Selection of Bacillus subtilis 168 Mutants with Deletions of the PBSX Prophage

By R. S. BUXTON

Division of Microbiology, National Institute for Medical Research, Mill Hill, London, NW7 1AA, England

(Accepted 10 September 1979)

SUMMARY

Heat-resistant derivatives of a Bacillus subtilis 168 strain carrying an xhi mutation, which causes heat-sensitive induction of the PBSX prophage, have been isolated and screened for the acquisition of auxotrophy. Two classes of auxotrophs were isolated, namely Pro− and Pro−Met−; they lacked the ability to produce PBSX, as shown by their resistance to mitomycin C-induced lysis. The proline and methionine requirements and the resistance to mitomycin C were shown to segregate together in phage PBS1-mediated transduction crosses and to be linked to thiB, which is known to be co-transducible with the PBSX prophage. It was therefore proposed that these strains had deletions which removed all or part of the PBSX prophage together with adjacent bacterial DNA encoding the pro(AB) and metC genes. The met mutation was shown to be metC in PBS1 transduction crosses; this gene is known to be co-transducible with the PBSX prophage. The proline requirement was probably due to the deletion of a pro gene which was demonstrated to lie between the PBSX prophage and metC and which was 90% co-transducible with metC.

These deletions have been transduced into a strain which was cured of phage SPβ, another bacteriophage carried by B. subtilis 168. No phage particles could be seen in mitomycin C-induced cultures of such strains.

The PBSX-deletion strains grew with the same generation time as the PBSX+ parent in L-broth (27 min at 35 °C) but they were slower in minimal medium (e.g. 72 min as against 51 min in the PBSX+ strain). Besides being resistant to mitomycin C-induced lysis, the deletion strains were also resistant to lysis induced by thymine starvation of thymine auxotrophs and the loss of viability of these strains after thymine starvation was 100-fold less than in the PBSX+ parent. The deletion strains had not, however, lost the bacterial autolytic enzymes, since they were still susceptible to lysis when placed under semi-anaerobic conditions.

INTRODUCTION

Bacillus subtilis 168 is lysogenic for a defective phage known as PBSX; its presence is recognized by the bacterium's ability to produce phage-like particles, with head and tail components, which have a killing activity on a sensitive strain, W23 (for reviews, see Garro & Marmur, 1970; Hemphill & Whiteley, 1975). PBSX production can be induced, for example, by treatment with mitomycin C or by thymine starvation, but the phage particles produced are non-infective, hence the designation of defective phage. In fact the DNA which is...
packaged has been shown to consist of fragments of the host chromosome, each of a size of 22S \((8.4 \times 10^6 \text{ mol. wt.}; \text{Okamoto et al. 1968})\). PBSX also fails to inject this DNA.

It has been suggested that PBSX provides some essential function (Subbaiah et al. 1965), since it has proved very difficult to obtain *B. subtilis* 168 strains cured of this phage. In contrast, a strain of *Escherichia coli* K-12 cured of the resident bacteriophage lambda was isolated as long ago as 1951 (Lederberg, 1951). The only reports of possible cured strains are one by Seaman et al. (1964), although this was later shown to be a W23 strain (A. J. Garro, personal communication), and two reports (Gross et al. 1968; Ephrati-Elizur et al. 1974) of mutants selected as insensitive to mitomycin C (MMC) or thymine starvation, which were on this basis, thought to be cured of PBSX. However, mutations in the PBSX repressor gene (Thurm & Garro, 1975) produce a similar phenotype and there is thus no positive evidence for curing.

It was thought at one time that the genome of PBSX was scattered around the *B. subtilis* chromosome, thus making curing difficult to achieve. This was supported by the isolation of the *tsi23* mutation (Siegel & Marmur, 1969), a probable PBSX mutation leading to heat induction of PBSX phage, since this mapped far from other PBSX specific markers (Garro et al. 1970). However, more recent work (Buxton, 1976) on another heat-sensitive mutant, *xhi-1479*, has shown that this mutation was closely linked to other PBSX markers and, moreover, only PBSX was induced in this mutant, whereas phage \(\phi_{105}\) was also induced in the *tsi23* mutant. *Tsi23* is therefore not a phage marker and all evidence for a scattered PBSX genome has been removed.

It could be, however, that the defective nature of PBSX makes the isolation of cured derivatives unlikely. Thus it seems that the derepressed PBSX prophage replicates *in situ* (Thurm & Garro, 1975), similarly to phage \(\phi_{105}\) (Armentrout & Rutberg, 1971), but in the case of PBSX the prophage DNA is not specifically excised; instead, the host chromosome is packaged. Curing, through increasing prophage excision, may therefore be impossible if there is no excision function there initially.

Attempts have in fact been made unsuccessfully (Buxton, 1976) to cure *B. subtilis* 168 by a short heat-pulse on a strain carrying the *xhi-1479* mutation. In the present paper I describe another approach to this problem of isolating *B. subtilis* 168 strains 'cured' of PBSX; this has been to select directly for heat-resistant derivatives of a strain carrying the *xhi-1479* mutation and to search among these for possible deletions extending from the PBSX prophage into the bacterial chromosome.

**METHODS**

*Bacterial strains.* The bacterial strains used are listed in Table 1.

*Media and chemicals.* The complex media usually used was L-broth (LB; Karamata & Gross, 1970). Penassay broth was used for the tests for lysis under semi-anaerobic conditions (Antibiotic medium no. 3; Difco manual). Minimal liquid medium for growth studies was MS medium (Sargent, 1973). MSA was MS plus 1% casamino acids (Difco). Recombinants were selected on T-S plates (Karamata & Gross, 1970). TM for transformation is based on Spizizen's minimal medium together with enhancing amino acids (Wilson & Bott, 1968). Mitomycin C (MMC) was from Sigma Chemical Co. (Poole, Dorset).

*Estimation of growth rates.* Samples (0.6 ml) taken at intervals from exponentially growing aerated (bubbled) cultures at 35°C were added to 0.6 ml 10% formalin on ice and the absorbance was measured at 450 nm using a Unicam SP500 spectrophotometer.

*Test of mitomycin C resistance.* To exponentially growing cultures of LB at 30°C, mitomycin C (0.5 \(\mu g/ml\)) was added and aeration (bubbling) continued. Growth was followed throughout using an EEL nephelometer (Evans Electroselenium Ltd., Halstead,
Deletions of PBSX prophage

Table 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Origin or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em> 168</td>
<td><em>purA</em>16 <em>metB</em>5 <em>ilvA</em>1 <em>xhi</em>-1479 <em>xki</em>-1479</td>
<td>Buxton (1976)</td>
</tr>
<tr>
<td>RB1052</td>
<td><em>metC</em> <em>pyrA</em> <em>argC</em></td>
<td>A. J. Garro (Thurm &amp; Garro, 1975)</td>
</tr>
<tr>
<td>GB64</td>
<td><em>metC</em> <em>purA</em> <em>xhl</em>-3</td>
<td>A. J. Garro (Thurm &amp; Garro, 1975)</td>
</tr>
<tr>
<td>GB64/159</td>
<td></td>
<td>PBSI. RB1052 × GB64/159 → Met⁺</td>
</tr>
<tr>
<td>RB1037</td>
<td><em>pyrA</em> <em>xhi</em>-1479 <em>xki</em>-1479</td>
<td>Heat⁺ derivative of RB1037. This paper.</td>
</tr>
<tr>
<td>RB1081</td>
<td><em>pyrA</em> Δ(PBSX-pro)</td>
<td>Heat⁺ derivative of RB1037. This paper.</td>
</tr>
<tr>
<td>RB1084</td>
<td><em>pyrA</em> Δ(PBSX-pro-<em>metC</em>)</td>
<td></td>
</tr>
<tr>
<td>QB879</td>
<td><em>argC</em>4 <em>metA</em>29 <em>thiB</em>4</td>
<td>J-A. Lepesant (Institut de Biologie</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moleculaire de la Faculté des Sciences</td>
</tr>
<tr>
<td></td>
<td></td>
<td>de Paris, Paris, France)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M. M. McConnell (Central Public Health</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Laboratories, London)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>168 <em>trp</em></td>
<td><em>trpC</em>2</td>
<td>P. J. Piggot (National Institute for</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medical Research, Mill Hill, London)</td>
</tr>
<tr>
<td>RB1099</td>
<td><em>metA</em>29 <em>thiB</em>4</td>
<td>DNA. 168 <em>trp</em> × QB879 → Arg⁺</td>
</tr>
<tr>
<td>QB943</td>
<td><em>pyrD</em> <em>ilvA</em>1 <em>thA</em> <em>thY</em> <em>thB</em> <em>trpC</em>2</td>
<td>H. E. Hemphill (Zahler <em>et al.</em> 1977)</td>
</tr>
<tr>
<td>RB1121</td>
<td><em>pyrD</em> <em>ilvA</em>1 <em>thA</em> <em>thB</em> <em>thiB</em>4</td>
<td>PBSI. RB1081 × RB1121 → Thi⁺</td>
</tr>
<tr>
<td>su*2⁺</td>
<td></td>
<td>PBSI. RB1084 × RB1121 → Thi⁺</td>
</tr>
<tr>
<td>RB1143</td>
<td><em>thr</em> <em>leu</em> <em>metB</em>5 <em>sup-3</em> <em>SPβ⁻</em></td>
<td>S. Baumberg (University of Leeds)</td>
</tr>
<tr>
<td>RB1144</td>
<td><em>pyrD</em> <em>ilvA</em>1 <em>thA</em> <em>thB</em> Δ(PBSX-pro)</td>
<td></td>
</tr>
<tr>
<td>146</td>
<td><em>pro</em>(AB)</td>
<td></td>
</tr>
<tr>
<td>RB1027</td>
<td><em>metC</em>3 <em>leu xhi</em>-1479 <em>xki</em>-1479</td>
<td>J. E. Fein (Fein &amp; Rogers, 1976)</td>
</tr>
<tr>
<td>FJ6</td>
<td><em>metC</em>3</td>
<td>J. E. Fein (Fein &amp; Rogers, 1976)</td>
</tr>
<tr>
<td>FJ8</td>
<td></td>
<td>J. E. Fein (McGill University, Montreal,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Canada)</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> W23</td>
<td></td>
<td>S. Baumberg (University of Leeds)</td>
</tr>
<tr>
<td>PTR7</td>
<td>F⁻pro(AB) <em>argD</em> <em>argR</em> <em>his</em> <em>ilv</em> <em>met</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>rpsL</em></td>
<td></td>
</tr>
<tr>
<td>EScherichia coI K-12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After 90 min, mitomycin C-sensitive bacteria began to lyse whereas resistant cultures continued to increase in mass.

Test of PBSX killing. This has already been described, using the sensitive strain W23 (Buxton, 1976).

Detection of plaques of phage SPβ. This was performed with strain su*²⁺ as indicator, as described by Zahler *et al.* (1977).

Thymine starvation. Cultures of thymine-requiring bacteria growing exponentially in MSA plus required amino acids (100 µg/ml), thymine (80 µg/ml) and uracil (40 µg/ml) were collected on sterile membrane filters (0.45 µm pore size; Millipore Corp.), washed and resuspended in the same volume of medium lacking thymine. Incubation was continued with bubbling aeration at 35 °C. Nephelometer readings and viable counts (on L-agar) were made every 30 min.

Lysis of whole cells under semi-anaerobic conditions. Bacteria were grown in Penassay broth until an A675 of approx. 0.8 was reached. The bacteria were centrifuged in a Sorvall centrifuge (10000 g for 10 min) at 4 °C, washed with buffer (0.005 M-sodium hydrogen maleate–NaOH, pH 5.5 or 0.05 M-glycine–HCl, pH 8.75) and resuspended so that the starting A450 was 0.5 to 0.6. The suspensions (4 ml) were added to fill optically matched tubes, covered with parafilm and kept on ice until the start of the experiment. Lysis was started by transferring the tubes to a 35 °C waterbath. The tubes were gently mixed periodically before taking absorbance readings at 450 nm in a Unicam SP600 spectrophotometer.

Genetic crosses. Transforming DNA was extracted as described by Karamata & Gross (1970). Competent bacteria were prepared according to Wilson & Bott (1968). Phage
PBSI-mediated transduction was carried out as described previously (Rogers et al. 1976). Recombinants were purified by single colony isolation before testing for the inheritance of unselected markers.

RESULTS

Isolation of heat-resistant derivatives of strain RB1037

Strain RB1037 has two mutations, which have been separated by recombination, within the PBSX prophage mapping between metA and metC: these are xhi-1479, a mutation which results in the heat induction of phage PBSX, and xki-1479, which renders the phage produced unable to kill sensitive strains (e.g. W23). This strain can grow at 30 °C but lyses at 45 °C due to PBSX production (Buxton, 1976). It has subsequently been found, however, that at 45 °C the xhi mutation was somewhat leaky, since not all colonies isolated at this temperature were actually heat resistant when later tested. Selection for heat resistance was therefore performed at 48 °C, on L-agar plates. The rate of mutation to heat resistance was approx. 4.6 × 10⁻⁵. These colonies were replica-plated to minimal plates to identify auxotrophs and the requirements of these were ascertained. A total of 30 auxotrophs, from a number of independent cultures, was tested. All of the auxotrophs tested were unable to produce PBSX as evidenced by their resistance to mitomycin C in L-broth at 35 °C (see later). Two classes only of auxotrophs were obtained, namely proline-requiring (Pro⁻) and proline and methionine-requiring (Pro⁻Met⁻). An example of each was studied further, strains RB1081 (Pro⁻) and RB1084 (Pro⁻Met⁻).

Characterization of the auxotrophic requirements of the heat-resistant mutants

All the auxotrophs isolated could have proline replaced by arginine, ornithine or citrulline. Also a strain of E. coli, PTR7, which is pro(AB) argD argR and which grows in the absence of proline or arginine, but excretes glutamate γ-semialdehyde (GSA) and possibly acetyl-GSA (see Itikawa et al. 1968), could cross-feed both RB1081 and RB1084 on plates lacking arginine and proline.

The strains retained the argC⁺ gene, which is quite closely linked to PBSX, since argC mutants require arginine or citrulline but do not respond to ornithine, and phage PBSI grown on RB1084 could transduce QB879 (argC) to arg⁺ at approximately the same frequency as PBSI grown on strain 168.

The most likely explanation of this phenotype is that the mutants are defective in the proline biosynthetic pathway, in the steps between glutamate and glutamate γ-semialdehyde (GSA). They can utilize arginine in place of proline because ornithine, produced via arginase, can be converted to GSA by ornithine aminotransferase. They are probably not blocked in the arginine pathway since such mutants cannot use proline, possibly because there is no direct route from proline to GSA or acetyl-GSA.

The methionine requirement of RB1084 was not satisfied by cystathione or homocysteine, indicating a lesion in metC or metD (see Garro et al. 1979). The identification of the mutation as metC was made using PBSI grown on RB1084 to transduce a metC mutant (GB64) to Met⁺. This cross gave no transductants, whereas PBSI grown on B. subtilis 168 did give rise to Met⁺ transductants, and, furthermore, the PBSI. RB1084 preparation was active in transducing a metA mutant (QB879) to Met⁺.

Genetic linkage of the auxotrophic requirements and mitomycin C resistance of the heat-resistant mutants

metC has already been shown to be quite closely linked to the xhi marker and hence the PBSX prophage (Buxton, 1976). Two pro mutations have been shown to be co-transducible with metC (Harwood, 1974; Gallori et al. 1978). The position of one of these pro markers
Deletions of PBSX prophage

Table 2. Three-point genetic cross involving the markers metC, pro and xhi

<table>
<thead>
<tr>
<th>Selected marker</th>
<th>Unselected markers</th>
<th>Indicated order</th>
</tr>
</thead>
<tbody>
<tr>
<td>met⁺</td>
<td>pro wonders</td>
<td>pro⁻ met⁺ xhi⁺</td>
</tr>
<tr>
<td>pro⁻</td>
<td>54</td>
<td>10</td>
</tr>
</tbody>
</table>

* Phage PBS1 was grown on the donor strain 146 (pro) and used to transduce the recipient strain RB1027 (metC xhi-1479) to Met⁺ at 32 °C. After purification by single colony isolation, the recombinants were tested for inheritance of pro and xhi⁺ (growth at 45 °C).

has been ascertained relative to metC and xhi in a transduction cross (Table 2). From the least frequent recombinant class, the order deduced is xhi-pro-metC, metC being 54 % co-transducible with xhi and 90 % co-transducible with pro.

Hence it seems most likely that the strains RB1081 and RB1084 contain deletions extending from the PBSX prophage into the adjacent bacterial chromosome, so that RB1081 is Δpro and RB1084 is Δ(pro-metC), especially as the only selection employed was for heat resistance. If this was so, all recombinants inheriting the auxotrophic marker(s) should also inherit mitomycin C resistance. Accordingly phage PBS1 was grown on strains RB1081 and RB1084 and used to transduce strain RB1121 (thiB) to thi⁻ since thiB is known to be closely linked to the site of the PBSX prophage (Buxton, 1976). The transductants were tested for their auxotrophic requirements and resistance to mitomycin C. With RB1081 as donor, 9 Thi⁺ recombinants were Pro⁻ and MMC⁺ and 40 were Pro⁺ and MMC⁺; with RB1084 7 Thi⁺ recombinants were Pro⁻ and Met⁻, and MMC⁺, and 23 were Pro⁺ Met⁺ MMC⁺. None of the MMC⁺ recombinants had any bactericidal activity on strain W23. Thus the auxotrophic markers segregated together with MMC⁺, and were linked to thiB.

No Met⁺ revertants could be isolated from RB1084, consistent with the expected phenotype of a deletion mutation. Pro⁺ revertants could, however, be isolated from RB1081 and RB1084, at a frequency of approx. 5 x 10⁻⁶. It could be that the pro mutations were point mutations and the Pro⁺ colonies were true revertants at this locus. However since no selection was involved in the isolation of the auxotrophs, except for heat resistance, this seems unlikely. It seems more likely that the Pro⁺ colonies are secondary-site revertants; they could be reverting to Pro⁺ because of leaky argC mutations - as this gene (presumably) encodes ornithine transcarbamylase, in these mutants there would be a build-up of ornithine, which could then be converted to GSA by the basal level of ornithine aminotransferase (S. Baumberg, personal communication). Alternatively, they could be reverting to Pro⁺ by virtue of an argD mutation; a similar situation has been shown to exist in E. coli (Itikawa et al. 1968). This is because N-acetylglutamic γ-semialdehyde accumulates, yielding glutamic γ-semialdehyde, a precursor of proline, and the low level activity of some transaminase other than acetylornithine δ-transaminase allows the formation of arginine.

Phage PBS1 grown on strain RB1037, the parent of RB1084, could not transduce RB1084 to Pro⁺ or Met⁺, although it could transduce strain GB64 (argC metC) to Arg⁺ and Met⁺. Either this deletion is too large to be transduced or zygotic induction is occurring, resulting in the killing of the recombinants.

Since B. subtilis 168 and its derivatives are resistant to PBSX because of non-adsorption, it was not possible to test for the retention of PBSX immunity. In fact tests were performed but all the auxotrophs isolated were still PBSX resistant whilst W23 was PBSX sensitive. We are therefore left with the somewhat unsatisfactory position of still not being sure that all of the PBSX prophage has been deleted. If the pro and metC loci were located on opposite
sides of the PBSX prophage, the deletion of both these genes would be circumstantial evidence for the deletion of the whole PBSX prophage. Since pro and metC both lie on one side of the PBSX prophage, it is very difficult to tell if any PBSX DNA has been retained in the deletion strains, and because the PBSX prophage markers, such as xil (Thurm & Garro, 1975) are not selective markers, it is not easy to detect the retention of these markers in the putative deletion strains.

Isolation of a strain lacking both phage PBSX and phage SPβ

*Bacillus subtilis* 168 is lysogenic for another phage, SPβ (Eiserling, 1964; Warner *et al.* 1977). The growth of this phage can also be induced by MMC, but the burst size is somewhat low (e.g. 28), and less than 1% of the bacteria in a culture release any plaque-forming phage particles although it is not a defective phage (Warner *et al.* 1977). Zahler *et al.* (1977) have discovered that a strain, su+3 was accidentally cured of SPβ and they have mapped the attachment site of the SPβ prophage near the terminus of replication, between ilvA and kauA.

The SPβ-cured strains still produce PBSX and a recombinant strain has been sought which lacks the ability to produce either of these phages. It was fortuitously found that strain RB1121, derived from 168 thy−, was non-lysogenic for SPβ. Thus when RB1121 was induced with MMC (1 μg/ml) no plaques of SPβ could be obtained on the best indicator strain (H. E. Hemphill, personal communication) for SPβ, su+3. In contrast, when MMC induction of RB1081 and RB1084 was performed, plating the resulting lysates on su+3 did result in plaques being formed. DNA hybridization experiments using SPβ DNA have also indicated that RB1121 and its derivatives do not contain the complete SPβ genome (M. G. Sargent, unpublished data). How RB1121 came to be ‘cured’ of SPβ is not known, but as 168 strains are occasionally spontaneously cured of SPβ (H. E. Hemphill, personal communication), it seems likely that this has occurred, especially as thymine starvation (RB1121 is thy−) may induce cultures if thymine becomes limiting, so that curing may take place. RB1121 was not actually sensitive to SPβ, but amongst SPβ non-lysogens, su+3 is the only really satisfactory host for SPβ (H. E. Hemphill, personal communication). Lysates of RB1121 could still kill W23, hence it was still lysogenic for PBSX.

Accordingly, strain RB1121 was transduced with PBSI grown on RB1081 and on RB1084, and thiB+ recombinants isolated, some of which, as mentioned earlier, were MMC-resistant, and had therefore inherited the ΔPBSX mutation and were thus cured of both SPβ and PBSX. MMC-induced lysates of these recombinants had no inhibitory activity on su+3 or W23, and no phage particles could be observed under the electron microscope (I. D. J. Burdett & A. Smallwood, unpublished observations). As far as can be ascertained therefore, those strains are not lysogenic for any known phages.

Properties of strains carrying deletions of the PBSX prophage

The growth rates of two thiB+ MMC+ recombinants, RB1143 and RB1144, obtained with PBSI. RB1081 and PBSI. RB1084 respectively, were compared with their parent RB1121. In LB plus thymine at 35 °C, both the parent strain, RB1121, and the recombinants had generation times of 27 min. In minimal medium (MS plus thymine, isoleucine, uracil, proline, vitamin B6 and glucose), however, the recombinants grew considerably slower than RB1121; the latter had a generation time of 51 min, whilst the times for RB1143 and RB1144 were 72 min and 66 min, respectively. Addition of casamino acids to minimal medium resulted in the difference in generation times being reduced; the times were: RB1121, 45 min; RB1143, 51 min; and RB1144, 54 min. It seemed likely, therefore, that the two recombinants grew more slowly because of their auxotrophic requirements, although the deletion of other, unknown genes, may also affect their growth rates.
Deletions of PBSX prophage

Fig. 1. Effect of addition of mitomycin C to PBSX+ and ΔPBSX strains. MMC was added to LB cultures of RB1037 (PBSX+) and RB1084 (ΔPBSX) at the times indicated by arrows, as described in Methods. ●●, RB1037; △△, RB1084. Scattered light readings by nephelometer.

Fig. 2. Effect of thymine starvation on PBSX+ and ΔPBSX strains. Starvation for thymine of the thymine requiring strains RB1121 (PBSX+) and RB1143 (ΔPBSX) was carried out as described in Methods. ●●, RB1121; △△, RB1143. (a) Scattered light readings by nephelometer. (b) Percentage survival.

As mentioned previously, many treatments which stop DNA synthesis, such as thymine starvation of thymine auxotrophs and treatment with mitomycin C, lead to the induction of the PBSX prophage. The deletion of the PBSX prophage was therefore manifested by a resistance to mitomycin C-induced lysis (Fig. 1). Addition of mitomycin C to an exponentially growing LB culture of RB1037 caused lysis to occur, starting after approx. 90 min. On the contrary, no such lysis occurred in the ΔPBSX-deletion strain RB1081. Similar resistance to lysis was observed in all PBSX-deletion strains. Electron microscopic examination of wild-type PBSX+ strains after MMC-induced lysis revealed the presence of complete phage particles resembling published photomicrographs of PBSX (Seaman et al. 1964),
whereas none could be seen in MMC-treated PBSX-deletion strains (I. D. J. Burdett & A. Smallwood, unpublished observations).

The PBSX-deletion strains also failed to lyse after thymine starvation, unlike the parental PBSX+ strain. Reduction in viable count after thymine starvation was also more marked in the PBSX+ strain, this losing viability to the level of approx. 0.01%, whereas in the PBSX− strains, viability was approx. 100 times higher (Fig. 2).

When placed under semi-anaerobic conditions, *B. subtilis* rapidly lyses due to the action of two autolytic enzymes, N-acetylmuramyl-L-alanine amidase and endo-β-N-acetylglucosaminidase. Both the PBSX-deletion strains RB1143 and RB1144 lysed when placed under such conditions, either at pH 5.5 (optimal for the glucosaminidase) or at pH 8.75 (optimal for the amidase) (unpublished data). Inability to lyse after MMC treatment or thymine starvation cannot therefore be attributed to loss of these bacterial lytic enzymes. Lysis under semi-anaerobic conditions was, however, reduced by about 50%, but this was probably because the ΔPBSX grew slightly more slowly than the PBSX+ strain. As a corollary to this experiment, a mutant, FJ6, 95% deficient in both autolytic enzymes (Fein & Rogers, 1976) was tested for MMC-induced lysis. Such lysis in this strain was comparable with that of its Lyt+ parent, FJ8. Also a recombinant of FJ6 carrying the *xhi*−1479 allele underwent heat-induced lysis. These results therefore make it most unlikely that lysis due to PBSX induction is mediated by the two bacterial autolytic enzymes.

**DISCUSSION**

The present work goes some way towards answering the question of whether the PBSX prophage provides some essential function to *B. subtilis*. Thus the PBSX-deletion mutants grew apparently as fast as their parental strains in rich media. They were also as competent in transformation with DNA as wild-type strains (unpublished data) and could sporulate normally. They did grow somewhat slower in minimal medium, but this is probably because of their nutritional requirements not being wholly satisfied. The concomitant deletion of other, unknown, genes may also affect their growth. Nevertheless, the limitations of the present study should be appreciated, since the evidence for deletion is circumstantial, being based on the simultaneous loss of genetic markers adjacent to the site of the PBSX prophage. Loss of genetic markers on both sides of the PBSX prophage would be much better evidence for deletion of the prophage and perhaps such deletions could be found if selection was made on a more nutritionally complete medium. Of course essential functions coded by bacterial DNA may be lost in such deletions, so that such strains would be inviable in a haploid organism.

Other mutants (Ephrati-Elizur *et al.* 1974) although described as behaving as PBSX non-lysogens, could, in fact, be point mutations in the prophage. The present PBSX-deletion mutants have the added advantage that the site of the locus determining resistance to MMC is known and can be transferred to other strains by transduction. This is particularly useful for studies involving thymine starvation, and recombinants have been obtained carrying the ΔPBSX marker together with a rod mutation, causing a shape change in the bacterium, in order to study their growth after such treatment (I. D. J. Burdett & R. S. Buxton, unpublished experiments).

As in other phages, it may be that lysis after PBSX induction is due to a phage-coded lytic enzyme. This is substantiated by the fact that in a lyt mutant of *B. subtilis* 95% deficient in the two autolytic enzymes, MMC-induced lysis could still occur (unpublished data), and PBSX-antibacterial activity was produced after such treatment (Fein & Rogers, 1976), and in a lyt xhi mutant, heat-induced lysis also still occurred (unpublished data). The nature of the PBSX-coded lytic enzyme is at present under investigation.
Deletions of PBSX prophage

It seems more reasonable to think of PBSX as a degenerate phage rather than as some kind of prototype evolving phage. For example, it could have lost the function necessary to cleave out the phage DNA upon induction, and it also has a rather small-sized head, somewhat like reported variants of phage P1 of *E. coli* (Anderson & Walker, 1960). Pyocins, which resemble phage tails (Ishii *et al.* 1965) could be an example of an even more degenerate bacteriophage.

If PBSX is an example of a degenerate bacteriophage, and if it does not play an essential role in bacterial metabolism, then its retention by the organism would indeed seem to point to it conferring some ecological advantage (see Lotz, 1976). The presence of defective phages resembling PBSX is quite widespread amongst species of *Bacillus* (Huang & Marmur, 1970; Steensma *et al.* 1978). These phages share a similar morphology, differing only in tail length and in the number of cross-striations in the tail (Steensma *et al.* 1978). They can be differentiated on the basis of their bacteriocidal effects. Thus producer strains are resistant to the phage for which they are lysogenic, that is phage adsorption does not occur, but they are sensitive to one or more other defective phages. Phages within a morphological group have a similar, but not identical killing range. The suggestion has therefore been made (Subbaiah *et al.* 1965) that the phages evolved from a prophage in a single bacterial strain. Selection favoured bacteria in which mutations altered the specificity of both the cell wall of the producer strain and the tail of the phage it produced. Altered tails allowed the phage to adsorb to previously resistant bacteria, affording an ecological advantage, whereas the altered receptor of the producer strain protected it from the killing activity of its own defective phage.

At present, the only known ecological advantage of lysogeny by defective phages is the ability to kill sensitive cells. Two more subtle advantages have also been proposed. One is that PBSX codes for an inducible genetic determinant for the modification of DNA (Arwert & Rutberg, 1974). Garro *et al.* (1978) have, however, demonstrated that in a PBSX induction-defective (Xin−) mutant, induction of phage modification by MMC still occurred. It thus seems that the inducible modification system is not controlled or associated with PBSX. The effect of MMC is, however, not specific to PBSX and leads also, for example, to derepression of chromosomal replication (Yoshikawa & Haas, 1968) independent of PBSX induction (Thurm & Garro, 1975). Therefore changes occurring during MMC induction cannot unequivocally be attributed to be associated with PBSX. Only with the use of the *xhl-1479* mutation (Buxton, 1976), specifically affecting PBSX induction, can this be done.

The other supposed advantage of PBSX lysogeny is that it confers resistance to infection by phage SP-10, cells only becoming sensitive when PBSX is induced. Thus, Goldberg & Bryan (1968) have shown that although *B. subtilis* 168 is not susceptible to infection with phage SP-10, growth of SP-10 did take place after induction of 168 with MMC. As pointed out above, however, addition of MMC has other effects besides induction of PBSX and other explanations could account for this phenomenon. Certainly phage SP-10 could not form plaques on the PBSX-deletion strains RB1081 and RB1084 isolated in the present study, although it could plaque on W23 (unpublished data). The PBSX-deletion strains may, of course, retain some PBSX DNA. Moreover, Saito *et al.* (1979) have mapped two mutations, the simultaneous presence of which is required for permissiveness for SP-10 multiplication. Neither mutation mapped near to the site of the PBSX prophage and thus it appears that links between PBSX lysogeny and non-permissiveness to SP-10 are unfounded. Perhaps screening of PBSX+ and PBSX-deletion strains with a wide range of bacteriophages may reveal some differences in bacteriophage sensitivity, although no difference in sensitivity to phages φ105, SPO2 or φ29 could be detected between PBSX-deletion and PBSX+ strains (unpublished data).

It would appear that no other phage, defective or otherwise, is carried by *B. subtilis* 168,
since no phage particles could be observed in the electron microscope after a ΔPBSX SPβ- strain was exposed to MMC. It cannot be ruled out, however, that small numbers of an unknown phage are produced.

I should like to thank Mr P. N. Sackett for his excellent technical assistance throughout this work, Dr I. D. J. Burdett and Mr A. Smallwood for the electron microscopic examinations and Professor H. J. Rogers for his comments on the manuscript. I am also very grateful to Dr S. Baumberg for much useful advice on proline metabolism, especially for explaining the defect in proline synthesis, to Dr H. E. Hemphill for his valuable help and advice about SPβ, and to Dr M. G. Sargent for the communication of unpublished data on hybridization experiments with SPβ.

REFERENCES


Deletions of PBSX prophage


(Received 25 June 1979)