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BIOCHEMICAL AND GENETIC ANALYSIS OF A DEFECTIVE *BACILLUS SUBTILIS* PHAGE, PBSX

by

PHYLLIS THURM

A dissertation submitted to the Graduate Faculty in Biomedical Science in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1975

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Science in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

1/30/78 — date — Chairman of Examining Committee

1/30/78 — date — Executive Officer

Supervisory Committee

The City University of New York

Abstract

BIOCHEMICAL AND GENETIC ANALYSIS OF A DEFECTIVE *BACILLUS SUBTILIS* PHAGE, PBSX

The defective phage PBSX produced by *Bacillus subtilis* 168 is non-infectious for any known host. A possible source of its defective nature had been the suggested scattering of its genes at three distant chromosomal locations, near the *pur*A, *pur*B and *met*C markers. To investigate this possibility, genetic and biochemical studies were performed on both newly and previously isolated *B. subtilis* mutants having lesions in PBSX genes.

Of the three newly isolated *B. subtilis* mutants with mutations in PBSX genes, one was a PBSX regulatory mutant and the others capsid mutants. The capsid mutants synthesized all of the seven proteins identified as the PBSX structural proteins as well as an eighth protein coinduced with PBSX structural proteins but absent in the mature phage. The PBSX specific proteins of the mutants, however, exhibited abnormal serological reactivity with anti-PBSX antiserum. In the mutant in which tails are not assembled (*xtl*-3), two proteins previously identified as tail proteins were not immunoprecipitable and a possible tail protein precursor remained uncleaved. In the mutant which failed to assemble heads (*xhd*) both of the proteins previously identified as the PBSX head proteins were missing from immunoprecipitates.

In the regulatory mutant (xin), PBSX was not induced by either ultraviolet irradiation or exposure to mitomycin C. The mutation was specific for PBSX as ϕ105 and SP02 lysogens of the mutant were inducible. All known PBSX specific mutations were mapped close to and to the left of metC, implying a single site for PBSX genes.

Evidence against the two other locations implicated as sites of PBSX genes is also presented. The site near purB was suggested by the adjacent location of a mutation responsible for the temperature sensitive induction of PBSX. However, it was shown that this mutation is not specific for PBSX and is probably a cellular mutation. The other site for PBSX genes, near purA, was suggested by the multiple replications of this region during mitomycin C induction of PBSX. Marker frequency analysis revealed that in cells carrying wild type PBSX, both metC and purA were replicated many fold during mitomycin C treatment, while in similarly treated cells non-inducible for PBSX, purA continued to be replicated independently of PBSX induction. Furthermore, replication of the metC marker in the apparent absence of prophage excision suggests that PBSX replicates in situ.

PBSX was also used to investigate the source of cross-links found in cellular DNA. One suggested source had been the mechanical shear forces acting on large DNA molecules during DNA isolation procedures. However,

chromosomal DNA packaged into PBSX heads which had been cleaved intracellularly to a size no longer susceptible to shear induced cross-linking was found to contain cross-links, eliminating this hypothesis. Evidence against another possible source of cross-links, the recombination pathway, is the finding that in *B. subtilis* strains carrying all known Rec type mutations normal amounts of cross-linked DNA were found.

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PART I

STRUCTURAL AND GENETIC ANALYSIS OF THE *Bacillus subtilis* DEFECTIVE PHAGE PBSX

CHAPTER I

INTRODUCTION

Diverse animal and bacterial species contain virus specific genetic information which does not lead to the formation of infectious viral particles either because of defects in the viral genome itself or because of the non-permissive nature of the host cells (Garro and Marmur, 1970; Heubner and Todaro, 1969). In bacteria, the presence of these determinants has been recognized by the ability of cells to produce phage-like particles which contain some or all of the normal phage components but which lack infectivity. These defective phages have been detected primarily by electron microscopy and by the bacteriocidal activity often associated with them.

The defective phages have been grouped into three classes on the basis of their structural organization and DNA content (Garro and Marmur, 1970). The first of these consists of phage-like particles containing random fragments of chromosomal DNA and includes the defective phages PBSX, PBSY,and PBSZ produced by Bacillus subtilis and the phage PBLB produced by Bacillus licheniformis. The second class consists of particles that contain phage specific DNA almost exclusively and includes one of the defective B. licheniformis phages PBLA as well as the phage ϕ15A produced by derivatives of Escherichia coli strain 15.

This class also includes a plasmid, P15B, which shows 90% homology to the DNA of the temperate E. coli phage P1, but has never been shown to code for phage structural proteins. The third and most commonly observed class of defective phages consists of particles that have the structural characteristics of phage tails and may not contain any DNA.

The defective B. subtilis phages PBSX, PBSY and PBSZ are produced by strains 168, S-31 and W23 respectively. They are morphologically similar but can be differentiated from one another by the density of the particles (Subbaiah et al., 1965), the number of cross striations in their tails (Rima and Steensma, 1971) and the 5' termini of the DNA found in phage heads (Huang and Marmur, 1970a). They can also be differentiated by their killing spectrum, each phage being able to absorb to and kill B. subtilis cells other than its producer strain (Subbaiah et al., 1965). Interestingly, B. licheniformis, a species with only limited genetic homology to B. subtilis, produces a defective phage, PBLB, which closely resembles PBSZ in its morphology and killing spectrum (Huang and Marmur, 1970b). Of the B. subtilis defective phages PBSX has been the most extensively studied phage and has served as a model in understanding the biochemistry of class I defective phages. PBSX has appeared in the literature under the following

designations: α (Eiserling, 1964), μ (Ionesco et al., 1964), "phage-like particles" (Stickler et al., 1965), GA2 (Azizbekyan et al., 1966; Bradley, 1965), and PBSH (Haas and Yoshikawa, 1969a). The molecular weight of PBSH DNA estimated by sedimentation analysis of the native molecule was greater than that of PBSX DNA, implying non-identity between the two phages. However, when other techniques to determine DNA molecular weight were used, similar values were obtained for both phages (Haas and Yoshikawa, 1969a).

Structurally, PBSX is characterized by an unusually small head attached by a narrow connector to a long contractile tail having both a base plate and tail fibers (Eiserling, 1964). The conclusion that the DNA in PBSX particles consists exclusively of fragments of chromosomal DNA was based on several lines of evidence. First, the inability, in early studies (Okamoto et al., 1968a) to detect preferential replication of a unique DNA species during induction of PBSX implicated the bacterial chromosome as being the source of the DNA packaged into phage particles. Secondly, the DNA isolated from PBSX had a buoyant density identical to its host's DNA and also showed strong sequence homology to it by DNA-DNA hybridization (Seaman et al., 1964). Finally, the DNA isolated from PBSX was shown by a transformation assay to contain genetic markers from all regions of the host chromosome (Haas and Yoshikawa, 1969b; Okamoto et al., 1968a; Seaman et al., 1964). The exclusive

packaging of host DNA is not unique to PBSX. It also occurs during replication of the DNA viruses polyoma and SV40 (pseudovirion formation; Aposhian, 1974) and in the production of the generalized transducing particles of bacteriophages SP10 (Okubo et al., 1963), PBS1 (Yamagishi and Takahashi, 1968) and P22 (Ebel-Tsipis et al., 1972). Unlike the generalized transducing phages, however, PBSX is not active in transduction probably because it is unable to inject its DNA (Okamoto et al., 1968a). This defect may be related to the observed backward contraction of the tail sheath away from the head.

Because the defective phages are non-infectious they must be propagated as prophages, either inserted into the host chromosome or existing extrachromosomally as plasmids. In the few cases which have been examined, most evidence points to a chromosomal site for defective phage genetic determinants. In the case of PBSX a tail gene had been localized adjacent to the host metC marker (Garro et al., 1970). Similarly genetic determinants for pyocin R, a class III defective phage, have been mapped near the Pseudomonas aeruginosa trp-1 marker (Kageyama, 1970a, b). Furthermore, cells carrying class III defective phages cannot be cured by agents known to eliminate plasmids (Clark-Walker, 1969; Kageyama et al., 1964; Traub, 1972) and plasmids have not been detected consistently in cells producing the class II defective phage ϕ15A (Ikeda et al., 1970). The E. coli plasmid P15B is the only known case of

defective phage existing extrachromosomally. However, this is not surprising, considering that this plasmid is related to the temperate phage P1 which also forms an extrachromosomal prophage (Ikeda et al., 1970).

Expression of PBSX determinants appear to be controlled by the same general type of repressor mediated negative control mechanism necessary for the maintenance of the prophage state in infectious phages such as λ. Both infectious and defective phages are produced in low levels spontaneously but host cells can be induced to produce higher levels by similar physical or chemical means. PBSX has been successfully induced by agents that damage DNA and/or interfere with its synthesis. These include the antibiotic mitomycin C (MC) and ultraviolet light (UV) (Eiserling, 1964; Seaman et al., 1964), thymine starvation (Brabander, 1963; Subbaiah, Unpublished Results), 5-bromodeoxyuridine (Boice, Unpublished Results; Subbaiah, Unpublished Results), naladixic acid (Subbaiah et al., 1964), hydrogen peroxide (Stickler et al., 1965) and 4-nitroquinoline-1-oxide (Hirokawa and Kallubar, 1969). The mechanism by which such treatments inactivate prophage repressors or interfere with their activity has not yet been clearly defined. Work with λ indicates that these agents initiate a sequence of events which includes repair of DNA damage utilizing enzymes of the Rec pathway (Fuerst and Simonvitch, 1965) and the elaboration of a factor(s) capable of inactivating the repressor protein

in vitro (Johnston and Echols, 1972). One step in the process appears to involve a derivative of adenine other than C-AMP or ATP (Kirby *et al.*, 1967; Ruff *et al.*, 1971).

Induction by direct thermal inactivation of a temperature sensitive repressor is well documented among infectious temperate phages (Calendar, 1970). However, to date, instances of thermally inducible PBSX have not been definitively traced to an alteration in the phage genes controlling repression. Yoshikawa and Haas (1968) isolated a *B. subtilis* mutant in which PBSH but not SP02 was induced by high temperature. However, subsequent analysis indicated that this phenotype involved a defect in membrane biosynthesis leading to cell lysis which was mistakenly taken as an indication of PBSH induction. Another mutation leading to the thermal inducibility of PBSX was mapped near the *pur*B marker by Siegal and Marmur (1969). Though SP02 was also induced at the non-permissive temperature, the mutation was presumed to be in the PBSX repressor gene because it could be transformed to the wild type phenotype only by DNA isolated from *B. subtilis* 168 which carries the homologous prophage. However, evidence will be presented here that this mutation is not in a PBSX prophage gene. Among the other classes of defective phage, the only documented case of a temperature inducible phage occurs in a *Ps. aeruginosa* mutant producing R type pyocin (Ikeda *et al.*, 1964). However, because induction appears to be mediated by inhibition of DNA synthesis at high temperature

(Liu *et al.*, 1969), it is also unlikely that this mutation is located in a prophage gene.

Induction of PBSX has been associated with an alteration in the pattern of cellular chromosome replication. Haas and Yoshikawa (1969b) showed that in MC treated *B. subtilis* 168 cells, the origin proximal region of the host chromosome undergoes multiple cycles of replication increasing the number of copies of origin proximal markers in the cells. As no evidence was found for the autonomous replication of DNA the size of PBSX DNA (22s or 8.3×10^6 molecular weight) early in induction , Haas and Yoshikawa suggested that if PBSX genes were located near the origin of chromosome replication the multiple reinitiations which occur in this region would provide a mechanism for PBSX replication during induction. They interpreted the unusual renaturation and biophysical properties of DNA derived from the chromosomal origin as support for this hypothetical location for PBSX genes (Haas and Yoshikawa, 1969c). However, the authors left open the possibility that autonomous replication of PBSX sized DNA may have occurred simultaneously with multiforked replication but could not be detected by their experimental procedure.

There is also evidence which indicates that DNA replication is not needed for PBSX production. Both Seaman *et al.* (1964) and Okamoto *et al.* (1968) demonstrated almost normal production of PBSX in the presence of a potent inhibitor of DNA synthesis, fluorodeoxyuridine. Further

evidence that little or no DNA synthesis is required for the production of PBSX is that the only observable effect of producing PBSX during restricted DNA replication (mediated by thymine starvation) is that a greater than normal proportion of PBSX DNA is of pre-induction origin (Haas and Yoshikawa, 1969b). A similar phenomenon is found in the case of colicin B in which replication of its coding element is not a necessary event for colicin production (Hausmann and Clowes, 1971).

A second chromosomal event associated with PBSX induction involves the breakdown of chromosomal DNA into uniform sized fragments which are packaged into PBSX heads. Following MC treatment, up to 50% of the host chromosome is fragmented into uniform 22s sized pieces having unique 5' nucleotide termini, dTMP and dGMP (Huang and Marmur, 1970b; Okamoto et al., 1968b). Though the time of appearance and the amount of both this fragmentation activity and PBSX killing activity in induced cells are almost identical, it is not known whether the enzyme responsible for fragmentation is a phage or a host gene product. All 22s fragments generated during induction appear in phage heads suggesting that the cutting and packaging mechanism are intimately associated. This association suggests a model for the PBSX packaging mechanism similar to the headful model proposed by Streisinger et al. (1967) for the packaging of T4 DNA. According to this model, the formation of uniform phage sized fragments from high molecular weight

DNA is accomplished by a mechanism in which the size of the DNA is determined by the volume of the phage head.

That the DNA found in PBSX consists of random fragments of chromosomal DNA was suggested by the observation that linkage distances between host markers are preserved in PBSX DNA (Siegel and Marmur, 1969). One consequence of such a random packaging mechanism is that the relative frequency of markers in PBSX DNA will reflect the frequency of these markers in cellular DNA at the time that it is packaged into phage heads. However, recently, Boice (Unpublished Results) has presented evidence for the preferential packaging of DNA presumably derived from the origin of replication. When inducing PBSX by germinating spores in the presence of bromouracil, Boice found that 90% of PBSX DNA was bifilarily labeled (HH) while only 67% of the DNA in the supernatant of the cell lysate was similarly labeled. The preferentially incorporated DNA was presumed to be derived from the chromosomal origin because growth in the presence of bromouracil causes multiforked initiations of this region. However, since germinating spores are known to extrude DNA (Borenstein and Ephrati-Elizur, 1969; Ephrati-Elizur, 1968), objection to the work may be raised on the grounds that the DNA in the supernatant probably included DNA from uninduced cells which appeared to make up a significant proportion of the cells in these cultures. This DNA which probably has a different

distribution of bromouracil than that of induced cells, will skew the real distribution of bromouracil in DNA that has been accessible to PBSX packaging.

Although the production of PBSX is lethal to the cell, the PBSX prophage may serve an important cellular function. This is indicated by the apparent inability to cure *B. subtilis* 168 cells of PBSX. Although several cases of cured *B. subtilis* 168 cells have been reported, none appear to be truly cured of PBSX. Seaman *et al*. (1964) isolated a *B. subtilis* strain believed cured of PBSX by dint of its newly acquired sensitivity to PBSX killing. Subsequently, however, this strain was shown to be a W23 contaminant (A. Garro, Unpublished Results). Recently Ephrati-Elizur *et al*. (1974) reported a *B. subtilis* strain insensitive to the inducing effects of MC. However, as numerous as yet unexplored mechanisms could underlie this phenotype, there is little reason to conclude that the strain is cured of PBSX.

PBSX may serve a beneficial function to the cell by eliminating ecologically competitive bacterial strains through its bacteriocin-like ability to kill a small group of related bacterial strains. As PBSX is unable to inject its DNA, the inhibition of cellular macromolecular synthesis (DNA, RNA and protein) responsible for cell death appears to be mediated from the cell surface. Studies with

pyocin R (Kaziro and Tanaka, 1965) indicate that inhibition of macromolecular synthesis is not a secondary effect of inhibition of cellular energy metabolism as occurs in the case of colicins E and K, but rather a primary effect of absorption of the defective phage. Similar effects occur during absorption of T4 ghosts to E. coli cells and the ensuing cell death is believed to occur through absorption mediated allosteric changes in cellular membrane proteins which inhibit essential membrane functions (Duckworth, 1970). A similar mechanism may underlie the killing activity of defective phages explaining their almost simultaneous and immediate effect on DNA and protein synthesis (Okamoto et al., 1968a) and on cell permeability (Kageyama et al., 1964).

The ability of PBSX to absorb to a cell is determined by the specificity of the cell surface receptor. Glaser et al., 1966 demonstrated that the chemical nature of the teichoic acids in the B. subtilis cell wall affects PBSX absorption; however whether teichoic acids form the actual receptor or whether the teichoic acid molecules on the cell surface control the availability and the activity of the true binding site remains uncertain (Young, 1967). There are several known instances in which a prophage can affect the specificity of cell wall polymers (Jonasson et al., 1969; Robbins and Uchida, 1962). It has been suggested that such lysogenic conversions may underlie the absorption and killing spectrum of the closely related B. subtilis defective phages (Subbaiah et al., 1965) as

well as their inability to absorb to their producer strains. A further prerequisite for absorption may involve the physiological state of the tail sheath. In the case of the pyocin R particle that is morphologically similar to the PBSX tail, it has been demonstrated that particles having contracted sheaths are incapable of absorbing to sensitive cells (Higerd et al., 1967; Coetzee et al., 1968).

There is little actual information on the nature and origin of naturally occurring defective phages. However, because the ubiquity of the phenomenon suggests that defective phages play a role in normal cell physiology and because of possible analogies between defective phages and partially derepressed viral genetic determinants involved in oncogenesis, the defective phages merit better understanding. Only in the case of PBSX has sufficient biochemical and genetic data been accumulated to suggest a possible cause for its defective nature. Various pieces of evidence suggested that PBSX genes were scattered at distant sites along the B. subtilis 168 chromosome. The only definitive localization of PBSX genes was by genetic mapping of a tail gene near the chromosomal metC marker (Garro et al., 1970). However, the presence of phage genes near purB was suggested by the adjacent location of the previously discussed mutation causing the thermal inducibility of both PBSX and SPO2 and another site for PBSX genes near the chromosomal origin marker purA was suggested by the multiple replications

of the origin proximal region occurring during MC induction of PBSX (Figure 1). If PBSX genes were scattered, all segments of PBSX specific DNA may not be derepressed during induction unless under regulon or cascade type control and certainly, phage specific DNA could not be excised as a unit.

The work to be described here was undertaken to determine if PBSX genes are indeed scattered. The task of definitively positioning PBSX genes was first approached by a transcriptional mapping technique in which the hybridizability of PBSX specific mRNA to fragments of chromosomal DNA carrying gene clusters derived from various parts of the chromosome was to be determined. Though PBSX specific mRNA could be isolated, due to technical difficulties the approach was abandoned in favor of biochemical and genetic analysis of both newly and previously isolated bacterial mutants having presumed prophage mutations.

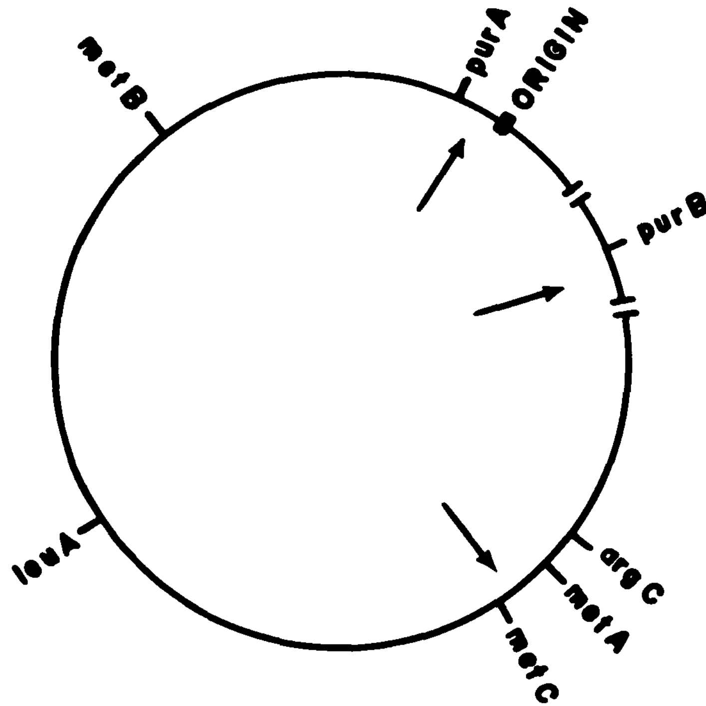


Figure 1

B. subtilis genetic map. Adapted from Young and Wilson (1972) and Hara and Yoshikawa (1973). The arrows indicate suggested sites of PBSX prophage genes.

CHAPTER II

MATERIALS AND METHODS

A. Bacterial and Phage Strains

The bacterial strains used are listed in Table 1. All strains except W23 are *B. subtilis* 168 derivatives and are lysogenic for PBSX. W23 is a PBSZ lysogen, sensitive to PBSX killing activity. Phage ϕ105 and the clear plaque mutant ϕ105c4 were obtained from L. Rutberg; SPO2, SPO2c and SP8* from J. Marmur; SPO1, β22, SP10, ϕ1 and ϕE from E. Hemphill and TSP1 from W.C. MacDonald.

B. Media

All minimal media contained Spizizen's salts (Anagnostopoulos and Spizizen, 1961) plus 0.5% glucose and 25 μg/ml aspartic and glutamic acids. Required amino acids were added to a final concentration of 50 μg/ml, purines and pyrimidines to 100 μg/ml and agar (Difco) to a concentration of 2%. In KS medium the basic minimal medium was supplemented with 0.1% Difco yeast extract; NaS is identical to KS except 6.07 g NaH_2PO_4 and 11.36 g Na_2HPO_4 are substituted for their respective potassium salts. CH-min is the basic minimal medium plus 0.05% casamino acids and 10^{-5} M $MnCl_2$. VY nutrient broth consists of 2.5% Veal Infusion plus 0.5% Yeast Extract in distilled water. TBAB plates (Difco Tryptose Blood Agar Base) were used for routine maintenance of cultures and for colony counts.

TABLE 1

BACTERIAL STRAINS

Strain	Properties[a]	Source
GB 1	hisA, trpC	E. Nester
GB 19	xtl-1[b], metC, pyrA	D. Dubnau
GB 21	metC	M. Comstock
GB 23	metC, leuA	P. Thurm
GB 26	trpC, metB, recA	C. Anagnostopoulos
GB 34	sacP, trpC	R. Dedonder
GB 64	argC, metC, pyrA	P. Thurm
GB 75	xin[b], metC, pyrA	P. Thurm
GB 159	pheA, ilvC, trpC, xtl-3	mutagenized derivative of BR 95
GB 219	pheA, ilvC, trpC, xin	mutagenized derivative of BR 95
GB 1001	leuA, metB, tsi-23	E. Siegel
GB 1323	pheA, ilvC, trpC, xhd	mutagenized derivative of BR 95
BD 71	argC, hisA, pyrA	D. Dubnau
BD 170	trpC, thr	D. Dubnau
BD 191	trpC, thr, recB	D. Dubnau
BD 193	trpC, thr, recD	D. Dubnau
BD 194	trpC, thr, recA	D. Dubnau
BD 224	trpC, thr, recE	D. Dubnau
BD 239	trpC, thr, recC	D. Dubnau
BD 246	trpC, thr, recG	D. Dubnau
BR 95	pheA, ilvC, trpC	L. Rutberg

TABLE 1

(CONTINUED)

Strain	Properties[a]	Source
44AO	xtl-1	K. Bott
MU8U5U16	purA, leuA, metB	N. Sueoka
W 23	str , PBSX^s	Spontaneous Str^r mutant

[a]xtl, PBSX marker leading to loss of bacteriocidal activity; xin, PBSX marker leading to noninducibility of PBSX; xhd, PBSX marker leading to formation of defective heads; PBSX^S, killed by PBSX; str, resistant to 5 mg/ml streptomycin sulfate.

[b]PBSX markers were introduced into strain GB64 by transformation at saturating DNA concentrations selecting argC$^+$ transformants and then assaying for the PBSX mutant phenotype.

The overlay plates used to assay PBSX killing activity consisted of 2.5 ml of the top agar described by Okubo and Romig (1965) seeded with a streptomycin resistant strain of W23, layered on CH-min plates. Both layers contained 3 mg/ml streptomycin sulfate. Similar plates, seeded instead with a streptomycin resistant 44AO strain were used for ϕ105 plaque assays. Overlay plates of tryptone broth plus 10^{-2}M $MgSO_4$ were used for SP8*, SPO1, β22, SP10, ϕ1 and ϕE plaque assays while overlay plate of Difco Brain Heart Infusion were used to assay TSP1.

C. Chemicals, Radioactive Materials and Enzymes

Mitomycin C (MC) was purchased from Hakko Kogyo Co. Ltd. Stock solutions were prepared every two weeks and stored in the dark at 4°C. Nitrosoguanidine (N-methyl-N'nitro-N-nitrosoguanidine) was obtained from Aldrich Chemicals (Cedar Knolls, N.J.), hydroxyapatite from Biorad Laboratories (Rockville Centre, N.Y.), spleen phosphodiesterase and micrococcal nuclease from Worthington Biochemical Corporation (Freehold, N.J.) and ^{14}C amino acid mix (0.1 m Ci/ml) and ^{3}H thymidine (methyl-^{3}H, 10-15 Ci/m mole) from New England Nuclear (Boston, Mass.).

D. Antisera

Anti-PBSX antiserum was prepared by immunizing rabbits with CsCl purified PBSX (500 μg phage protein/ml) in complete Freund's adjuvant. The rabbits received four toe pad injections once a week for four weeks and were bled one

week after the final innoculations. Sheep antiserum to rabbit γ globulin was generously donated by B.F. Erlanger.

E. Preparation of PBSX

Throughout the procedures to be described, cells were grown at 37°C with vigorous aeration and culture densities monitored with a Klett-Summerson colorimeter equipped with a No. 66 red filter. One Klett unit is approximately equivalent to 1 x 10^6 cfu/ml.

1. Induction and Labeling

B. subtilis 168 cells were grown in VY to early exponential phase (23-25 Klett units) and MC was added to a final concentration of 0.4 μg/ml. Thirty minutes later the cells were harvested and resuspended in an equal volume of prewarmed KS supplemented with 0.4 μg/ml MC and 1.5 μc/ml ^{14}C amino acid mix. After 120 min of additional incubation the culture had lysed. The lysate was incubated for 30 min at 37°C with 1 μg/ml of both pancreatic deoxyribonuclease and pancreatic ribonuclease and made 0.5 M with respect to NaCl before removal of cell debris by low speed centrifugation (10 min at 7,000 revs/min in a Sorvall SS-34 or GSA rotor).

2. Concentration and Purification

PBSX particles were concentrated by polyethylene glycol precipitation and purified in CsCl step gradients as described by Yamomoto *et al*. (1970). The phage band, identified by its density of 1.375 g/cm^3, was dialyzed

against TMK (10^{-2} M Tris-HCl, 5 x 10^{-3} M $MgCl_2$, 0.3 M KCl, pH 7.2). For further purification, the phage was rebanded in a CsCl gradient of 1.375 g/cm^3 average density, centrifuged for 48 hr at 35,000 revs/min in a Spinco type 50 rotor and then dialyzed against TMK.

3. Isolation of PBSX DNA

DNA was isolated from purified PBSX particles by the method described by Okamoto *et al.* (1968a).

F. Mutagenesis

BR 95 cells were mutagenized by a modification of a procedure described by Adelberg *et al.* (1965). Cells grown in VY to a density of 100 Klett units were resuspended in an equal volume of Spizizen salts adjusted to pH 6.0 and incubated for 10 min at 37°C with 100 μg/ml nitrosoguanidine. After thorough washing, the culture was grown in VY to stationary phase to allow cell division and segregation of mutant genotypes before plating on TBAB.

G. Screening of Mutants

Single colonies were inoculated into 1 ml VY and grown overnight to stationary phase. Ten μl of these cultures were inoculated into 1 ml VY which contained 250 μg/ml deoxyadenosine and 8 μCi/ml ^{3}H thymidine to label the bacterial DNA which would be packaged by the phage during induction. The cultures were grown until the cells in each tube reached a visually estimated density of 25 Klett units

(approximately 2-3 hr). MC was added to a final concentration of 0.5 µg/ml and incubation was continued for 3 hours.

Clones potentially defective for PBSX gene functions were identified by monitoring the MC induced cultures for three phage associated activities: cell lysis, PBSX mediated killing of B. subtilis W23 and packaging of chromosomal DNA into PBSX heads. Clones defective for PBSX induced cellular lysis (Lys^-) were identified by sustained high turbidity of the culture at 3 hours post induction. In cultures of MC treated cells carrying wild type PBSX turbidity normally increases for only 90 min but then falls rapidly as the cells lyse. Clones defective for the production of PBSX killing activity (Kil^-) were identified by the inability of the induced culture to cause a zone of clearing on a lawn seeded with sensitive W23 cells. The loss of killing activity is associated with defects in the PBSX tail. Chromosomal DNA packaged into intact PBSX heads was found to be resistant to degradation by nucleases. Mutants defective for PBSX head formation were expected to be found among those clones which failed to protect (Prt^-) ^3H labeled chromosomal DNA from exogenously added nucleases. To detect such clones lysates were treated with 100 µg/ml lysozyme for 30 min at 37°C, then adjusted to 0.1 M Tris, 5×10^{-3} M $CaCl_2$, pH 7.6 and incubated for 45 min at 37°C with 10 µg/ml spleen phosphodiesterase and 10 µg/ml micrococcal

nuclease. The percent protection was calculated from the ratio of acid insoluble counts, measured by the method of Li and Felmly (1973), after and before nuclease treatment. The Prt^- phenotype was ascribed to lysates which afforded less than 40% protection. This is the average amount of bacterial DNA packaged into PBSX particles. Kil^- cells also exhibited the Prt^- phenotype probably because defective tails allow nuclease to penetrate otherwise normal heads.

H. SDS Polyacrylamide Gel Electrophoresis

1. Preparation of Samples and Immunoprecipitation

0.2 Ml aliquots of cells, grown and MC induced in either KS or NaS were pulse labeled with ^{14}C amino acid mix. In samples taken for direct analysis by electrophoresis, the pulse was terminated by 2 volumes of cold 0.02 M NaN_3 and immediately centrifuged (15 min, 5,000 revs/min in a Sorvall SS34 rotor). The pelleted cells were lysed by a 30 min incubation in 20 μl of a 0.02 M NaN_3 solution containing 5 mg/ml lysozyme, followed by the addition of 20 μl double strength cracking buffer (0.05 M Tris-HCl, pH 6.8, 1% SDS, 1% mercaptoethanol, 0.002 M EDTA, 10% glycerol). To prepare samples for immunoprecipitation, the pulse was terminated by a 30 min incubation in an equal volume of 500 μg/ml lysozyme, 0.02 M NaN_3. The lysate was incubated first with anti-PBSX antiserum for 60 min at 37°C and then with sheep anti-rabbit γglobulin for 60 min at 37°C. After overnight incubation at 4°C the immuno-

precipitates were washed with cold 0.9% saline and dissolved in cracking buffer. All samples were placed in boiling water for 3 min and cooled before being applied to the gel.

2. Electrophoresis

Samples were analyzed on slab gels as described by Studier (1973) using the discontinuous buffer system of Laemmli (1970). In the immunoprecipitated sample only 1-3 μl of the dissolved precipitate was placed in each slot to avoid distortion of the bands by excessive immunoglobulins. Kodak no-screen X-ray film was used for autoradiography of dried gels. The pattern of grain densities converted into absorbence was recorded on a Canalco microdensitometer.

I. Transduction and Transformation

Preparation of competent cells and transformation were carried out as described by Rudner and Remeza (1973). Preparation of PBS1 transducing lysates and transductions were carried out as described by Dubnau et al. (1967).

J. Isolation of DNA

DNA from exponentially growing cultures was isolated by the method described by Okamoto et al. (1968a). Spore DNA was isolated from BR95 spores which had been germinated for two hours in the presence of 100 μg/ml chloramphenicol and then heated for 10 min at 60°C (Yoshikawa, 1965). The germinated spores were lysed with 1 mg/ml lysozyme

for 30 min at 37°C. 1% Sodium lauryl sulfate was added and the procedure for cellular DNA followed.

K. Genetic Mapping

PBS1 transducing lysates were used to mediate three factor genetic crosses. Since it is not possible to select directly for PBSX markers, primary selection was for the more origin proximal auxotrophic marker argC or for metC. Prior to scoring for unselected markers, prototrophic transductants were purified by streaking them on appropriately supplemented selective plates.

L. UV Killing Assay

To minimize the proportion of cells growing in chains, cultures were grown in CH-min media to a cell density of 25 Klett units. Cells, transferred to an equivalent volume of Spizizen salts in a Petri dish, were aerated by a magnetic spinbar and irradiated in a darkened room with an 8 watt G.E. Germicidal lamp at an approximate dose rate of 25 ergs/mm^2/sec. Samples were taken at intervals during the irradiation and divided into two parts, one of which was used to determine surviving colony forming units and the other, diluted 1/5 in VY, was incubated for 120 min to allow production of PBSX.

M. Preparation of ϕ105 and SPO2 Lysogens

Strains to be lysogenized were used as lawns for plaquing ϕ105 and SPO2. Prospective lysogens, picked from

the center of turbid plaques, were tested for immunity by cross-streaking against ϕ105c4 and SPO2c respectively.

N. Electron Microscopy

Samples for electron microscopy were either resuspended in a solution of 1% ammonium acetate, 10^{-2} M $MgCl_2$ (AAM) or dialyzed against it. A drop of the solution was placed on carbon coated formovar covered grids and negatively stained with 2% uranyl acetate. Specimens were examined on a Hitachi or AEI electron microscope.

O. Hydroxyapatite Chromatography

Hydroxyapatite columns were prepared according to the procedure of Bernardi (1969) using a sodium phosphate pH 6.8 buffer system. DNA was alkali denatured in the following manner: equal volumes of 1.0 M NaOH and 20 μg/DNA/ml in 0.01 M sodium phosphate buffer were mixed gently at 0°C for 10 min and neutralized with an equivalent volume of cold 1.0 M NaH_2PO_4. Both native and alkali denatured DNA samples were dialyzed against 0.001 M phosphate buffer before being applied to the column. The DNA was eluted with a linear gradient of sodium phosphate buffer ranging from .001 M to 0.5 M. Three ml samples were collected and the amount of DNA in each was determined spectrophotometrically using the conversions 1 OD_{260} = 50 μg double stranded DNA and 1 OD_{260} = 42.5 μg single stranded DNA. All transformations utilized 0.1 μg transforming DNA/ml competent cells.

CHAPTER III

RESULTS

A. PBSX Specific Proteins

Prior to attempting a genetic analysis of PBSX, the phage structural proteins were analyzed to enable subsequent identification of mutations affecting PBSX gene products. SDS gel electrophoresis of disrupted purified phage revealed seven distinct bands designated X1 through X7 (Figure 2). The molecular weights of these proteins and their relative proportions in the purified particles are presented in Table 2.

The kinetics of phage protein synthesis was examined using lysates prepared from cells which had been pulse labeled at various times during induction. Synthesis of most of the previously identified structural proteins (X1, X2, X3, X4 and X6) was first detectable at approximately 56 minutes post-induction and their rate of synthesis appeared to increase progressively during the latent period (Plate IA, B). Two structural proteins X5 and X7 could not be identified in these gels. X7 is not seen because of the poor resolution of low molecular weight proteins in these gels; it is readily detectable among the proteins of total cell lysates resolved on 13% gels (Figure 3). Protein X5, however, is never seen in pulse labeled total cell lysates. A direct comparison of induced and uninduced cultures shown in Plate II reveals more clearly than Plate I

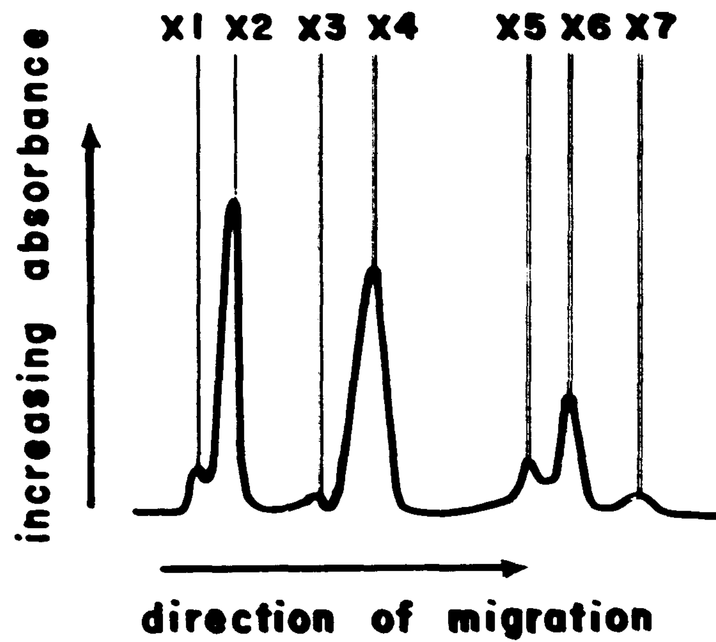


Figure 2

Proteins of purified PBSX. CsCl purified PBSX labeled with ^{14}C amino acids were electrophoresed on 13% SDS polyacrylamide gels.

TABLE 2

STRUCTURAL PROTEINS OF PBSX

PBSX Protein	Molecular weight[a] (daltons)	% Total Protein in Phage[b]
X1	83,000	3
X2	71,500	28
X3	47,200	2
X4	30,600	37
X5	17,700	10
X6	13,700	15
X7	10,800	5

[a]Approximate molecular weights were determined by the method of Weber and Osborn (1969).

[b]The amount of protein in each band of radioautographs was determined by the area under each peak measured by automatic integrator function of a Canalco microdensitometer.

Plate I

Autoradiograph of (A) a 10% and (B) a 20% SDS gel showing the kinetics of protein synthesis after MC induction. BR95 was grown in NaS medium and induced with 0.4 μg/ml at 0 min. At the times indicated 0.2 ml samples were removed and pulsed for 3 min. with 2.5 μCi ^{14}C amino acids. The pulse was terminated by 0.4 ml cold 0.02 M NaN_3 and the cells concentrated by centrifugation. The pellets were lysed and processed for electrophoresis as described in Materials and Methods.

(A)

Min. after MC addition

0 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72 76 80 84 88

X 1 2 3 4 8

(B)

0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72 76 80 84 88

X 1 2 3 4 8 6 7

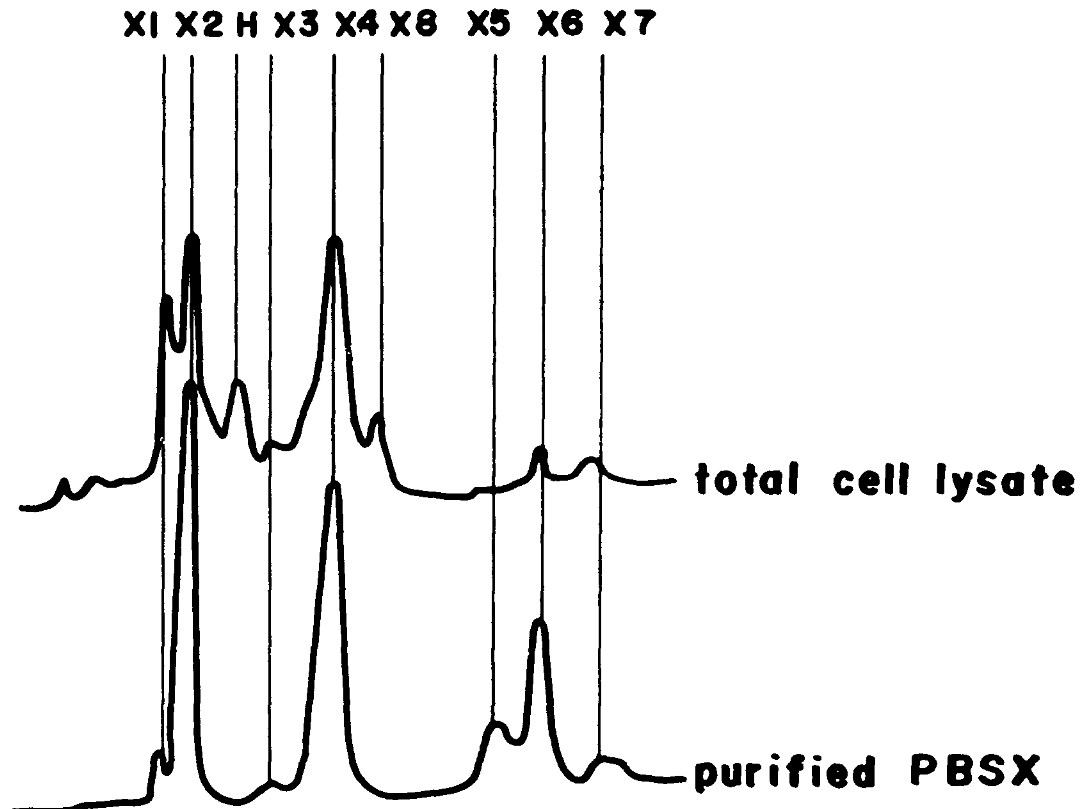
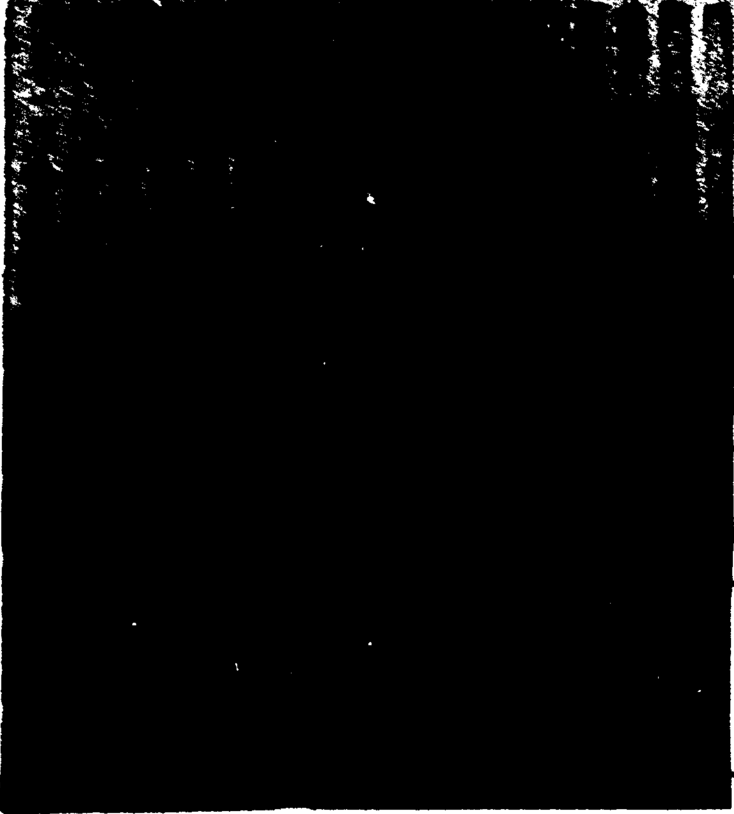


Figure 3

Comparison of the proteins found in purified PBSX and those inducible by MC in *B. subtilis*. BR95 cells were pulse labeled at 75 min. post induction and electrophoresed on a 13% SDS-polyacrylamide gel.

Plate II

Autoradiograph of a 10% gel showing a direct comparison of proteins synthesized in a culture induced for PBSX(+) and a non-induced (-) culture. Samples were prepared as described in Plate I.



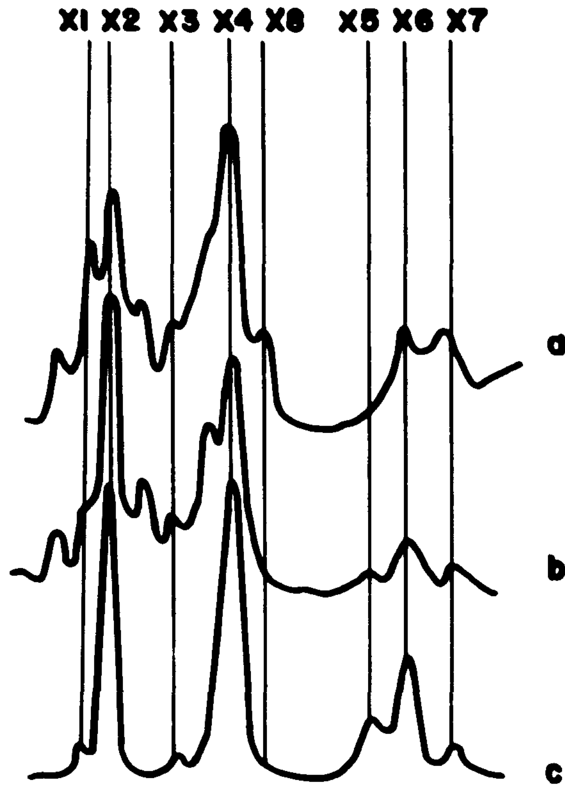
0'
15'
30'
45'
60'
75'
90'
105'
- + - + - + + - + - + - + - +
X1
X2
H
X3
X4
X8
X6

that some of the normal host proteins were turned off during the course of induction, though many continued to be synthesized throughout. Some of these, such as protein H, were synthesized in relatively large quantities. Because of this high background of host specific protein synthesis it is impossible to unambiguously identify all MC inducible proteins, some of which may be non-structural PBSX proteins. However the induction of a protein termed X8, that is not incorporated into the mature particle, can be detected. It appears at 60 minutes post induction and its kinetics of synthesis are similar to PBSX structural proteins.

The previously identified MC inducible proteins labeled by a short pulse differ from those found in purified phage in two respects: (1) compared to other phage proteins, relatively greater amounts of X1 and X7 are synthesized than are incorporated into mature particles, and (2) protein X5 could not be identified in pulse-labeled total cell lysates while protein X8 was consistently observed. In an attempt to determine if there was a product-precursor relationship between X5 and one of the other proteins, particularly X8, a pulse chase experiment was performed. As shown in Figure 4,the amount of radioactivity in X5 does increase during the chase period. However radioactivity is lost from two of the previously identified MC inducible proteins, X1 and X8, obscuring the origin of X5.

Figure 4

Proteins labeled during a pulse chase experiment. At 75 min. post induction two aliquots of BR95 cells in NAS were pulse labeled for 3 min. with 12 μCi, ^{14}C-amino acids. The pulsed sample (a) was processed as described in Plate I. Cells in (b) were harvested and washed by filtration on a nitrocellulose membrane filter, resuspended in an equal volume of fresh NaS containing 0.4 μg/ml MC and incubated for 60 min. The lysate was then treated for 30 min. at 37° with 20 μliters of a 5 mg/ml lysozyme, 0.02 M NaN_3 solution. An equal volume of double-strength cracking buffer was added and the sample heated at 100° for 3 min. Approximately equivalent amounts of radioactivity from a and b were electrophoresed on 13% SDS-polyacrylamide gels. Sample (c) is purified PBSX.


X1 X2 X3 X4 X8 X5 X6 X7
a
b
c

It was possible to determine which of the proteins were associated with the head and tail structures by analyzing the proteins present in isolated PBSX tails. A fraction (Fraction 2 of Figure 5) consisting almost exclusively of phage tails, with some contaminating flagella (Plate IIIA) was isolated by fractionating concentrated radioactively labeled PBSX on CsCl gradients of an average density less than that of intact phage (density of 1.330 units rather than 1.375 units). In contrast to the tails of purified intact phage (Fraction 1 of Figure 5 and Plate IIIB) many of the isolated tails are partially disintegrated, disjointed and contracted. This may explain the low level absorption and killing activity of the isolated tail fraction (Table 3). Electropherograms of this fraction (Figure 6) reveal the presence of proteins X1, X2, X3, X5 and X6 in PBSX tails. The two PBSX structural proteins absent in the tail fraction are assumed to be the head proteins. This assumption was later confirmed by work with a mutation affecting PBSX head assembly. The flagella protein (FL) was identified both by its enrichment in other fractions consisting primarily of flagella and by its characteristic molecular weight of 40,000 daltons (Martinez <u>et al</u>., 1967).

B. Biochemical Characterization of PBSX Mutants

In screening approximately 1,500 mutagenized clones, one was isolated with a phenotype (Prt^-, Kil^+, Lys^+) expected for cells carrying a prophage mutated in a PBSX head gene,

Figure 5

Fractionation of a PBSX preparation on CsCl density gradients. BR95 cells were grown in KS containing 8 μCi/ml ^{3}H thymidine and 250 μg/ml deoxyadenosine. When the density reached 33 klett units, 0.4 μg/ml MC was added and 30 minutes later 1.5 μCi/ml ^{14}C amino acid mix. After the culture lysed, it was concentrated as described in Materials and Methods. The concentrated phage were spun to equilibrium on a CsCl gradient of 1.330 density units (48 hrs at 35,000 rpm). 5 μliter aliquots of each of the 0.2 ml fractions collected was spotted on a glass filter and counted. Based on the resultant radioactivity profile, the samples were pooled into the 7 fractions indicated and each was immediately dialyzed against either TMK (5×10^{-3} M $MgCl_2$ + 0.3 M KCl, buffered with 0.01 M Tris-HCl, pH 7.2) or AAM. —O—O—, ^{3}H; —●—●—, ^{14}C.

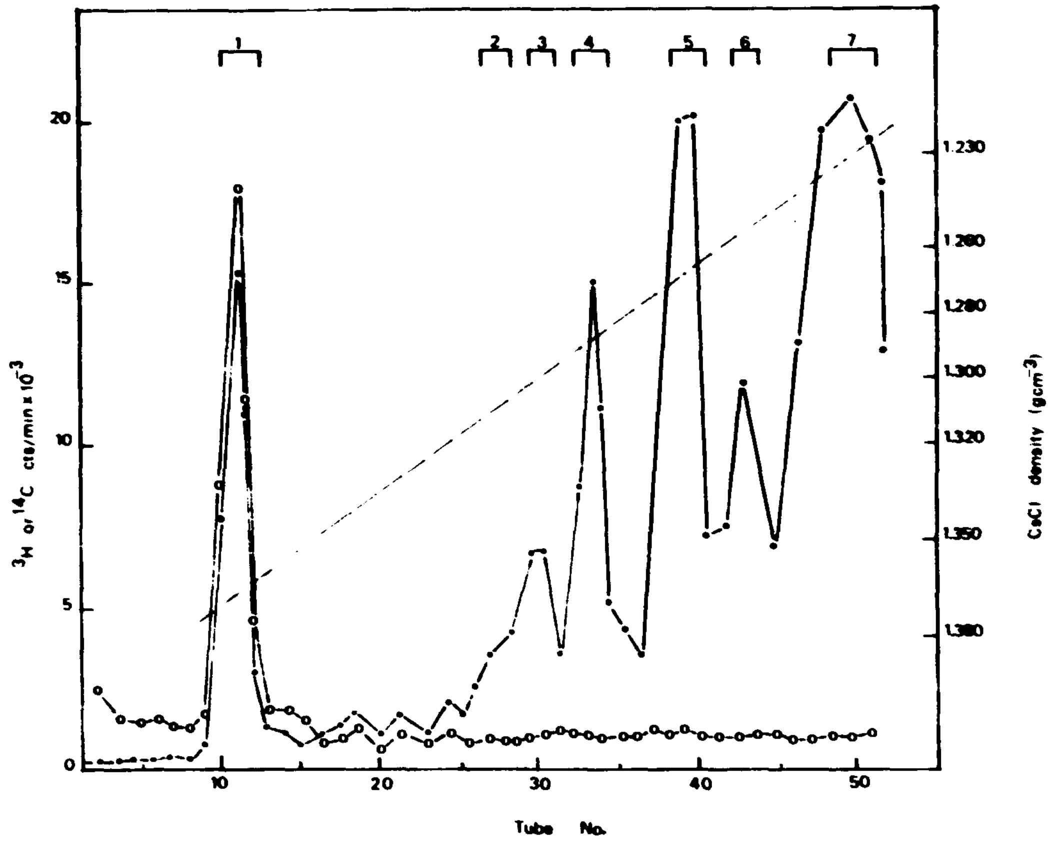
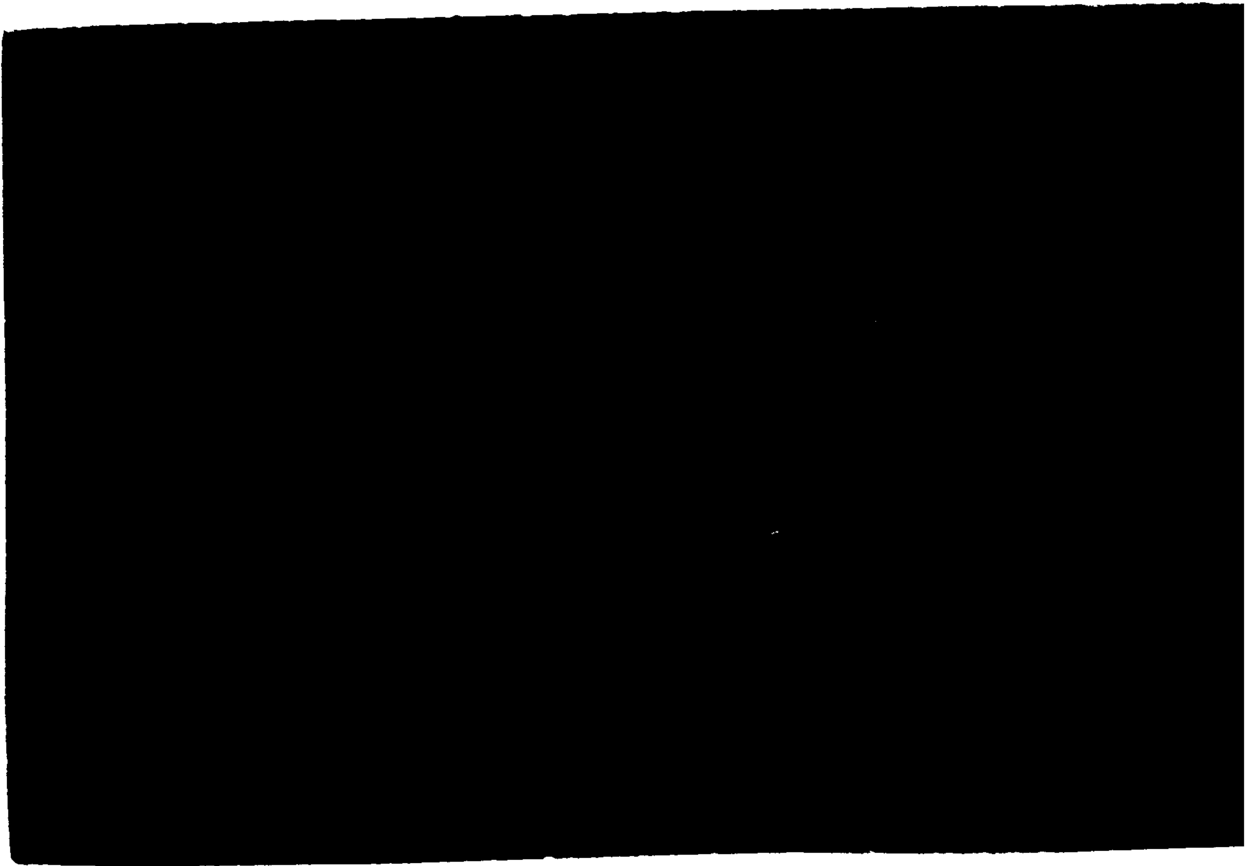

1
2
3
4
5
6
7
3H or 14C cts/min x 10-3
0
5
10
15
20
CsCl density (gcm-3)
1.230
1.280
1.300
1.320
1.350
1.380
Tube No.
10
20
30
40
50

Plate III

Electron micrographs of isolated PBSX tails. Fractions 1 and 2 of the CsCl density gradient presented in Figure 5 were dialyzed against AAM. A. Fraction II, isolated tails. The arrows indicate tail fragments. B. Fraction I, whole phage. The scales in A and B represent 450 Å and 410 Å respectively.



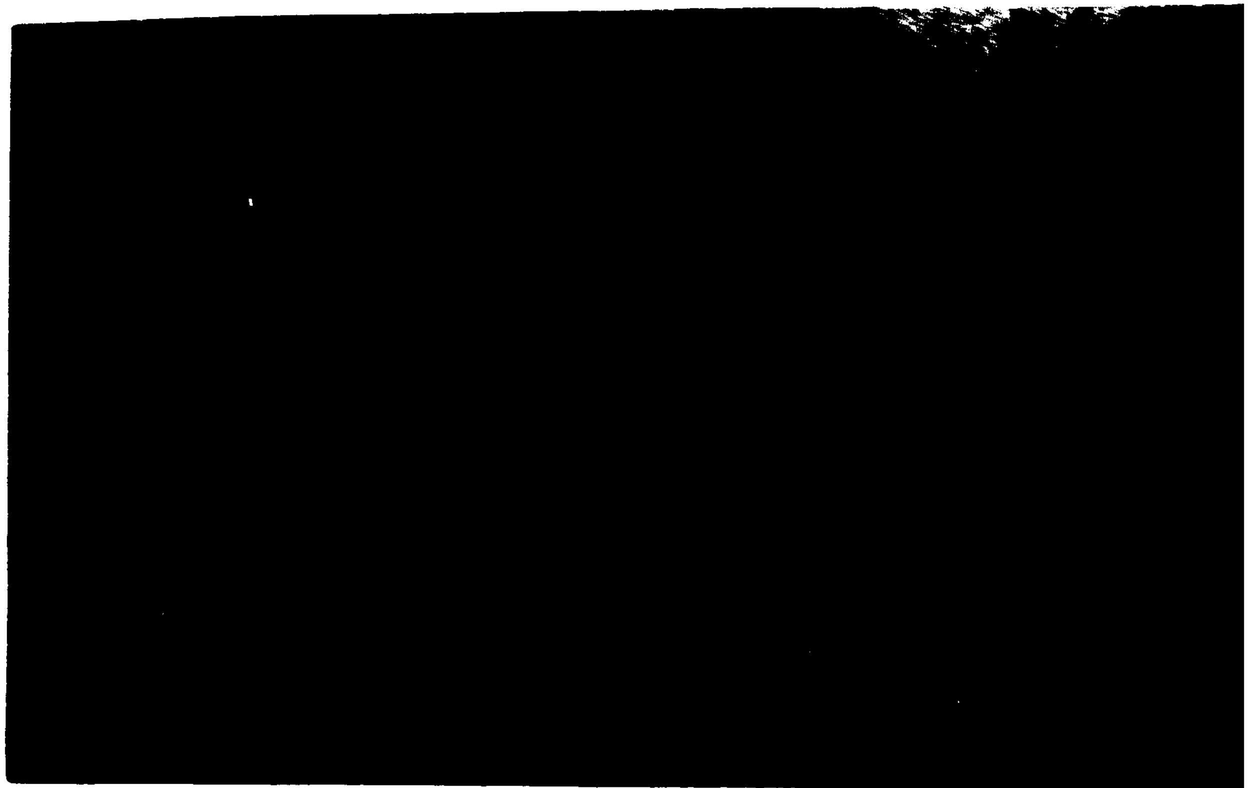


TABLE 3

BIOLOGICAL ACTIVITY OF FRACTIONATED PBSX PREPARATIONS

Fraction No.	Killing Activity[a]	Specific Absorption To W23 Cells[b]
1	1224	4000
2	4	300
3	4	300
4	4	0
5	4	0
6	0	0
7	0	0

[a]The killing activity is the reciprocal of the highest dilution which produces a clear zone on a lawn of W23 cells.

[b]Absorption to W23 cells was determined by the method of Okamoto et al. (1968a). Samples from each fraction, containing equivalent numbers of ^{14}C counts, were mixed with both W23 and 168 cells. The difference in counts between the absorption to W23 and 168 cells represents absorption specific for W23 cells.

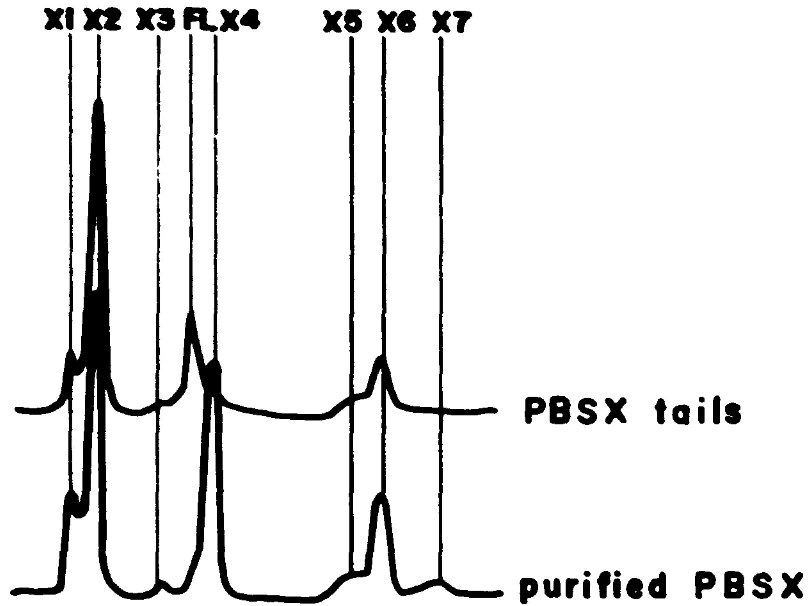


Figure 6

Structural proteins of isolated PBSX tails. Gradient fraction 1 (purified phage) and fraction 2 (isolated tails) shown in Fig. 5 and Plate III were electrophoresed on 13% SDS-polyacrylamide gels.

Xhd^-, and twelve clones with a phenotype ($Prt^{\pm}$ Kil^-, Lys^+) expected for cells carrying a prophage mutated in a PBSX tail gene, Xtl^-. Since the Xhd^- and Xtl^- phenotypes potentially reflected mutations in PBSX structural components, the pattern of phage specific protein synthesis in induced cultures of the single Xhd^- isolate, GB 1323, and a randomly chosen Xtl^- isolate, GB 159, were examined.

As seen in Figure 7, both mutants appear to produce all the previously identified PBSX proteins. However, since this approach would only detect gross alterations in either the synthesis or molecular weight of a particular protein, the serological reactivity of the proteins synthesized was examined. Labeled proteins present in total cell lysates capable of complexing with rabbit anti-PBSX antibodies were analyzed by SDS gel electrophoresis. The results presented in Figure 8 show that the immunoprecipitate of the wild type strain BR95 contains at least 6 of the 7 PBSX structural proteins in approximately the same proportions present in purified phage. Though several faint bands appear at the approximate position of X5, it is difficult to conclusively establish if any of these are identical to X5. The immunoprecipitate of the Xhd^- mutant, 1323, is conspicuously missing proteins X4 and X7, previously identified as head proteins. In the case of the Xtl^- mutant, 159, proteins X6 and possibly X5 are not immuno-

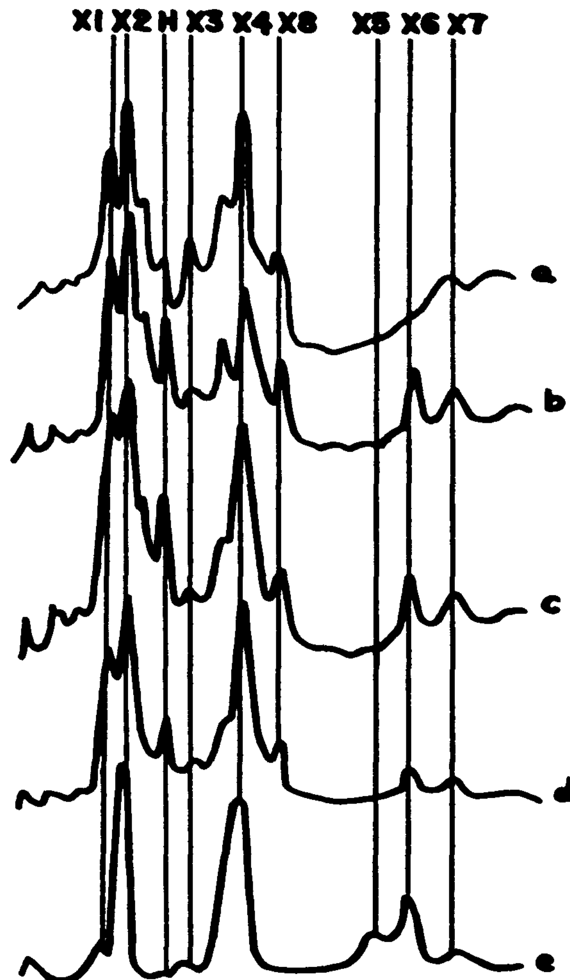


Figure 7

PBSX specific proteins in total cell lysates of B. subtilis mutants. The lysates were prepared as described in Plate I after pulse labeling the cells for 3 min. at 75 min. post induction and electrophoresed on a 13% SDS gel. a, xtl-1, 44AO; b, Xhd$^-$ isolate 1323; c, Xtl$^-$ isolate 159; d, BR95; e, purified PBSX.

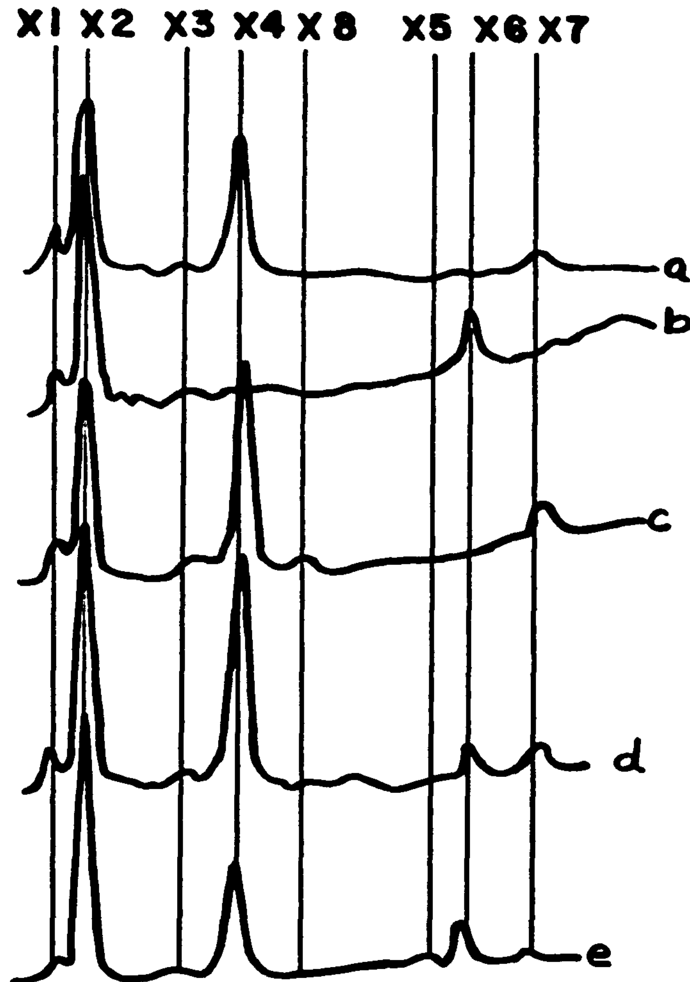


Figure 8

Proteins immunoprecipitated from lysates of B. subtilis mutants by anti-PBSX antiserum. At 75 min. post induction, 0.2 ml aliquots of cultures growing in KS were pulsed for 6 to 10 min. with 5 μCi ^{14}C-amino acids and processed for immunoprecipitations and gel electrophoresis on 13% SDS gels. a, xtl-1, 44AO; b, Xhd^- isolate 1323; c, Xtl^- isolate 159; d, BR95; e, purified PBSX.

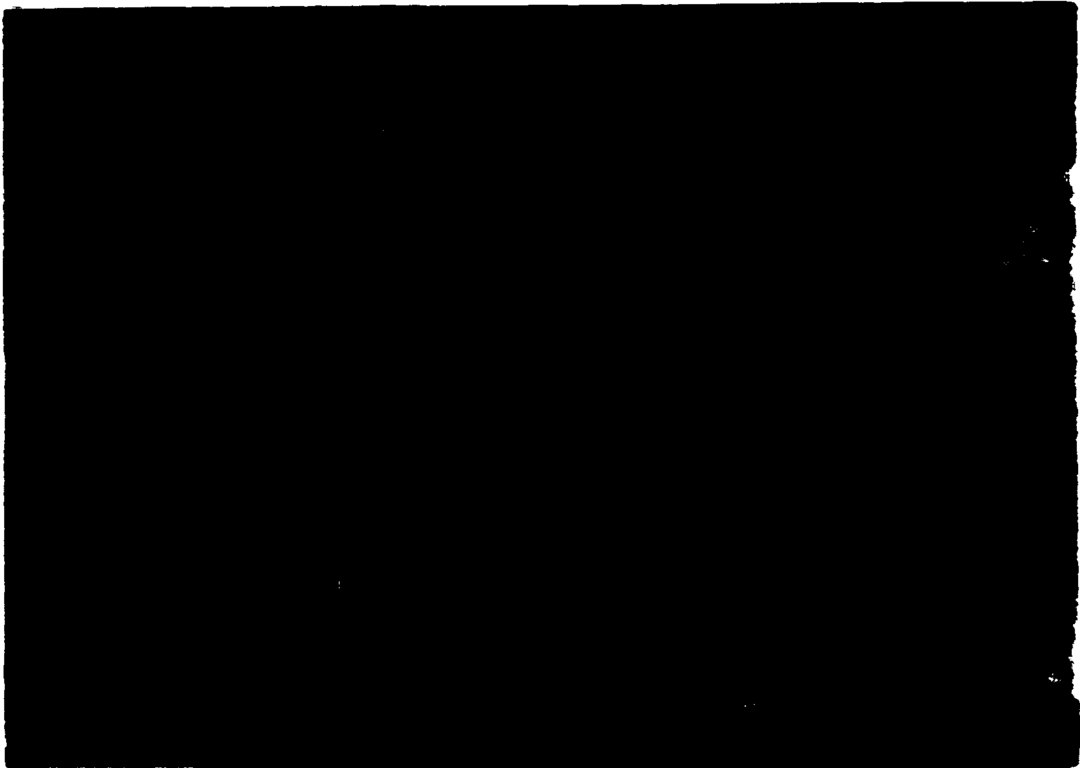
precipitable while a protein banding at the position of X8 is precipitated. In an independently isolated B. subtilis mutant, 44AO, carrying the PBSX tail gene mutation xtl-1, protein X6 is not synthesized at all(Figure 7). Immunoprecipitation of total cell lysates of 44AO are missing X6 as expected and possibly X5 as well (Figure 8).

Electron micrographs were taken of pellets prepared by high speed centrifugation (43,500 x G for 180 min.) of the MC induced lysates of the mutants. As seen in panels A and B of Plate IV, the pellet of the Xhd^- mutant contained assembled tails without heads and that of the Xtl^- mutant contained head structures without apparent tail structures. In the latter case, any tail substructures present may go unrecognized because they would be easily obscured by flagella and cell debris. The inability to assemble complete phage tails has also been observed in cells carrying the xtl-1 mutation (J. Mangan, Personal Communication).

The screening for PBSX mutants also detected six clones (Prt^-, Kil^-, Lys^-) which failed to lyse or release PBSX particles following exposure to MC. This non-inducible phenotype (Xin^-) is expected for mutations in PBSX regulatory genes involved in maintenance of repression. However, mutants with altered cellular functions affecting prophage induction such as certain Rec^- mutants (Rutberg and Rutberg, 1971) or mutants with altered permeability to MC would also be expected to exhibit this phenotype. Rec^- mutants could be

PLATE IV

Electron micrographs of particles produced by induced cultures of (A) the Xhd^- and (B) the Xtl^- mutant strains. The lysates were cleared of cell debris by centrifugation for 10 min at 3,000 x G and then centrifuged for 3 hr. at 43,500 x G. The pelleted material was resuspended in 1/30 the original volume AAM. The arrows in A indicate flagella. The circled structures in B are heads. The scales in A and B represent 300 Å and 270 Å respectively.



differentiated from PBSX mutants by their enhanced sensitivity to killing by UV irradiation. Of the six potential mutants only one, isolate 219, did not exhibit UV sensitivity but was actually more UV resistant than wild type Rec^+ cells (Figure 9). Mutant 219 in contrast to wild type strains, also failed to produce PBSX after exposure to the various UV doses tested. Resistance to UV as well as MC induction implies that impermeability to MC is not involved in generation of the Xin^- phenotype.

The mutation carried by isolate 219 is specific for PBSX. ϕ105 and SPO2 lysogens of this strain are inducible by MC (Table 4); thus the mutation appears to block MC or UV mediated derepression of PBSX. When isolate 219 was examined for MC induction of PBSX specific protein synthesis by SDS gel electrophoresis, no structural proteins were detected even by immunoprecipitation.

Mutant 219 is not cured for PBSX. The presence of the wild type PBSX xtl-1 allele was demonstrated by the ability of mutant 219 to act as the donor for PBS1 mediated transduction of an xtl-1 strain to $xtl\text{-}1^+$ (Table 5). Between 14 and 43% of the metC recombinants were converted to $xtl\text{-}1^+$. A more precise determination of the contransduction frequency of xtl-1 and metC could not be made in this cross since 29% of the metC recombinants also became noninducible for PBSX. The high frequency of conversion of

Figure 9

UV dose dependence of cell survival and PBSX induction. The PBSX titers are expressed as the reciprocal of the highest dilution which produced a cleared zone on a lawn of W23 cells. Survival of Xin⁻ isolate 219, ▲; BR95, ●; GB26 recA, o; PBSX induction in BR95, Δ; Xin⁻ isolate 219, ◘.

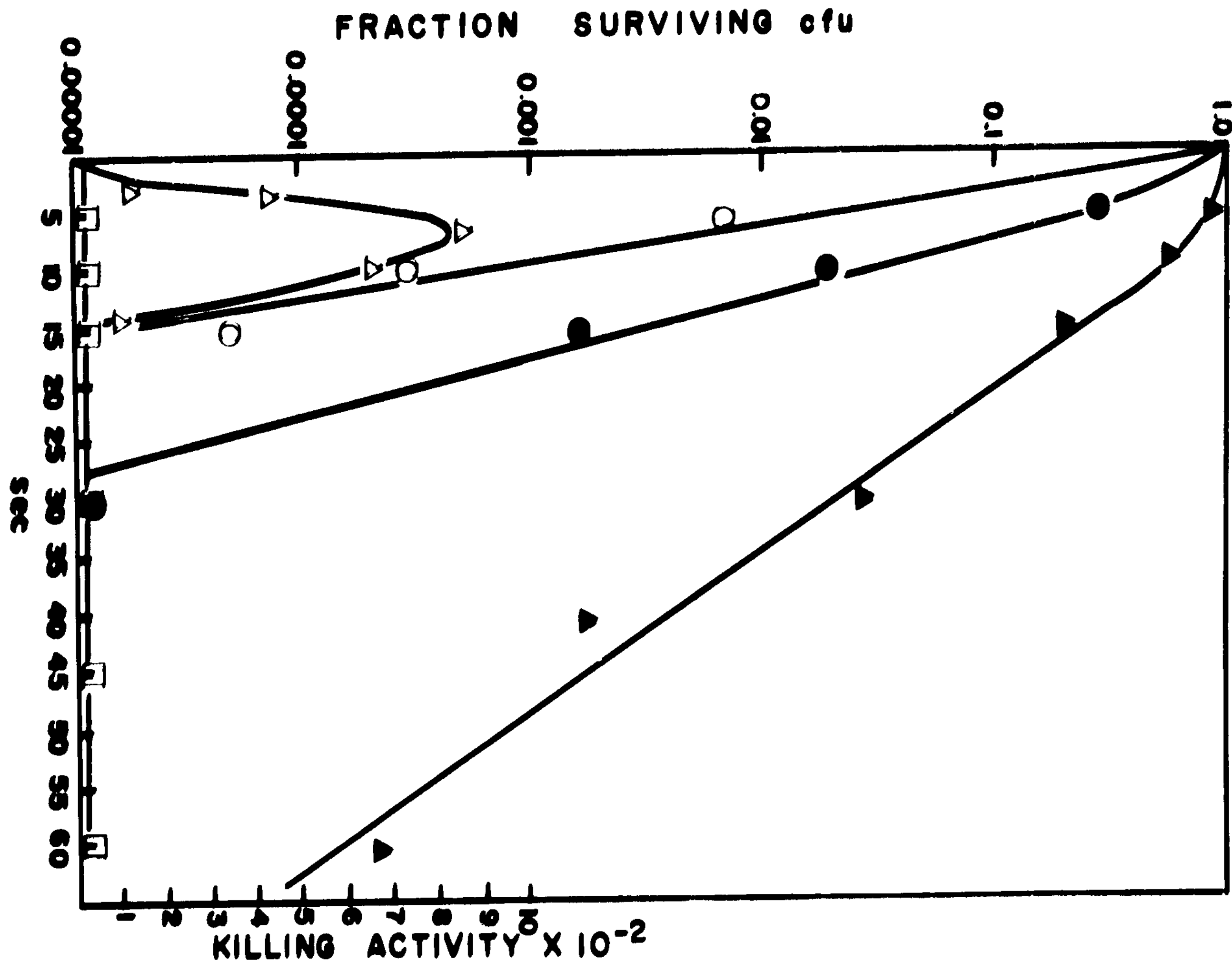

FRACTION SURVIVING cfu
0.00001
0.0001
0.001
0.01
0.1
1.0
sec
5
10
15
20
25
30
35
40
45
50
55
60
KILLING ACTIVITY X 10^-2
1
2
3
4
5
6
7
8
9
10

TABLE 4

INDUCTION OF SPO2 AND φ105 PROPHAGES

IN STRAIN 219

Strain	MC Induction[a]	
	Yielders[b]	Burst
BR 95 (φ105)	1.2	109
GB 219 (φ105)	1.8	79
BR 95 (SPO2)	1.4	108
GB 219 (SPO2)	1.1	86

[a]Cultures of φ105 and SPO2 lysogens, grown in VY to a density of 25 klett units, were treated with 0.4 μg/ml and 0.3 μg/ml MC respectively. After 90 min. lysates containing SPO2 were titered while cultures of φ105 lysogens were incubated for 30 minutes with $CHCl_3$ before titering.

[b]Yielders: ratio of infectious centers, after MC addition but before burst, to viable cells present before MC.

TABLE 5

PBS1 MEDIATED TRANSDUCTION OF THE

xtl-1 MARKER FROM ISOLATE 219

Donor: GB 219 pheA, ilvC, tryC, xin

Recipient: GB 219 metC, pyrA, xtl-1

Selected marker: metC

metC	xtl-1	xin	No. of Recombinants
1[a]	0	0	37
1	1	0	9
1	N. S.	1	19
			Total 65

Minimal percentage converted to Xtl^{+} 9/65 = 13.9%

Maximum percentage converted to Xtl^{+} 28/65 = 43.1%

[a]"1" and "0" refer to donor and recipient phenotypes respectively. N. S. means that the marker could not be scored because of the noninducible phenotype of the recombinant.

Xin^{+} to Xin^{-} demonstrates that the mutation responsible for the noninducible phenotype contransduces with *metC*. This observation was confirmed by determining the cotransfer frequency of mutation 219 with *metC* (Table 6).

C. Genetic Mapping of PBSX Mutants

The mutation responsible for the Xhd^{-} phenotype of isolate 1323 and the Xtl^{-} phenotype of isolate 159 are also linked to *metC*. Three factor crosses using PBS1 mediated transduction were performed to order the mutations with respect to *argC* and *metC*. The *xtl*-1 mutation was also remapped at this time since previous work (Garro *et al*., 1970) had positioned this marker to the right of *metA* on the *B. subtilis* map but failed to resolve its location with respect to *metC*. All the PBSX specific markers mapped between *argC* and *metC* (Table 7a, b, c, d). The cotransfer frequency (CF) between *argC* and *metC* varied from .12 to .23 for individual transducing lysates. By correcting the contransduction frequency between *argC* and the PBSX markers for this variation [CF = (CFx to *argC*)(.23/CF *metC* to *argC*)], and subtracting the corrected values from 1.0, relative recombination frequencies can be calculated (Table 8). The following map order was deduced:

argC *xin* *xtl*-3 *xhd* *xtl*-1 *metC*

TABLE 6

LINKAGE VALUES BETWEEN MUTATION 219 AND AUXOTROPHIC MARKERS OBTAINED BY TWO FACTOR CROSSES MEDIATED BY PBS1 TRANSDUCTION

Selected Marker	% Cotransfer of Mutation 219
purA	0 (0/67)[a]
metC	67 (54/80)

[a]The fraction in parenthesis represents the number of cells converted to prototrophy over the number of cells tested.

TABLE 7

ANALYSIS OF THREE FACTOR TRANSDUCTION CROSSES

INVOLVING THE xhd, xtl and xin MARKERS

7a

Donor: GB 1323, xhd, pheA, ilvC, trpC
Recipient: GB 64, argC, metC, pyrA
Selected phenotype: $argC^+$

argC	xhd	metC	No. of Recombinants
1	0	0	73
1	1	0	12
1	0	1	0
1	1	1	11
			Total 96

Frequency of cotransfer of xhd with argC: 23/96 = 0.24

Frequency of cotransfer of metC with argC: 11/96 = 0.12

Suggested order of markers: argC, xhd, metC

7b

Donor: GB 159, xtl-3, pheA, ilvC, trpC
Recipient: GB 64, argC, metC, pyrA
Selected phenotype: $argC^+$

argC	xtl	metC	No. of Recombinants
1	0	0	81
1	1	0	21
1	0	1	0
1	1	1	14
			Total 116

Frequency of cotransfer of xtl-3 with argC: 35/116 = 0.30

Frequency of cotransfer of metC with argC: 14/116 = 0.12

Suggested order of markers: argC, xtl-3, metC

TABLE 7

(CONTINUED)

7c

Donor: GB 75, xin, metC, pyrA
Recipient: BD 71 argC, hisA, pyrA
Selected phenotype: $argC^{+}$

argC	xin	metC	No. of Recombinants
1	0	0	52
1	1	0	37
1	0	1	0
1	1	1	21
			Total 110

Frequency of cotransfer of xin with argC: 58/110 = 0.53

Frequency of cotransfer of metC with argC: 21/110 = 0.19

Suggested order of markers: argC, xin, metC

7d

Donor: 44AO, xtl-1
Recipient: GB 64, argC, metC, pyrA
Selected phenotype: $argC^{+}$

argC	xtl-1	metC	No. of Recombinants
1	0	0	54
1	1	0	16
1	0	1	2
1	1	1	19
			Total 91

Frequency of cotransfer of xtl-1 with argC: 35/91 = 0.39

Frequency of cotransfer of metC with argC: 21/91 = 0.23

Suggested order of markers argC, xtl-1, metC

[a]"1" and "0" refer to donor and recipient phenotypes respectively.

TABLE 8

RECOMBINATION FREQUENCIES

BETWEEN *argC* AND PBSX MARKERS

	Corrected CF[a]	Recombination Frequencies
argC and:		
xin	.636	.364
xtl-3	.480	.520
xhd	.456	.544
xtl-1	.390	.610

[a]CF was computed as described in the text.

D. Other Sites Suggested for PBSX Genes

As mentioned in the Introduction, the presence of PBSX genetic determinants at a site in the vicinity of the origin proximal purA marker and also at a site linked by PBS1 transduction to purB has been suggested. It has already been shown that the purB linked mutation which causes the thermal induction of PBSX also causes induction of SPO2 (Siegel and Marmur, 1969). It was of interest therefore to examine the effect of this mutation on other B. subtilis temperate phages such as ϕ105. As seen in Figure 10, ϕ105 is also induced at the restrictive temperature in this strain indicating that other temperate phages of B. subtilis 168 are also affected by this mutation.

The suggested localization of PBSX genes near the origin of host chromosome replication was based on marker frequency analysis which demonstrated that MC treated cells induced for PBSX were also derepressed for replication of the origin proximal purA marker (Haas and Yoshikawa, 1969b). To demonstrate that this derepression and the resultant enrichment of purA marker DNA in PBSX heads is a result of multiforked initiations at the chromosomal origin, the frequency of another origin marker in PBSX DNA was examined. Table 9 indicates that PBSX DNA is enriched for the origin marker sacP as well as for purA. In all previously reported experiments, the frequency of the PBSX proximal marker metC was not determined. Therefore marker frequency

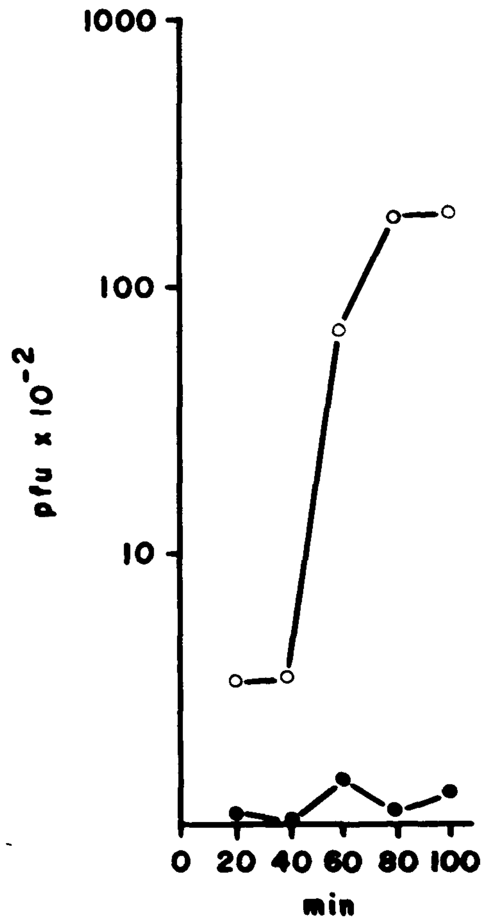


Figure 10

Thermal induction of ϕ105 from GB1001(ϕ105). A culture growing exponentially in VY at 32° was diluted 1:100 into VY at 42°. Samples were taken at 20 min. intervals and assayed for phage. -o-o-, shifted to 42°C; -●-●-, maintained at 32°C.

TABLE 9

MARKER FREQUENCY ANALYSIS OF THE DNA FOUND IN PBSX HEADS[a]

	sacP[c]	purA	leuA
Source of DNA			
GB 1 cells[b]	4.2	2.3	1.3
PBSX produced by GB 1 cells	32.3	59.0	2.7

[a]Marker frequency analysis has been described by Yoshikawa et al. (1964). The marker frequency of spore DNA was used to normalize all values. The efficiency of transformation was standardized to transformation of the metB marker. Transformations were performed with 0.05 μg DNA/ml competent cells.

[b]DNA was isolated from a culture of GB-1 cells that was treated with 0.4 μg/ml MC and immediately poured over an equivalent volume of ice, 0.4 M NaN_3.

[c]The sacP marker was monitored as described by Lepesant and Dedonder (1968).

analysis was performed using cellular DNA isolated at various times during the course of induction. Figure 11 indicates that the relative number of copies of the _metC_ marker, as well as the _purA_ marker, increases during induction of _xin_$^+$ strains (BR95). However, in MC treated _xin_$^-$ strains (Table 10) only the _purA_ marker is present in multiple copies, indicating that _purA_ replication occurs independently of PBSX induction. The absence of _metC_ replication in this strain confirms the notion that amplification of the _metC_ marker is uniquely related to induction of PBSX.

E. Prospective PBSX Mutants

One of the isolates, 440, had a phenotype (Prt$^-$, Kil$^+$) suggestive of a PBSX head mutant. It differed however from the known head mutant 1323 in that cell lysis was slow and incomplete. In addition strain 440 was found to be defective for another MC inducible activity that may be associated with phage heads; that is, fragmentation of the host chromosome into pieces of uniform molecular size (22s). Figure 12 indicates that the DNA in MC treated strain 440 consists of fragments of varying molecular size as opposed to the discrete 22s pieces found in induced cells having the wild type 440 allele. To study the molecular basis of the phenotype associated with mutation 440, the synthesis of PBSX structural proteins was analyzed by SDS gel electrophoresis (Figure 13B). During induction many host specific

Figure 11

The frequency of auxotrophic markers in MC induced B. subtilis cells. DNA was isolated from aliquots taken from an MC induced culture at the times indicated. Less than saturating levels of DNA were used in all transformations (0.05 μg transforming DNA/ml competent cells). The values obtained were normalized to spore DNA and the efficiency of transformation standardized to transformation of the leuA marker. —●—●—, metC; —X—X—, purA; —□—□—, metB.

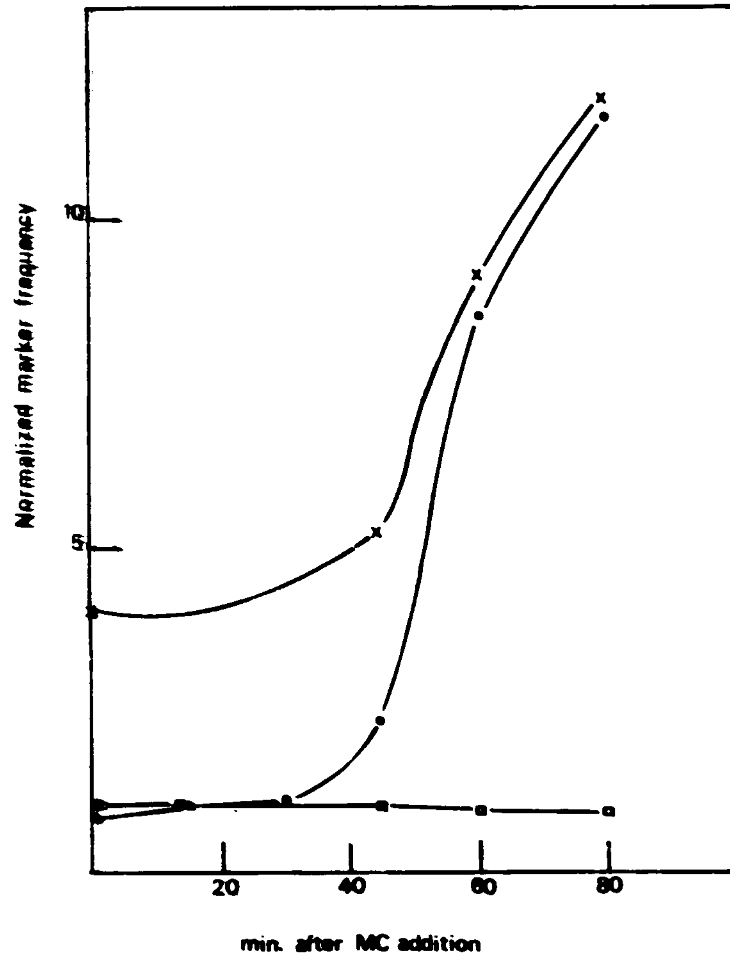

Normalized marker frequency
10
5
20
40
60
80
min. after MC addition

TABLE 10

MARKER FREQUENCY ANALYSIS OF MC TREATED

xin⁻ AND xin⁺ STRAINS

Strain[a]	Normalized Values[b]			
	purA	metC	leuA	metB
BR 95 (xin^+) T = 0	4.0	1.3	1.0	1.0
BR 95 (xin^+) T = 80	12.0	10.0	1.0	0.9
GB 219 (xin^-) T = 0	3.5	0.9	1.0	0.8
GB 219 (xin^-) T = 80	9.0	0.9	1.0	1.0

[a]Cells were grown in VY to a cell density of 23 klett units and induced with 0.4 µg/ml MC. DNA was isolated immediately after induction, T = 0 and after 80 min. of incubation, T = 80.

[b]The efficiency of transformation was standardized to transformation of the leuA marker. Values obtained with spore DNA were used for normalization. All transformations were at non-saturating concentrations of DNA.

Figure 12

Fragmentation of chromosomal DNA during induction of PBSX. ^{3}H DNA isolated from MC treated BR95 and 440 cells were prepared and processed as described by Okomato _et_ _al_. (1968). ^{14}C label T7 DNA (—O—O—), prepared according to Armentrout _et_ _al_. (1971), served as a molecular size marker (32s). BR95 DNA: (a) 0 min., (b) 90 min., (c) 120 min. post induction. 440 DNA: (d) 0 min., (e) 90 min., (f) 120 min., (g) 190 min. post induction. The arrow indicates the 22s position.

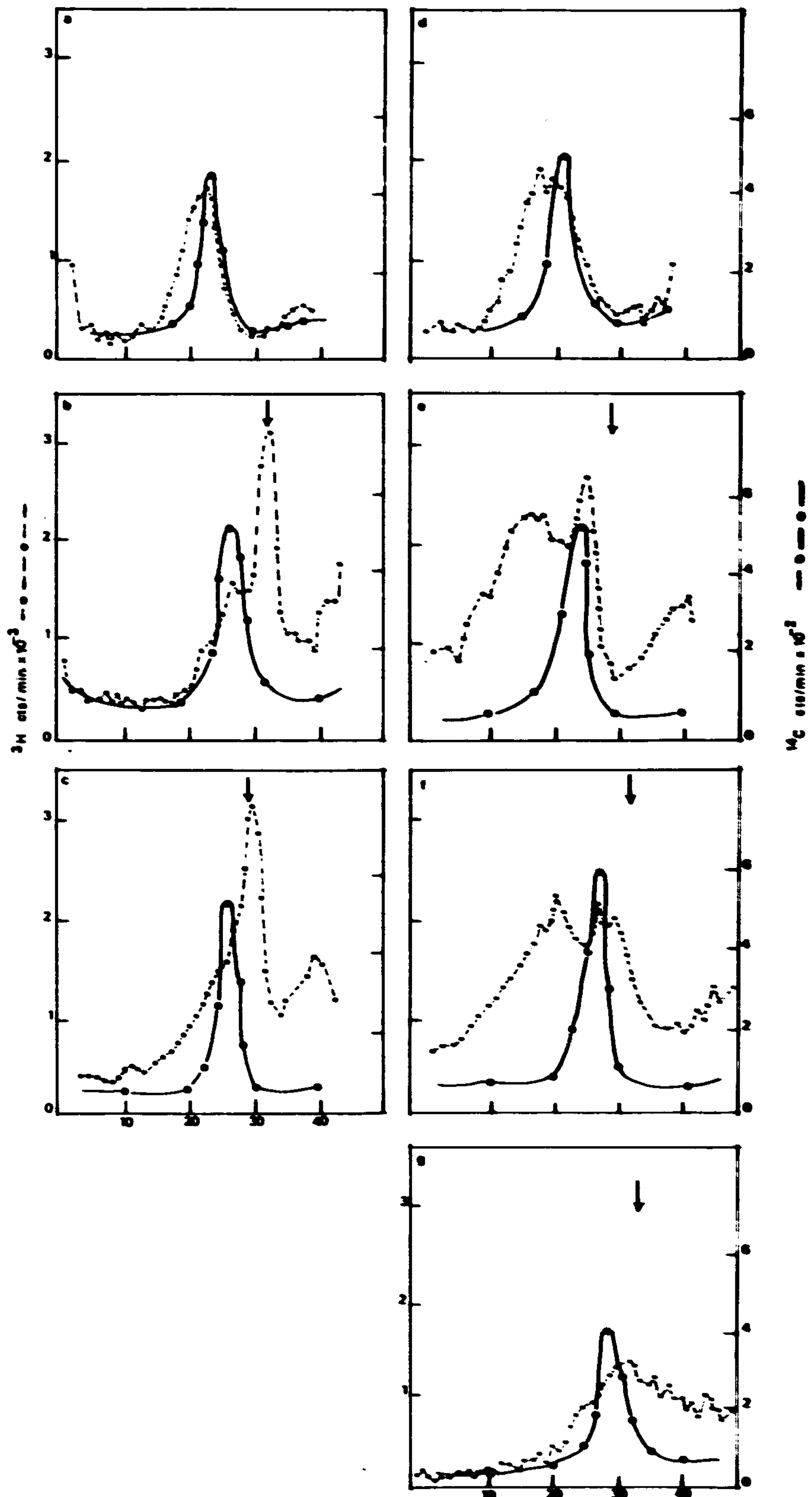
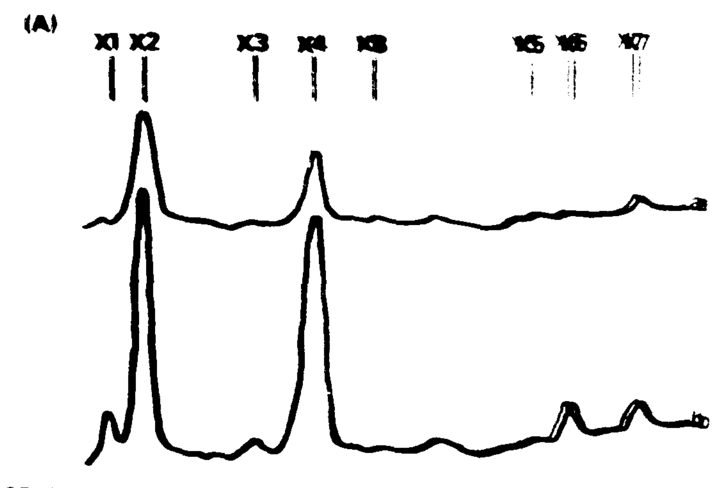


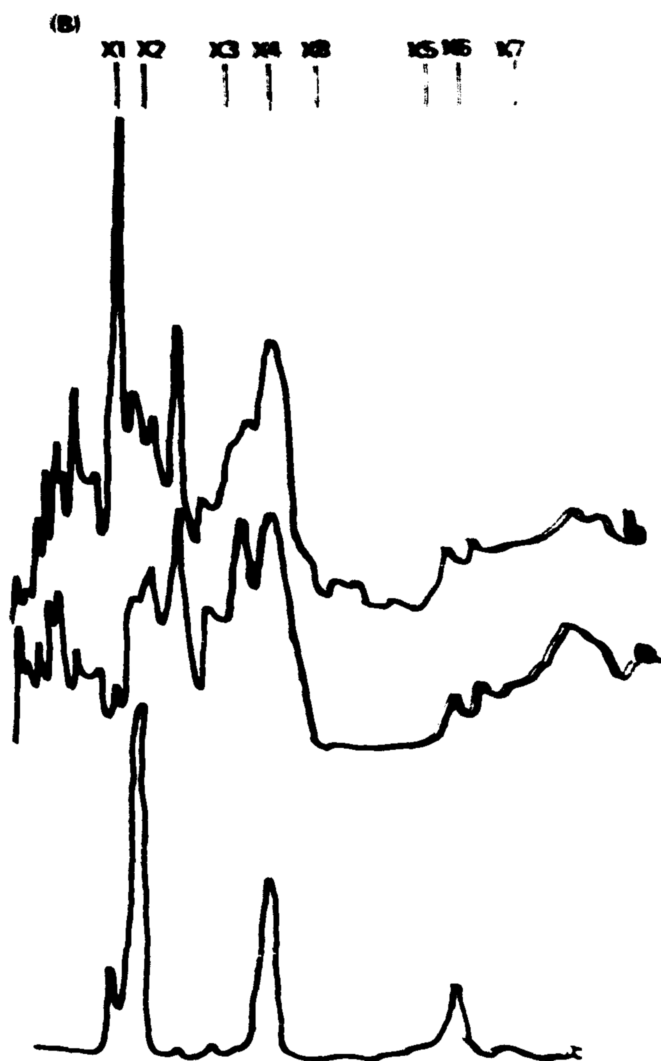
Figure 13

Proteins synthesized during MC induction of lysate 440.

A. Proteins immunoprecipitated from lysates of (a) isolate 440 and (b) BR95. MC treated cells were labeled for 10 min. with ^{14}C amino acids just before cell lysis began and PBSX proteins were immunoprecipitated as described in Materials and Methods. Proteins were resolved on 13% SDS gels.

B. Whole cell lysates of isolate 440 pulse labeled at (a) 80 min. and (b) 180 min. post induction. (c) purified PBSX.


(A)
X1
X2
X3
X4
a
b


(B)
X1
X2
X3
X4
b
a
c

proteins normally shut off, continue to be synthesized. This background obscures PBSX structural proteins which are produced in small amounts in strain 440. At 80 minutes post induction, only X6 can be identified while 100 minutes later (180 minutes post induction) synthesis of large amounts of protein X1 make it readily observable. The presence of all other structural proteins was detected by immunoprecipitation of cell lysate, radioactively labeled just before lysis began (Figure 13A). Interestingly, protein X6, identified in total cell lysates, could not be readily identified in immunoprecipitates. Electron microscopy of phage pelleted from lysates of isolate 440 revealed the presence of both PBSX tails and empty heads.

Strain 440 also shares some of the characteristics of cells having Rec type mutations. These include enhanced sensitivity to the killing effects of MC and UV light (Figure 14) and low level transformability. To determine if further analogies exist between 440 and Rec^- strains, the inducibility of ϕ105, SPO2 and PBSX prophages from strain 440 and four strains carrying different Rec mutations was compared. Preliminary data indicate that the pattern of prophage induction in strain 440 differs from all the Rec^- strains tested (Table 11).

F. Relationship of PBSX to Other B. subtilis Phages

Although the origin of the defective phages remains unknown, the possibility exists that they evolved from

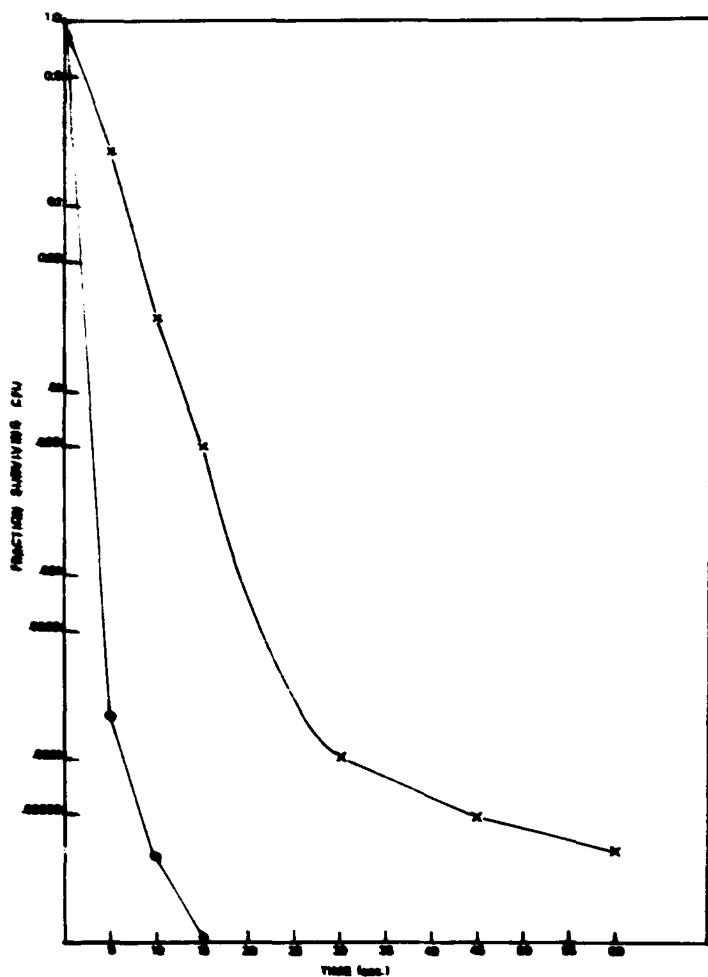


Figure 14

UV sensitivity of isolate 440. —●—●—, isolate 440; —X—X—, BR95.

TABLE 11

THE EFFECT OF Rec⁻ MUTATIONS AND MUTATION(S) 440 ON THE INDUCTION OF PBSX, ϕ105 and SPO2 PROPHAGES

	Bacterial Strains				
	BD170 (Rec$^+$)	BD194 (recA)	BD191 (recB)	BD246 (recG)	Isolate 440
Induction of:					
ϕ105					
Cell lysis[b]	+	-	+	-	±, lysis slow
Burst[c]	50	2	45	28	20
SPO2					
Cell Lysis	+	+	+	+	+
Burst	50	1	40	15	20
PBSX					
Cell Lysis	+	-	+	-	-
Titer/5λ Sample	512	128	256	16	512

[a]Induction was carried out as described in Table 4.

[b]Lysis of the host cell was defined by a gradual decline in the turbidity of the culture.

[c]Plaque forming units/infective center.

[d]Titering of PBSX samples was described in Figure 9.

infectious phages by loss of some function essential for infectivity. To study this possibility in the case of PBSX its infective counterpart was sought by testing the ability of anti-PBSX antiserum to neutralize plaque formation by a number of B. subtilis phages. The non-specific neutralizing effect of serum was eliminated by calculating the percentage reduction in plaque forming units as follows:

> [(plaque forming units (pfu) in the presence of normal rabbit serum -pfu in the presence of anti-PBSX antiserum) ÷ pfu in the presence of phage diluent] x 100

As indicated in Table 12, of the phage infectious for strain 168, SP8* showed significant cross neutralization. β22 had a similar percentage reduction in pfu; however the reliability of this result is questionable because of the poor visability of β22 plaques. Phage SP10, infecting W23 strains, also shows a low level of cross neutralization.

G. Relationship of PBSX to Another B. subtilis Defective Phage, PBSZ

The defective phage PBSZ produced in B. subtilis W23 strains is closely related to PBSX and has been shown to react with anti-PBSX antiserum (Huang and Marmur, 1970b; Subbaiah, Unpublished Results). Immunoprecipitates formed by reacting anti-PBSX antiserum with MC induced lysates of W23 cells were found to contain seven proteins designated Z1, Z2, Z3, Z4, Z8, Z6 and Z7, presumed to be the structural proteins of PBSZ. Of these, five proteins band at positions

TABLE 12

NEUTRALIZATION OF *B. subtilis* PHAGES BY ANTI-PBSX ANTISERUM[a]

Phage	SP8*	SPO1	β22	SP82	SP10	φ1	φε	TSP1
Host: *B. subtilis*	168	168	168 or W23[b]	168	W23	168	168	168
% Reduction of pfu	28	<0.01	28	<0.01	8	<0.01	<0.01	<0.01

[a]Samples of each phage were diluted in phage diluent (10^{-2} M Tris HCl, pH 7.4, 5×10^{-3} M $MgCl_2$, 0.001% gelatine), to a concentration appropriate for plating and treated with either phage diluent, anti-PBSX antiserum, or normal serum for 1 hr. at 37° C before plating. The percentage reduction of pfu was calculated as described in the text.

[b]*B. subtilis* 168 cells were used in this study to plaque β22

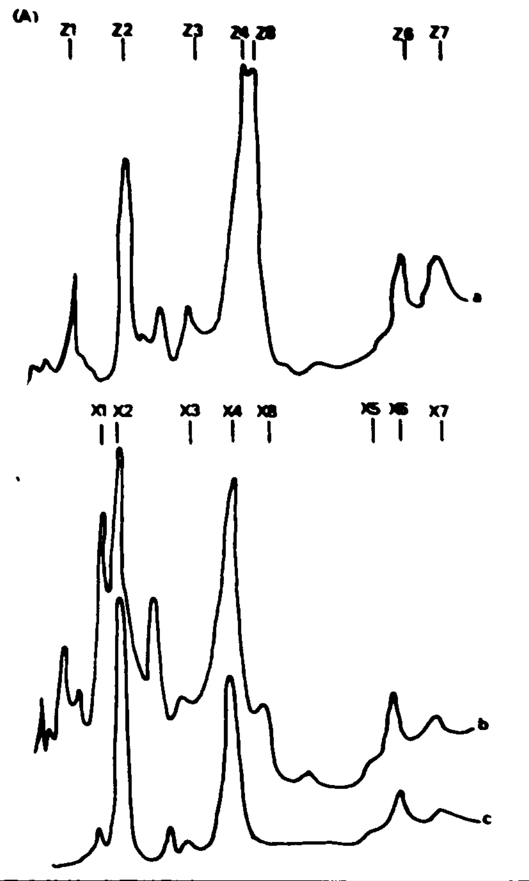
identical to PBSX structural proteins while Z1 and a minor band Z8 are found to have higher molecular weights than their presumed PBSX counterparts (Figure 15a). It is difficult to discern whether a protein analogous to X5 is present. Figure 15b indicates that compared to other PBSZ proteins greater amounts of Z1, Z7 and Z8 are synthesized during induction than are immunoprecipitated. Similar observations for PBSX proteins X1 and X7 and for X8 produced in mutant 159 suggest that Z1 and Z8 may be the structural counterparts of X1 and X8.

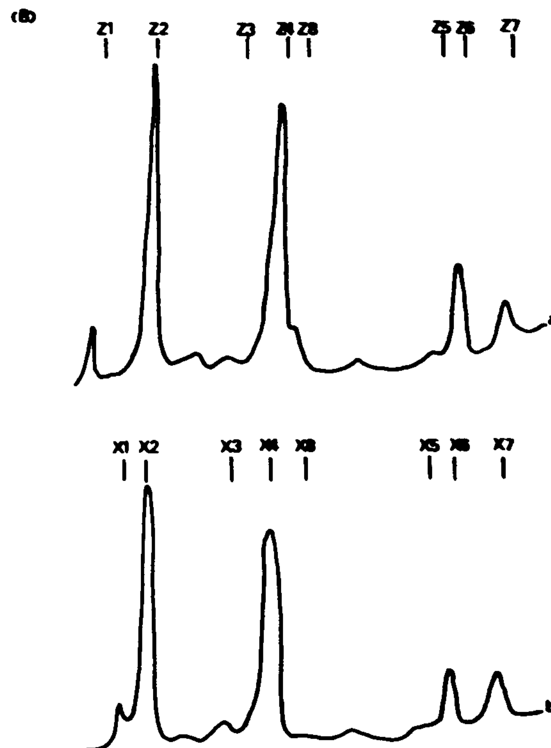
Figure 15

Proteins synthesized during induction of PBSZ.

A. Whole cell lysate of (a) strain W23 and (b) strain BR95 pulse labeled with ^{14}C amino acids for 3 min. at 75 min. post induction; (c), purified PBSX.

B. Immunoprecipitate of (a) strain W23 and (b) strain BR95 labeled for 10 min. with ^{14}C amino acids at 75 min. post MC induction.


(A)
Z1
Z2
Z3
Z4
Z8
Z6
Z7
a
X1
X2
X3
X4
X8
X5
X6
X7
b
c


(B)
Z1
Z2
Z3
Z4
Z8
Z5
Z6
Z7
a
X1
X2
X3
X4
X8
X5
X6
X7
b

CHAPTER IV

DISCUSSION

A. Structural Proteins of PBSX and PBSZ

Seven PBSX structural proteins, X1 through X7, were resolved by SDS gel electrophoresis of purified particles. An eighth protein, X8, which appears to be unstable is induced by MC along with the PBSX structural proteins. Although X8 could not be unequivocally identified as a phage coded protein, it seems likely that it is either a non-structural protein or a precursor of a PBSX structural protein. Two lines of evidence suggest a product-precursor relationship between X8 and X5 similar to that reported for some of the structural components of a number of DNA phages (Laemmli, 1970; Zweig and Cummings, 1973). First, X8 is only seen under pulse labeling conditions in which X5 is not detected; second, in the xtl-3 mutant, a protein banding at the position of X8 is immunoprecipitated by anti-PBSX antiserum while X5 is not. However an unequivocal relationship between X5 and X8 was not established because the origin of X5 is obscured by the presence in induced cells of two unstable proteins, X1 and X8.

Proteins X4 and X7 were presumed to be head proteins since they could not be detected in preparations of isolated PBSX tails. The validity of this classification is contingent on the assumption that none of the tail proteins were lost

prior to or during the isolation procedure. Although the killing activity in the tail preparation was significantly lower than that expected from the amount of PBSX specific protein present, it need not be attributed to loss of a tail protein. The more likely reason for this low level of activity is the large proportion of disintegrating, disjointed and contracted tails in the preparation. That the contracted state of the PBSX tail can interfere with its killing activity is suggested by the fact that the morphologically similar pyocin R particle is rendered biologically inactive through contraction of its tail sheath (Coetzee et al., 1968; Higard et al., 1967). Further evidence that both X4 and X7 are head proteins is that they are simultaneously affected by the xhd mutation.

Five of the structural proteins of PBSX and the related B. subtilis phage PBSZ have identical molecular weights as determined by their migration in SDS polyacrylamide gels. This observation, taken together with the known serological cross-reactivity between PBSX and PBSZ, suggests that at least some of these proteins may be similar and serve similar structural functions in the two phages. However which of them is responsible for the immunoprecipitability of PBSZ by anti-PBSX antiserum remains unknown. Two of the PBSZ proteins, Z1 and Z8, differ in

their molecular weights from any of the PBSX specific proteins. Their presumed PBSX counterparts are X1 and X8 (X5?) suggesting that Z1 and Z8 are tail proteins. Variations in the tail proteins of PBSX and PBSZ are expected on the basis of their different absorption and killing spectra. These observations on the structural relationship between PBSX and PBSZ lend support to a theory of evolution of the three related *B. subtilis* defective phages proposed by Subbaiah *et al.* (1965). According to this theory, all three prophages evolved in a single bacterial strain. Selection favored bacterial strains in which mutations in the prophage altered the specificity of both the cell wall receptor of the producer strain and the tail of the phage it produced. Altered tails allowed the phage to absorb to previously resistant cells thus eliminating ecologically competitive strains while the altered receptor of the producer strain protected it from the killing activity of its own defective phage.

B. Mutations *xhd*, *xtl* and *xin*

The *xhd* and *xtl*-3 mutations interfere with the assembly of PBSX head and tail structures respectively. Although no alterations of the structural proteins produced by the two mutants was evident by SDS gel electrophoresis, serological differences were observed. Antisera to PBSX failed to precipitate proteins X6 and possibly X5 from lysates of the strain carrying mutation *xtl*-3 while a

protein at the position of X8 was evident. Whether this latter protein is identical to X8, which may be normally cleaved during maturation of tail, is uncertain. In lysates of strains carrying the xhd mutation the two proteins X4 and X7 were not immunoprecipitable. The fact that the serological reactivity of several proteins is simultaneously affected does not necessarily imply that the strains are carrying multiple closely linked mutations. Although this possibility cannot be eliminated, a more likely explanation is that the antiserum, raised against whole PBSX particles, does not in all cases recognize individual structural polypeptides but recognizes instead antigenic determinants formed by structural polypeptides that have been processed and/or assembled during the long labeling period. Non-immunoprecipitability of an individual polypeptide therefore does not necessarily imply that the mutation lies in the gene coding for that polypeptide as mutations in other phage genes may indirectly affect its processing and assembly into a serologically reactive entity.

The xin mutation interferes with MC and UV induced derepression of PBSX prophage and may be analogous to the λ Ind$^-$ mutations. These λ mutations have been localized in the phage repressor gene and lead to the production of an altered repressor protein no longer derepressible by UV or MC (Jacob and Campbell, 1959; Ogawa and Tomizawa, 1967; Ptashne, 1967). As in the case of λ Ind$^-$ lysogens,

strains carrying the xin mutation are more UV resistant than xin^+ strains presumably because of elimination of prophage induction which is lethal to the cell.

C. Potential PBSX Mutants

Preliminary genetic analysis of strain 440 (A. Garro, Personal Communication) has indicated that it is carrying multiple mutations. This was shown by the ability to separate the mutations responsible for the UV sensitivity and the non-transformability of the strain. The difference in the induction pattern of ϕ105, SPO2 and PBSX prophages in strain 440 and in the Rec^- strains may therefore be the result of the combined effects of two or more mutations. Pol^- and Uvr^- mutations are also known to increase sensitivity to UV and MC (Gass et al., 1971; Laipus and Ganesan, 1972). λ Lysogens of E. coli strains carrying either Pol^- or Uvr^- mutations are inefficiently induced by UV doses normally used for inducing λ from wild type strains (Witkin and George, 1973). If the UV sensitivity of strain 440 is due to either of these mutations, MC doses routinely used for induction may have been too high to efficiently induce PBSX. This would account for the defects in PBSX associated activities such as the inefficient fragmentation of the host chromosome and the poor induction of PBSX specific protein synthesis observed in this strain. The possibility still exists, however, that the defects in PBSX associated activities are due to a separate mutation in the PBSX

prophage. The possibility also exists that the defect in PBSX mediated fragmentation of the host chromosome and the non-transformability of the strain are pleiotrophic effects of a single mutation. This is based on a possible association between transformability of a strain and the presence of a PBSX prophage suggested by the similar size of PBSX DNA and the double stranded DNA intermediate believed to be involved in the transformation process (Dubnau and Cirigliano, 1972).

D. Genetic Site(s) of PBSX Genes

No evidence was found for wide scattering of PBSX prophage genes across the *B. subtilis* chromosome. The three mutations, affecting both structural and control functions are presumed to span a significant portion of the PBSX prophage. Yet they all mapped in the same region of the chromosome, *met*C, as does the previously described mutations *xtl*-1 and *xtl*-2. The latter mutations, definitively localized to the right of *met*A on the *B. subtilis* map, have cotransfer frequencies to *met*C similar to the PBSX mutations described here. This suggests that PBSX prophage genes are clustered between *met*A and *met*C. The recombination frequencies observed in PBS1 mediated crosses indicate a map order of *xin*, *xtl*-3, *xhd*, *xtl*-1. However, it is not possible at the present time to confirm this order by fine structure mapping as recombinants for these markers cannot be selected directly in genetic crosses.

The results presented here provide alternative explanations for the data suggesting that PBSX genes are located in the vicinity of either purB or the origin proximal purA markers. The observation that the purB linked tsi mutation causes the thermal inducibility of ϕ105 as well as the previously reported induction of PBSX and SPO2 indicates that this mutation is analogous to the E. coli cellular mutation tif, which produces thermal derepression of inducible coliphages (Kirby et al., 1967). With respect to the purA locus, marker frequency analysis of DNA in MC treated xin mutants demonstrates that MC induced multiforked initiations at the chromosome origin occurs independently of PBSX induction. Furthermore the lack of involvement of purA in PBSX induction is supported by the apparent absence of multiforked initiations during thermal induction of a mutant temperature sensitive for PBSX induction (Seigel and Marmur, 1969). Depression of chromosome replication appears to be one of many functions inducible by UV and presumably MC (Bridges, 1972).

E. Replication of PBSX

The ten fold increase in the frequency of the metC marker following exposure of xin^+ (but not xin^-) cells to MC suggests that derepression of PBSX enables enhanced replication of adjacent bacterial genes. This can either occur if the prophage detaches from the bacterial chromosome

with the adjoining bacterial genes and then replicates autonomously or if the derepressed PBSX prophage replicates *in situ* enabling the replication machinery to read through to adjacent markers. The former possibility seems unlikely as Haas and Yoshikawa (1969b) were unable to detect DNA fragments of the presumed prophage size until late in induction when fragmentation of chromosomal DNA for packaging into PBSX heads had already begun. *In situ* replication is known to occur in the *B. subtilis* phage ϕ105 and also results in amplification of adjoining bacterial genes during phage induction (Armentrout and Rutberg, 1971).

F. Possible Evolution of PBSX

That PBSX may be related to infectious phages is suggested by the serological relationship between PBSX and the infectious *B. subtilis* phages SP8* and SP10 and by the apparent sensitivity of SP10 to the PBSX repressor (Goldberg and Bryan, 1968). However, it is unclear whether PBSX (as well as the other defective phages) is the evolutionary antecedent of infectious phages or whether it is derived from infectious phage by physical and functional loss of genetic information. The ability to generate defective phage from infectious ones lends support to the latter hypothesis. A defective form of λ, λ docL, has been generated by inducing lysogens under restrictive conditions causing the incorrect excision of an otherwise

intact prophage. Particles containing this DNA are rendered non-infectious by the absence of the right arm of the λ chromosome (Little and Gottesman, 1971). Interestingly, the absence of the right cohesive DNA end also results in a PBSX-like inability to inject DNA.

Another model for the generation of defective phage also involves malfunctioning of prophage excision process. Smith (1966) isolated a mutant of the temperate phage P22 having a defect in a gene involved in prophage excision. Like PBSX, induction of the P22 mutant prophage leads to production of predominantly non-infectious particles containing almost exclusively host DNA, specifically enriched for prophage proximal markers. These characteristics are attributed to the absence of phage specific integrase function which leads to both in situ replication and subsequent inability to excise intact prophage genomes. The striking physiological similarities of PBSX induction to the P22 mutant and to the less well characterized λ docL suggests that PBSX may be derived from an infectious phage ($SP8^*$? SP10?) by loss of functional integrase system. Superimposed on the defective excision process or perhaps integral to it may be the effects of PBSX's small head capacity. That the PBSX head may indeed be unable to contain a full complement of PBSX genetic information is suggested by the unusually large proportion of the coding capacity (85%) of DNA of the size packaged in PBSX heads needed to account for PBSX specific proteins (Adolph and Haselkorn, 1972; Mendez et al., 1971).

PART II

ON THE NATURE OF CROSS-LINKS IN CELLULAR DNA

CHAPTER I

INTRODUCTION

It is well documented that when DNA isolated from various sources is denatured, a fraction of the molecules spontaneously regain native conformation upon removal of denaturing conditions. The double stranded nature of this fraction has been established on the basis of its residual transforming activity (Marmur and Lane, 1961; Ginoza and Zimm, 1961), density in CsCl gradients (Rownd et al., 1968), elution from hydroxyapatite (Bernardi, 1969), distribution in dextran polyethylene-glycol polymer systems (Alberts and Doty, 1968) and visualization by electron microscopy (Barzalai and Thomas, 1970). Using biophysical techniques, Alberts and Doty (1968) demonstrated that the unusual renaturation properties are the result of a covalent link which appeared to be located near one end of the double stranded molecule.

Cross-links may either be of biological origin or they may be extracellularly introduced artifacts. Although any metabolic functions involving enzymes capable of cross-linking DNA may be the source of naturally occurring cross-links, research has focused on DNA replication because the hairpin structure of cross-linked DNA is similar to the DNA intermediate predicted by the knife and fork model for DNA replication (Guild, 1968; Kornberg, 1969). In this model

the problem of replicating the DNA strand with 5' to 3' polarity in the absence of known polymerases capable of 3' to 5' synthesis, is surmounted by postulating that the polymerase replicating the strand with 3' to 5' polarity continues 5' to 3' synthesis on the exposed complementary strand. The resultant hairpin is eventually cleaved to two complementary strands. As predicted by this model, the most newly synthesized DNA was found to form the cross-linked fraction in both T7 (Barzilai and Thomas, 1970) and *E. coli* DNA (Pauling and Hamm, 1969).

There are, however, several observations which argue against DNA replication being the source of cross-links. Cross-links are not found preferentially in replicating markers of *B. subtilis* DNA and they are found in non-replicating spore DNA (Alberts, 1968). Alberts (1968) has proposed that cross-links are artifacts induced by the mechanical shear forces which fragment large DNA molecules during DNA isolation. This proposal was based on the observations that the proportion of cross-linked DNA molecules was constant even when the DNA was derived from cells grown under a wide range of metabolic conditions or from cells of diverse procaryotic and eucaryotic species. Furthermore, λ DNA which had not been sheared during isolation because of its relatively low molecular weight was free of cross-links. However, because cross-links could not be induced experimentally by shearing λ sized DNA molecules

to smaller pieces, Alberts suggested that only shear forces acting on large DNA molecules (>200 x 10^6 molecular weight) efficiently generate cross-links. The position of the cross-link near the ends of the molecules suggested a model in which shear induced covalent bond rupture produced transient formation of either free radicals or ion pairs which by further reaction produced a new covalent union connecting opposite strands.

PBSX is uniquely suited to determine if the cross-links found in cellular DNA are shear induced artifacts because cellular DNA found in PBSX has been cleaved intracellularly to a size no longer susceptible to shear induced cross-linking. Furthermore as PBSX can be thermally induced in a B. subtilis mutant temperature sensitive for PBSX production the complication of MC induced cross-linking can be avoided. The aim of the studies presented here was to determine whether cross-links are present in cellular DNA isolated from PBSX heads and to examine other possible sources of naturally occurring cross-links in DNA.

CHAPTER II

RESULTS

A. Detection of Cross-links in DNA Isolated from PBSX

The DNA isolated from purified PBSX particles was alkali denatured and the residual transforming activity examined after neutralization at 0°C (Table 13). Both DNA isolated from whole cells (chromosomal DNA) and the cellular DNA isolated from PBSX particles (PBSX DNA) have similar residual transforming activities. The lower values obtained at less than saturating concentrations of transforming DNA are a better indication of the percent cross-linked molecules, since, under these conditions, the number of transformants produced is linearly related to the DNA concentration in both native and denatured samples.

Hydroxyapatite chromatography was used to confirm the double stranded nature of the cross-linked molecules. Figure 16 demonstrates that the majority of molecules responsible for the residual transforming activity in chromosomal and PBSX DNA eluted at a salt molarity characteristic of their respective double stranded DNAs. Native-like DNA amounted to approximately 3.7% and 3.2% of the recovered DNA of chromosomal and PBSX DNA respectively. Assuming that neither double nor single stranded DNA species are preferentially retained by the column and cannot be recovered, the percent of native-like molecules in the input DNA is identical to that of the recovered DNA.

TABLE 13

RESIDUAL TRANSFORMING ACTIVITY OF ALKALI DENATURED CELLULAR AND PBSX DNA

DNA Source	Native DNA Concentration	trp^+ Transformants/ml Native DNA	trp^+ Transformants/ml Denatured DNA	Residual Transforming Activity[b] (%)
GB 21*	Saturating	138 x 10^3	300 x 10'	2.2
GB 21*	<Saturating	67 x 10^3	67 x 10'	1
PBSX*	Saturating	917 x 10^3	292 x 10^2	3.2
PBSX*	<Saturating	335 x 10^3	513 x 10'	1.5

[a]GB 21*, a mutagenized derivative of GB 21, is temperature inducible for PBSX. The mutation(s) underlying this phenotype have not been further characterized. PBSX* was induced from GB 21* by shifting the culture to 48°C when it reached a cell density of 25 klett units. After cell lysis, PBSX was harvested and purified as described in the Materials and Methods section.

[b]The residual transforming activity was determined by comparing the transforming activities of native DNA and an equivalent amount of denatured DNA.

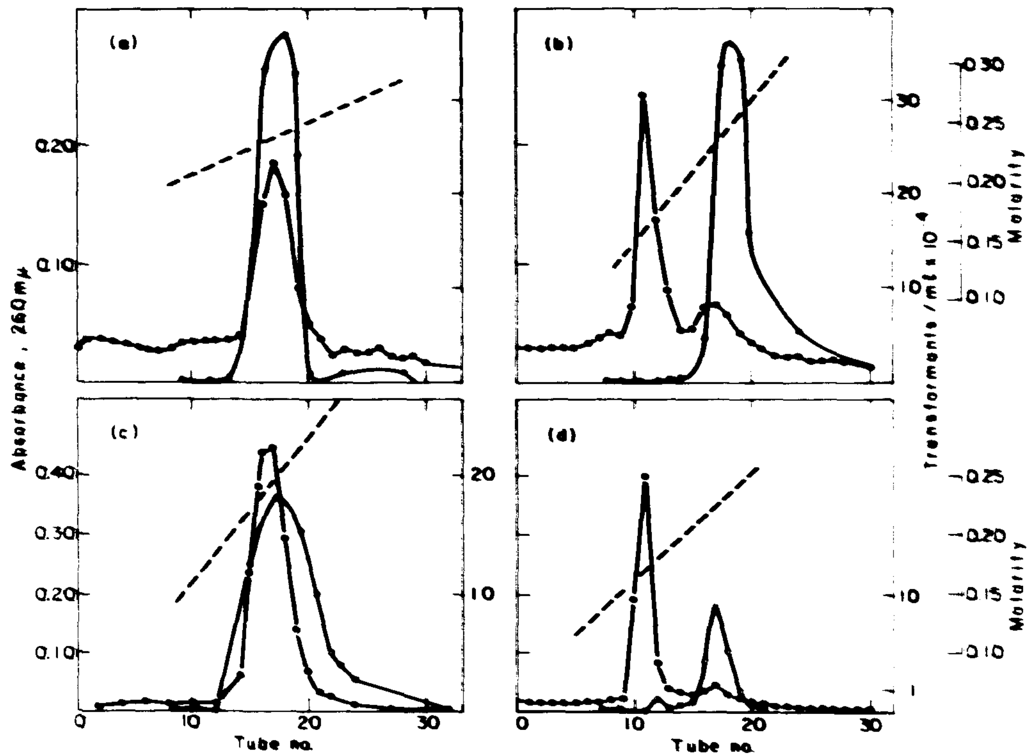


Figure 16

Hydroxyapatite chromatography of cellular and PBSX DNA. 200-300 μg of either native or alkali denatured DNA in .001 M sodium phosphate buffer pH 6.8 were applied to each column. A linear gradient ranging from 0.001 M to 0.5 M sodium phosphate buffer, pH 6.8 was used to elute the DNA. Molarities of the eluted samples, determined by a Zeiss refractometer, are indicated by the broken line. (a) GB21* DNA, native; (b) GB21* DNA, alkali denatured, 70% recovery; (c) PBSX* DNA, native; (d) PBSX* DNA, alkali denatured, 80% recovery, —O—O—' O. D. 260; —●—●—, transformation of the trpC marker.

B. Detection of Cross-links in DNA Isolated from Rec$^-$ Strains

Cells defective for recombination functions (Rec$^-$) are potentially missing enzymes capable of cross-linking DNA. To test the association between these functions and naturally occurring cross-links, several Rec$^-$ strains were examined for residual transforming activity. Table 14 indicates that cells carrying *recA*, *recB*, *recC*, *recD*, *recE* and *recG* mutations have residual transforming activities ranging from 0.8-2.2%. Based on its elution properties from hydroxyapatite the molecules responsible for residual transforming activity in *recA* strains are double stranded and represent approximately 1% of the DNA (Figure 17).

TABLE 14

RESIDUAL TRANSFORMING ACTIVITY OF DNA ISOLATED FROM B. SUBTILIS STRAINS CARRYING Rec MUTATIONS[a]

Strain	Locus	Class	DSF	SSF	DRC[b]	Transformation	Transduction		Residual Transforming Activity (%)[c]
							Homologous	Heterologous	
GB 26	recA1	I	+	+	-	-	+	-	0.8
BD 191	recB	IIa	+	+	+	-	-	-	2.1
BD 193	recD	IIb	+	+	-	-	-	-	1.5
BD 224	recE	IIb	+	+	-	-	-	-	1.5
BD 239	recC	III	+	+	+	-	+	+	1.2
BD 246	recG	IV	+	+	+	+	-	-	1.0
BD 170	Rec$^+$		+	+	+	+	+	+	3.1

[a]The Rec$^-$ mutations involved in this study have been previously described (Dubnau et al., 1973; Dubnau and Cirigliano, 1974).

[b]DSF, SSF and DRC refer to postulated intermediates in the transformation process (Dubnau and Davidoff-Abelson, 1971; Dubnau and Cirigliano, 1972). They stand for double-stranded fragments, single-stranded fragments and donor recipient complex, respectively.

[c]All transformations were carried out with less than saturating levels of transforming DNA and the residual transforming activity was determined as in Table 13.

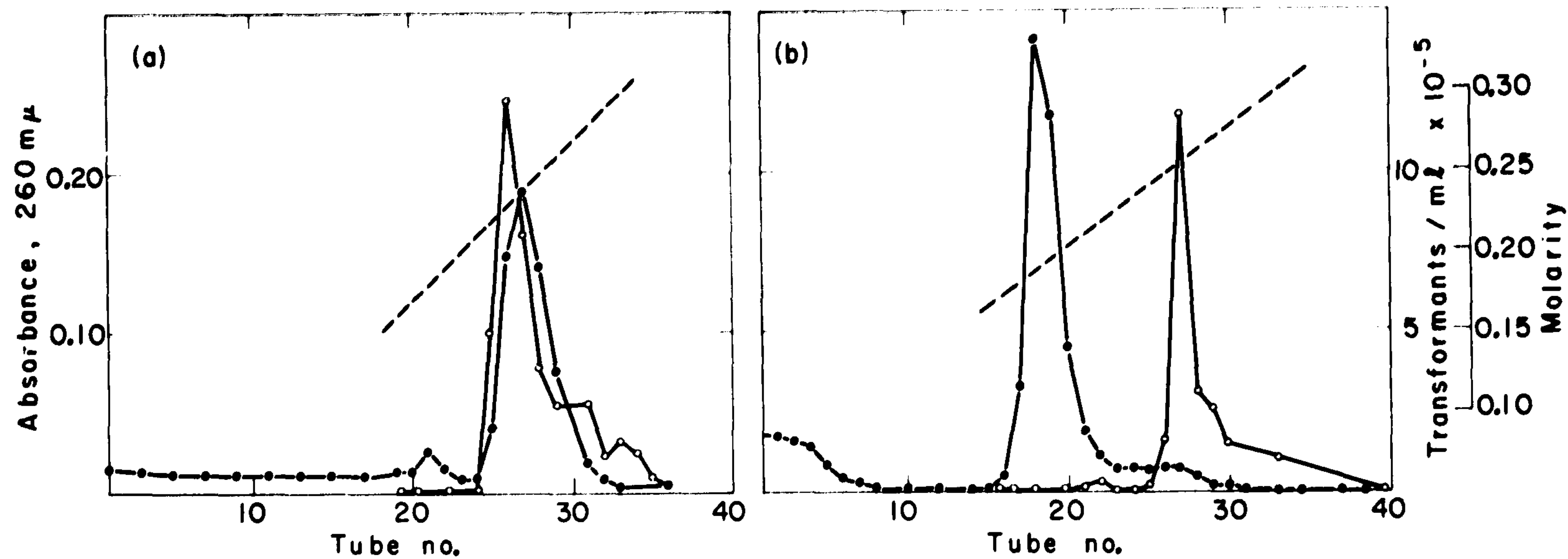


Figure 17

Hydroxyapetite chromatography of DNA isolated from GB26 (recA-1).

a. GB26 DNA, native; b. GB26 DNA, alkali denatured, 90% recovery; —●—●—, O. D. 260; —O—O—, transformation of the hisA marker.

CHAPTER III

DISCUSSION

Cellular DNA in PBSX heads appears to contain cross-links. As this DNA has been cleaved intracellularly to a size that Alberts (1968) suggests is no longer susceptible to shear induced cross-linking, mechanical shearing forces are probably not the source of naturally occurring cross-links. The possibility exists, however, that intracellular double stranded enzymatic cutting of DNA during PBSX production could lead to cross-linking by producing the same DNA intermediates presumed responsible for shear induced cross-linking. The percentage of cross-linked molecules in DNA isolated from both PBSX and from whole cells is lower than the 6% previously reported for _B. subtilis_ DNA. However, this may be attributed to the alkali denaturation method used in the experiments presented here. It has been shown that single stranded DNA in alkali is extremely sensitive to shearing. One effect of such scissions is to reduce the amount of DNA present in cross-linked molecules (Alberts and Doty, 1968).

No association was found between naturally occurring cross-links and the known elements of the Rec pathway. Cells carrying the _rec_A, _rec_B, _rec_C, _rec_D and _rec_G markers as well as the strain carrying the mutation responsible for the most recombination deficient phenotype, _rec_E, all contain cross-linked DNA. The variations in the percent of cross-linked molecules in these various strains are

within the normal range of values obtained for DNA of Rec^+ strains. Again, denaturation by alkali treatment may have contributed to the small percentage of cross-linked DNA molecules found in the Rec^- strains. As all elements of the Rec pathway may not have been defined as yet, its involvement in the generation of cross-links remains a possibility.

Finally, by a process of elimination, the results presented suggest reconsideration of DNA replication as the source of cross-linked DNA. Although recent evidence on the fine structure of the DNA growing point in T7 and studies of kinetics of incorporation of H^3 thymidine into cross-linked DNA seems to eliminate the knife and fork model, alternate mechanisms of DNA replication may account for the generation of naturally occurring cross-links in DNA (Burger, 1971; Wolfson and Dressler, 1972). Other cellular processes, such as DNA repair, which involve enzymes capable of cross-linking DNA, also remain potential sources of cross-links in DNA.

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