy *ade-16*-containing phage DNA when induction takes place with 5-BUdR. A second possible mechanism is that abnormal chromosomal DNA replication takes place only at the origin region. This would also result in a preferential increase of the *ade-16* marker and in a density shift of *ade-16* phage and phage DNA.

The following experiment was designed to distinguish between these two possibilities. DNA was extracted from a whole culture during its induction with MC. The DNA samples were fractionated on a neutral sucrose gradient to determine the molecular weight of the DNA pool

FIG. 8. Growth curves (A) and DNA synthesis (B) in MC-induced cultures in medium C+GB. Intracellular synthesis of DNA after induction with MC was followed in three flasks each containing 200 ml of synthetic medium C+GB (7) in the presence of TdR (+T), without TdR (-T), and with 5-BUdR (+BU) replacing TdR. Cells were prelabeled with (per ml) 2 μC (3 μg) of ^3H -TdR; in early exponential phase (Klett reading of 18), they were resuspended in an equal volume of prewarmed medium C+GB without isotope or TdR in order to reduce intracellular TdR pools, and were induced with 4 μg of MC per ml. After 15 min of incubation with MC, the cells were resuspended in three flasks each containing 200 ml of medium with 2 mc of $^{32}\text{PO}_4^{2-}$ postlabel, one flask without TdR (-T), one flask with 2 μg of 5-BUdR per ml, and one flask with 2 μg of TdR per ml. Lysis occurred in all three cultures, and lysates were treated as usual (7). Samples (1 ml) were taken during induction to determine DNA synthesis.

FIG. 9. CsCl density gradient of phage harvested from induction experiments in synthetic medium C+GB in the absence of thymine (-T), in the presence of 5-BUdR (+BU), and in the presence of thymine (+T) as control. On the left (A), the incorporation of the ^3H prelabel and the incorporation of ^{32}P postlabel into the phage bands are seen. On the right (B), the transduction frequencies of the same gradients are seen for *ade-16* (origin marker) and *met* (terminus marker). Symbols: ○, optical density at 260 nm; ×, ^3H prelabel; □, ^{32}P postlabel; ●, *ade-16* transducing activity; △, *met* transducing activity.

during phage development. If the increase in *ade-16* activity is due to phage detachment and replication, the activity of this marker will appear with a molecular weight similar to that of PBSH DNA, assuming that the replicating phage genome has a molecular weight equal to that of the DNA found in whole-phage particles. If, on the other hand, the *ade-16* activity is due to multiple reinitiation of the bacterial chromosome, this activity will appear in a DNA fraction having the sedimentation properties of bacterial DNA. The data confirm the latter mechanism.

Seven samples of DNA obtained during MC induction were fractionated on a 5 to 20% neutral sucrose gradient (Fig. 12). The first sample at 35 min was taken immediately after resuspension and ^{32}P postlabel addition. ^{32}P incorporation increased as a function of time, whereas the total ^3H prelabel in the samples remained constant (see Table 6). The DNA samples were isolated with an average sedimentation value of 40S,

TABLE 4. Incorporation of ^3H and ^{32}P into purified phage and into DNA in the induced lysate^a

Determination	Label or marker	Without TdR		With 5BUdR		With TdR	
		Counts/min	$^3\text{H}/^{32}\text{P}$	Counts/min	$^3\text{H}/^{32}\text{P}$	Counts/min	$^3\text{H}/^{32}\text{P}$
Counts/min in the purified phage peak	^3H	197,000	6.35	183,000	2.15	122,000	.56
	^{32}P	31,000		85,000		217,000	
Counts/min in DNA in the induced lysate (1-ml samples)	^3H	16,500	.64	19,500	.49	12,000	.12
	^{32}P	26,000		40,000		100,000	
Transduction per 0.1-ml phage sample from CsCl gradient	<i>ade-16</i>	67,000		112,000		268,000	
	<i>met</i>	4,700		8,100		10,000	
Transduction ratio ^b	<i>ade-16/met</i>	8		8		15	

^a The experimental procedures are given Fig. 8.

^b Normalized with spore DNA.

FIG. 10. CsCl density gradient of phage PBSH induced in a 1-liter batch. 5-BUdR and ^{32}P were added after MC induction of a culture in medium C+GB. ^3H -TdR was used as prelabel. Phage were purified in the CsCl density gradient ($\rho = 1.35$) shown. The minor peak seen in fractions 30 to 38 represents phage with contracted tails, with increased density. Symbols: $\bigcirc$, optical density at 260 nm; $\square$, ^3H prelabel; $\times$, ^{32}P postlabel.

corresponding to a molecular weight of some 23×10^6 . Both the ^3H prelabel and the ^{32}P postlabel appeared essentially with equal molecular weight in a single peak in all samples through the 65-min sample. This showed that the molecular weight of postinduction DNA was at least as high as that of the preinduction cellular DNA. This ruled out the "detachment and replication" theory, since no trace of 25S DNA (12×10^6 daltons) was seen prior to 65 min after induction. Even if detached prophages were replicated by forming long concatenated molecules (6), one would ex-

pect a difference in molecular weight development between the concatenate molecules and cellular DNA as a function of time, since breakage of DNA during isolation is a function of the molecular weight of the nonbroken molecules (2).

Packaging of the DNA pool started 65 min after the addition of MC. There was a shift in the molecular weight of all of the available DNA in the pool towards a sedimentation value of 25S. This was also the sedimentation value of DNA extracted from purified phage particles (95-min sample). During the packaging step, no change could be detected in the behavior of the newly replicated and the prelabeled DNA in the pool. During the induction and outgrowth period, the *ade-16* activity remained associated with the peak of bacterial DNA. Part of this activity was packaged into phage particles and was present in the phage particles as transforming DNA. No evidence for replication of phage DNA was found; rather, bacterial DNA was packaged into phage heads. These results supported the premise that postinduction DNA replication took place preferentially at the origin of the bacterial chromosome.

DISCUSSION

The data presented demonstrated that treatment of strain 168LTT with MC caused abnormal chromosomal DNA replication and induction of phage-specific proteins which led to phage production and lysis of the host. The observed increase in the *ade-16* marker frequency during MC treatment is not by itself sufficient evidence for multiple reinitiation of the bacterial chromosome at the origin region. This is because the specific transforming activity of phage DNA was

FIG. 11. *CsCl* density gradient ($\rho = 1.70$) of DNA extracted from phage grown in the presence of 5-BUdR in synthetic medium C+GB. (A) Distribution of ^3H prelabel and ^{32}P postlabel into heavy (HH), fully 5-BUdR-substituted DNA, light-heavy (LH), hybrid 5-BUdR-substituted DNA, and light (LL) DNA. (B) Biological activity as determined by transformation. The normalized ratios of transforming activity are as follows. Light peak: $\text{ade-16}/\text{met} = 4.8$; $\text{leu}/\text{met} = 1.8$. Intermediate peak: $\text{ade-16}/\text{met} = 20$; $\text{leu}/\text{met} = 2.7$. Heavy peak: $\text{ade-16}/\text{met} = 35$. Symbols: $\circ$, optical density at 260 nm; $\square$, ^3H prelabel; $\times$, ^{32}P postlabel; $\bullet$, ade-16 transforming activity; $\triangle$, met transforming activity.

lower than that found for bacterial DNA extracted from noninduced cells. The specific transforming activity was lower for all markers, including *ade-16*. This decrease may be due in part to a decrease in molecular weight of the transforming DNA and in part to binding of MC to DNA (12).

The experiment with 5-BUdR (Fig. 11) showed that most of the phage DNA was replicated at least once [intermediate density (LH)] or more than twice (HH). Only the *ade-16* marker was preferentially replicated more than twice. The specific transforming activity of the heavy DNA for *ade-16* was not significantly higher than that of the intermediate density or the light DNA. DNA labeled with 5-BUdR is generally found to possess low specific transforming activity, which is attributed to its high frequency of single-strand

TABLE 5. Summary of Fig. 11 B^a

Determination	HH ^b	LH ^b	LL ^b
^{32}P counts/min	1,586	1,630	
<i>ade-16</i> Transformants	26,000	75,000	15,000
<i>ade-16</i> Transformants per ^{32}P counts per min	32	46	
<i>met</i> Transformants	425	2,000	1,750

^a The total number of transformants and the ^{32}P activity in each of the three peaks were added, and the specific transforming activity (transformants per counts per min) was determined. The reason for the low transforming activity of heavy (HH) DNA is discussed in the text.

^b Abbreviations: LH, light-heavy DNA; LL, light DNA; HH, heavy DNA.

interruptions (25; L. C. Kanner and P. C. Hanawalt, *Federation Proc.*, p. 872, 1967). The relatively low specific transforming activity of the fully substituted (HH) 5-BUdR DNA, however, does not affect the conclusion of the preferential replication of the *ade-16* marker, unless all other markers in this (HH) DNA were preferentially inactivated by an unknown mechanism. This is unlikely, because most of the transforming activity of the other markers was found to be unreplicated or replicated only once after MC treatment. From these observations, we concluded that the increase in the *ade-16* marker frequency during MC treatment was not due to differential marker inactivation but to preferential replication of the *ade-16* marker.

Autonomous replication of phage DNA was not detected during the induction period. We cannot rule out the rather remote possibility that a phage genome was induced with a molecular weight different from the DNA found in the phage particles, and following its replication this phage DNA was cut to fit into the synthesized PBSH heads. Such a possibility seems unlikely, as elaborate trials at curing the lysogen were unsuccessful (8; unpublished data). It is also possible that only a small portion of the phage particles (less than 10%) contain true phage DNA which had replicated autonomously. This cannot be ruled out, since the experimental methods used would not have detected it (Fig. 12). However, if this were the case, one would expect to find some nonlysogenic strains of *B. subtilis* on which the induced phages could grow plaques. Extensive trials at finding such a host, with some 40 unrelated strains obtained from various investigators, were all negative. In addition, the experiment in Fig. 12 clearly ruled out the possibility that the increase in *ade-16* marker frequency

FIG. 12. Sucrose gradient fractionation of purified DNA extracted during MC induction of strain MY2YIU5. Strain 168LTT in low-phosphate Penassay Broth with (per ml) $1 \mu\text{C}$ ($2 \mu\text{g}$) of ^3H -TdR was grown until approximately 10^7 cells/ml, resuspended in an equal volume of medium without TdR, and induced. After 15 min of incubation with MC, cells were resuspended in medium containing $20 \mu\text{g}$ of TdR per ml and $10 \mu\text{C}$ of ^{32}P - PO_4^{3-} per ml. Immediately, a 50-ml sample was withdrawn and poured into 10 ml of ice-cold 0.6 M EDTA-NaCl, pH 8.2, containing 0.1 M sodium azide. Six more samples were withdrawn at 10-min intervals. At the time the seventh sample was withdrawn, the culture had lysed and it therefore contained mature phage. All samples were centrifuged for 60 min at $80,000 \times g$ and were resuspended in 2 ml of 0.1 M EDTA-NaCl, pH 8.2, with 1 mg of lysozyme per ml. After 30 min of incubation at 37°C , 1 mg of solid Pronase per ml was added, and the samples were incubated at 37°C overnight. Next, DNA was extracted from the samples by the pH 9-phenol method of Miura (14). By this method, DNA is extracted simultaneously from phage and from the cellular fraction. The resulting doubly labeled seven DNA samples were precipitated in ethyl alcohol and were dissolved in 0.9 ml of $0.1 \times \text{SSC}$ (0.15 M NaCl plus 0.015 M sodium citrate); 0.3 ml of each sample was subsequently put on top of 5 to 20% sucrose gradients in SSC and run for 4.5 hr at $37,000 \text{ rev/min}$ and 10°C . Gradients were fractionated and diluted with 0.3 ml of sterile SSC. An 0.1-ml amount of each sample was precipitated, and ^3H and ^{32}P activity was counted. An 0.05-ml amount of each sample was used for transformation on recipient strain MU8U5U16. Symbols: $\circ$, ^3H prelabel; $\bullet$, ^{32}P postlabel; dashed line, ade-16 activity. The last sample contained DNA extracted from phage purified from the 95-min sample.

TABLE 6. Incorporation of ^{32}P postlabel and stability of ^3H prelabel in purified DNA samples from an induced culture^a

Determination	Samples at various times (min)						
	35	45	55	65	75	85	95
^3H prelabel	2,860	3,090	3,560	3,170	3,370	3,230	1,660
^{32}P post-label	3,700	6,900	18,720	36,070	52,240	56,270	30,210
$^{32}\text{P}/^3\text{H}$	1.3	2.2	5.2	11.3	15.5	17.3	18.2

^a The 95-min sample contained only phage, and data are derived from this sample after phage purification in a CsCl density gradient.

might be due to autonomous replication of a phage genome that contains the *ade-16* marker within it. We conclude, therefore, that PBSH is defective in DNA replication.

In the absence, thus far, of an isogenic, cured strain, we have not studied the induction process itself. Some *B. subtilis* strains were found to be noninducible with MC but produced some phage particles upon ultraviolet induction; inducibility of the phage is therefore not a good criterion for curing. On the basis of the evidence presented above and based on the general features of some well-studied lysogenic systems, phage PBSH may be induced and phage particles may be produced by one of the following mechanisms. (i) The prophage genes map at any point along the bacterial chromosome other than the origin region. Replication of phage genes and their expression are induced simultaneously with multiforked replication of the bacterial chromosome at the origin. These two replication events may be linked or independent, although after MC action they occur simultaneously. In this mechanism, an undetectable amount of autonomous phage DNA replication may take place. Most of the DNA in phage particles is bacterial of either pre- or postinduction origin, which has been fragmented by a phage-directed enzyme and packaged into phage heads. (ii) The prophage genes map at a point near the chromosomal origin. Multiple reinitiation by MC treatment may be unrelated to the existence of the prophage, although we suggest the possibility that induction of prophage gene expression may result entirely from the multiple reinitiation of the bacterial chromosome. If the prophage genes are located near the origin, extensive replication of this region may result in an abnormal increase of these phage genes in the cell, which may in turn induce expression of "late" phage genes without autonomous phage-gene replication. Expression of late genes of coliphage λ and T4 is dependent on phage-specific DNA replication (11, 18). After induction of PBSH late genes, phage development takes place while bacterial DNA is now fragmented and packaged into phage heads.

We cannot decide at the moment which of the two models is right. However, we have obtained evidence that the *ade-16* marker in PBSH DNA has different physicochemical properties from the *ade-16* marker in bacterial DNA (*in preparation*). This suggests that the prophage genes are indeed located at the origin of the bacterial chromosome near the *ade-16* marker. The physicochemical and biochemical structure of the initiation region of the bacterial chromosome is currently being studied in this laboratory.

ACKNOWLEDGMENTS

The authors are grateful to Estelle Cook, Joyce Cerwin, and Barbara Jansen for their technical assistance and to Thomas H. Jukes for his interest and support of this investigation.

This investigation was supported by grant NGR-05-003-020 from the National Aeronautics and Space Administration and by grant E-517 from The American Cancer Society to the University of California, Berkeley.

LITERATURE CITED

1. Anagnostopoulos, C., and J. Spizizen. 1961. Requirements for transformation in *Bacillus subtilis*. *J. Bacteriol.* 81:741-746.
2. Burgy, E., and A. D. Hershey. 1963. Sedimentation rate as a measure of molecular weight of DNA. *Biophys. J.* 3:309-321.
3. Campbell, A. M. 1968. Techniques for studying defective bacteriophages, p. 279-320. In K. Maramorosch and H. Koprowski (ed.), *Methods in virology*, vol. 4. Academic Press Inc., New York.
4. Dubnau, D., C. Goldthwaite, I. Smith, and J. Marmur. 1967. Genetic mapping in *Bacillus subtilis*. *J. Mol. Biol.* 27:163-185.
5. Ephrati-Elizur, E. 1968. Spontaneous transformation in *Bacillus subtilis*. *Genet. Res.* 11:83-96.
6. Frankel, F. R. 1968. Evidence for long DNA strands in the replicating pool after T4 infection. *Proc. Natl. Acad. Sci. U.S.A.* 59:131-138.
7. Haas, M., and H. Yoshikawa. 1968. Defective bacteriophage PBSH in *Bacillus subtilis*. I. Induction, purification, and physical properties of the bacteriophage and its deoxyribonucleic acid. *J. Virol.* 3:233-247.
8. Ionesco, H., A. Ryter, and P. Schaeffer. 1964. Sur un bacteriophage hébergé par la souche Marburg de *Bacillus subtilis*. *Ann. Inst. Pasteur* 107:764-776.
9. Iyer, V. N. 1966. Mutations determining mitomycin resistance in *Bacillus subtilis*. *J. Bacteriol.* 92:1663-1669.
10. Kammen, H. O., R. J. Wojnar, and E. S. Canellakis. 1966. Transformation in *B. subtilis*. II. The development and maintenance of the competent state. *Biochim. Biophys. Acta* 123:56-65.
11. Levinthal, C., J. Hosoda, and D. Shub. 1967. Control of protein synthesis after phage infection. p. 71-87. In J. S. Colter and W. Paranchych (ed.), *Mol. Biol. Viruses*. Academic Press Inc., New York.
12. Mahler, I. 1966. Effects of mitomycin C on five excision-repair mutants of *B. subtilis*. *Biochem. Biophys. Res. Commun.* 25:73-79.
13. Marmur, J. 1961. A procedure for the isolation of DNA from microorganisms. *J. Mol. Biol.* 3:208-218.
14. Miura, K. 1967. Preparation of bacterial DNA by the phenol-pH 9-RNases method, p. 543-545. In A. L. Grossman and K. Moldave (ed.), *Methods in enzymology*, vol. 12, part A. Academic Press Inc., New York.
15. Morse, M. L., E. M. Lederberg, and J. Lederberg. 1956. Transduction in *Escherichia coli* K-12. *Genetics* 14:142-156.
16. O'Sullivan, A., and N. Sueoka. 1967. Sequential replication of the *B. subtilis* chromosome. IV. Genetic mapping by density transfer experiment. *J. Mol. Biol.* 27: 349-366.
17. Ptashne, M. 1965. The detachment and maturation of conserved lambda prophage DNA. *J. Mol. Biol.* 11:90-96.
18. Skalka, A., B. Butler, and H. Echols. 1967. Genetic control of transcription during development of phage lambda. *Proc. Natl. Acad. Sci. U.S.A.* 58:576-583.
19. Subbiah, T. V., C. D. Goldthwaite, and J. Marmur. 1965. Nature of bacteriophages induced in *Bacillus subtilis*, p. 435-436. In V. Bryson and H. J. Vogel (ed.), *Evolving genes and proteins*. Academic Press Inc., New York.
20. Sueoka, N., and H. Yoshikawa. 1965. The chromosome of *Bacillus subtilis*. I. Theory of marker frequency analysis. *Genetics* 52:747-757.

21. Yoshikawa, H. 1965. DNA synthesis during germination of *Bacillus subtilis* spores. *Proc. Natl. Acad. Sci. U.S.* **53**: 1476-1483.
22. Yoshikawa, H. 1966. Mutations resulting from the transformation of *Bacillus subtilis*. *Genetics* **54**:1201-1214.
23. Yoshikawa, H. 1967. The initiation of DNA replication in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. U.S.* **58**:312-319.
24. Yoshikawa, H. 1968. Chromosomes in *Bacillus subtilis* spores and their segregation during germination. *J. Bacteriol.* **95**:2282-2292.
25. Yoshikawa, H., and M. Haas. 1968. On the regulation of the initiation of DNA replication in bacteria. *Cold Spring Harbor Symp. Quant. Biol.* **33**: *in press*.
26. Yoshikawa, H., and N. Sueoka. 1963. Sequential synthesis of the *Bacillus subtilis* chromosome. I. Comparison of marker frequencies in exponential and stationary growth phases. *Proc. Natl. Acad. Sci. U.S.* **49**:559-566.
27. Yoshikawa, H., and N. Sueoka. 1963. Sequential synthesis of the *Bacillus subtilis* chromosome. II. Isotopic transfer experiments. *Proc. Natl. Acad. Sci. U.S.* **49**:806-813.