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Productive Infection of Bacillus subtilis 168, with Bacteriophage SP-10, Dependent upon Inducing Treatments

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Strains of Bacillus that harbor defective phage PBSX were found to be insensitive to SP-10(C), although the phage adsorbed to these insensitive strains. Strains that did not carry the phage were sensitive to SP-10(C). B. subtilis 168 ind−, which can be transduced by SP-10(C) but is nonpermissive for the phage, was rendered phage-sensitive after treatment with ultraviolet (UV) light or mitomycin C. After induction with UV light, maximal sensitivity to SP-10(C) was obtained at a multiplicity of infection (MOI) of approximately 14; with mitomycin C induction, an MOI of approximately 1.0 was required. Phage maturation in sensitized cells was followed by plating infected streptomycin-sensitive cells in the presence of streptomycin at various stages during phase development. The latent period was estimated to be 60 to 75 min. We suggest that the resistance of B. subtilis 168 to SP-10 is controlled, at least in part, by the presence of a defective prophage.

Phage SP-10 mediates generalized transduction of Bacillus subtilis 168, B. subtilis W-23, B. licheniformis 9945A, and their derivatives. However, it neither produces plaques nor propagates on B. subtilis 168 or its derivatives; plaquing and propagation are normal on the 9945A and W-23 strains. The experiments of Okubo et al. (21) with SP-10 and of Mahler, Cahoon, and Marmur (18) with phage PBS2 of Takahashi (30) demonstrated that incorporation of bacterial deoxyribonucleic acid (DNA) into these phages occurred without inclusion of phage and bacterial DNA in the same particle. Thus, the fate of the injected phage DNA should be independent of the transferred bacterial DNA.

In this paper, we report experiments suggesting that the inability of SP-10 to propagate on B. subtilis 168 is the result of an immunity conferred by a defective prophage.

Materials and Methods

Organisms. B. subtilis 168 ind−, B. subtilis W-23-Sr (a streptomycin-resistant mutant of wild-type 23), B. licheniformis ATCC 9945A, and B. subtilis var. atterrimus ATCC 6460 were from the Fort Detrick culture collection. B. subtilis 168 ind−Sr and B. licheniformis 9945A-Sr, mutants resistant to 1 mg of streptomycin sulfate per ml, were isolated after exposure of spores to ultraviolet (UV) light. B. subtilis strains 231 NRS and Marburg (Yale) were obtained from G. Ivanovics. B. subtilis 168a (168 wild type resistant to six antibiotics) and B. subtilis 168Xa [a strain derived from 168a by Seaman, Tarney, and Marmur (25) that is sensitive to the defective phage PBSX] were obtained from Edna Seaman. B. subtilis μ-resistant 32 was obtained from Luis Glaser. It is resistant to the defective particle μ produced upon induction of B. subtilis strain Marburg (13).

Phage SP-10(C) was isolated as a spontaneously occurring clear-plaque variant of the temperate phage SP-10 (32). This mutant was identical to the parent SP-10 in terms of host range, efficiency of plating (EOP), and inactivation by antiserum prepared against the wild-type phage.

Media and cultural conditions. TY broth consisted of 10 g of Difco tryptone, 5 g of Difco yeast extract, and 10 g of NaCl per liter (23). The diluent contained 10 g of Difco peptone per liter (32). The diluent was supplemented with 250 μg of streptomycin sulfate per ml for assays in agar containing streptomycin. Minimal 10 agar was prepared as described by Thorne and Stull (33). Soft minimal 10 agar containing 7.5 g of agar per liter was made by diluting 1.5% agar with an equal volume of double-strength minimal 10 broth. When phage were to be assayed in the presence of streptomycin, both the hard and soft agar were supplemented with 250 μg of streptomycin sulfate per ml. Phage assay (PA) agar, hard and soft, was prepared as described by Taylor and Thorne (31). Triple-distilled water was used in the preparation of all media.

Spores were produced on potato extract agar, which consisted of potato extract medium (32) plus 2.5% Difco agar.

Viable counts were determined by plating on nu-
trient broth plus yeast extract (NYB) agar (32). Lysis was followed by means of a Klett-Summerson colorimeter (no. 54 filter).

**Phage propagation.** Phage SP-10(C) was propagated on *B. subtilis* W-23-S' in shaken flasks of TY broth. Flasks were inoculated with 5 ml of a 16-hr culture and incubated on a shaker at 37 C for 2.5 hr. Cells were infected at a multiplicity of infection (MOI) of 0.03 to 0.3, and incubation was continued until lysis occurred (3 to 4 hr). The lysates were pooled and treated with deoxynuclease (0.01 μg/ml), ribonuclease (0.05 μg/ml), and lysozyme (0.2 μg/ml) at 37 C for 1 hr. The lysate was clarified by centrifugation at 10,400 × g for 20 min in an RC-2 Sorvall centrifuge and was filtered through 0.65-μm membranes (Millipore Corp., Bedford, Mass.). The resulting lysate contained 3 × 10⁹ to 5 × 10⁹ plaque-forming units (PFU) per ml when assayed in PA agar with *B. subtilis* W-23-S' as the indicator. It was then centrifuged in the no. 21 rotor of a Spinco model L preparative ultracentrifuge at 44,300 × g for 1 hr. The pellet was resuspended in 0.2% peptone containing 10⁷ dimethylsulfoxide (34) to yield a preparation containing approximately 10⁹ PFU/ml when assayed in PA agar against *B. subtilis* W-23-S'. The concentrated phage preparations were stored at −20 C.

**Assay of phage and phage-infected cells.** SP-10(C) and *B. subtilis* 168 ind− infected with SP-10(C) were assayed by the soft-agar overlay method (1) according to the protocol of Thorne (32) on a lawn containing 10⁸ spores of a suitable indicator. Plaques were counted after 16 to 24 hr of incubation at 37 C. Samples to be assayed for infected cells were incubated with sufficient antiserum to inactivate all free phage prior to dilution in peptone. Unless otherwise indicated, phage and infected cell assays were done in minimal 10 agar with *B. licheniformis* 9945A-S' as indicator (12). The inability of the minimal 10 agar to support the growth of the auxotrophic recipient coupled with the lower bacteriocin activity of *B. subtilis* on *B. licheniformis* make this procedure for assaying infected cells preferable to the use of PA agar with *B. subtilis* W-23-S' as indicator. Plaques of SP-10(C) can easily be differentiated from bacteriocins by morphol ogy on the minimal 10 medium. However, ESF values obtained by this procedure are one-third to one-fourth of those obtained with W-23-S' in PA agar.

In experiments designed to differentiate conclusively between free phage and infected cells, a modification of the streptomycin procedure of Symonds (28) was used to follow intracellular phage development. Phage were adsorbed to cells for 8 min, and antiseraum was added for 5 min to inactivate free phage. After removal of the antiseraum by centrifugation, the cells were resuspended in the same volume of fresh broth. Samples were removed at various times, diluted in peptone containing streptomycin, and plated in minimal 10 agar containing streptomycin. The infected *B. subtilis* 168 ind− cells were streptomycin-sensitive, and the *B. licheniformis* 9945A-S' indicator spores were streptomycin-resistant. The infected cells were inhibited by the streptomycin, thus preventing further phage development. However, infected bacteria that contained mature particles at the time of plating were able to produce plaques.

**Induction of *B. subtilis* 168 ind− by UV light.** An actively growing culture was prepared by transferring 1.0 ml of an overnight culture (15 to 16 hr) to 50 ml of fresh TY broth (250-ml flask). TY medium was used in induction studies in place of Penassay medium (25) because of the poor adsorption of SP-10(C) to cells grown in the latter. After 2.5 hr of incubation, the cells (5 × 10⁹ to 7 × 10⁹/ml) were usually concentrated 7- to 10-fold in TY broth prior to irradiation. Portions (10 ml) were placed in the bottom of a glass petri dish and exposed to the light produced by two 15-w General Electric germicidal bulbs for 60 sec at a distance of 41 cm. This treatment inactivated 70 to 80% of the cell population in terms of colony-forming units. For postirradiation incubation, 15-ml portions were shaken in 250-ml flasks at 37 C. Precautions were taken to avoid possible photoreactivation.

**Induction of *B. subtilis*** 168 ind− by mitomycin C (MC). Cells were induced with MC by a procedure similar to that described by Seaman et al. (25). They were grown in the manner described previously for UV induction. MC (3 μg/ml) was added to 15-ml portions of cells that had been subcultured for 2.5 hr. After 10 min of incubation at 37 C, the MC was removed by a 2-min centrifugation at 17,300 × g. This treatment inactivated 60 to 70% of the cells in terms of colony-forming units. The cells were resuspended in an equal volume of TY broth that had been presuscinated at 37 C. Postinduction incubation was at 37 C.

**Spot test for susceptibility to PBSX-like particles.** *B. subtilis* 168 ind− was induced with MC or UV light. After the maximal drop in turbidity had occurred, portions were centrifuged at 10,400 × g to remove cells and debris, and filtered through Millipore membranes (0.45 μ). The particles were concentrated sevenfold by centrifugation at 78,410 × g in the no. 30 rotor of a Spinco model L preparative ultracentrifuge and then were resuspended in 1.0% peptone diluent. Dilutions of the concentrated particles were spotted onto PA plates previously spread with the test organisms. The plates were incubated for 16 to 24 hr at 37 C and scored for lysis (clearing) in the spotted areas.

**Phage antiserum.** High-titer antiserum was obtained from Darrel D. Owen of this laboratory, who prepared it in rabbits by subcutaneous injections of concentrated SP-10(C). He determined its K value to be approximately 5,000 by the procedure of Adams (1).

**Materials.** Deoxyribonuclease, ribonuclease, and lysozyme were obtained from Worthington Biochemical Corp., Freehold, N.J. Streptomycin sulfate was purchased from E. R. Squibb and Sons, New York, N.Y. MC was obtained from Calbiochem, Los Angeles, Calif.

**Results**

**Relationship between sensitivity to SP-10(C) and inducibility for PBSX-like phages.** Several strains of *B. subtilis* have been reported to be inducible for defective phages that are able to kill but not plaque upon sensitive strains. PBSX (25, 27), μ (13), and ε3610 (26) are designations...
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for very similar, and possibly identical, phages. Similar particles were described by F. A. Eiserling and W. R. Romig (Bacteriol. Proc., p. 118, 1964). We shall refer to these particles as PBSX-like or simply as defective phages. An examination of Table 1 reveals a strong correlation between inducibility for PBSX-like phages and susceptibility to SP-10(C). The first column represents a compilation of the published data on the inducibility of the strains listed plus our own data for B. subtilis strains 168 ind- and W-23-S'. It should be noted that W-23-S' is inducible for another type of defective phage that is active on the Marburg strain (13; Eiserling and Romig, Bacteriol. Proc., p. 118, 1964), which we are not concerned with here. In general, those strains that harbor the defective prophage, as revealed by their inducibility for and resistance to PBSX-like particles, are insensitive to SP-10(C), even though the phage adsorbs well to them (Table 1, column 4). On the other hand, strains that are free from the defective prophage are sensitive to SP-10(C). Strain μ-resistant 32 was isolated as a mutant of W-23, to which the defective phage was unable to adsorb (L. Glaser, personal communication). The sensitivity of this mutant to SP-10(C) suggests that different receptor sites are involved in the adsorption of SP-10(C) and the defective phage. These results implicate prophage-determined immunity as a factor in the resistance of B. subtilis 168 ind- to SP-10(C).

Sensitization of B. subtilis 168 ind- to SP-10(C) after induction with UV light or MC. If prophage-determined immunity were responsible for insensitivity to SP-10(C), one should be able to lift the immunity via induction, thereby sensitizing the cells to the phage. Preliminary attempts to demonstrate an increase in phage yield over input after adsorption of SP-10(C) to induced cells were unsuccessful. Although greater numbers of free phage could be demonstrated in the filtrates of induced cells than in those of non-induced cells, yields were not reproducibly greater than input. This failure could be explained on the basis of adsorption of the newly synthesized phage to the noninduced cells and debris in the culture. However, one should be able to detect an increase in infected cells following the addition of phage to induced cells. Such experiments are shown in Fig. 1. Phage were added to cells at various times after induction. After adsorption, the phage-cell complexes were treated with antiserum to inactivate free phage. After induction, the turbidities of the uninfected control cultures increased for 120 to 135 min and then decreased. With UV light, a slow rise commenced at 210 min, probably as a result of growth of noninduced cells. After the initial drop following induction, the viable count (not shown) changed little, if any, for at least the first 130 min of postinduction incubation. Although the kinetics of defective phage release were not followed, spot tests of induced lysates on B. subtilis W-23-S' revealed their presence after lysis (tested 265 min after MC induction, 180 min after UV induction). Samples assayed prior to lysis (45 min with MC, 30 min with UV light) showed little, if any, activity. The number of cells susceptible to SP-10(C) increased as a function of time, reaching a peak at approximately 120 to 180 min after induction. We attribute the subsequent apparent drop in sensitivity to the lysis of susceptible cells before infection. Samples infected earlier than 45 min after induction were not included in these curves because of poor adsorption (approximately 60% at zero-time).

TABLE 1. Host ranges of SP-10(C) and PBSX-like phages

<table>
<thead>
<tr>
<th>B. subtilis strain</th>
<th>Inducible for defective phages PBSX, p. 118.</th>
<th>Sensitivity to PBSX-like particles produced on induction of ind-</th>
<th>EOP of SP-10(C)</th>
<th>Adsorption of SP-10(C) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W-23-S'</td>
<td>-</td>
<td>+</td>
<td>1.0</td>
<td>92</td>
</tr>
<tr>
<td>168X-</td>
<td>-</td>
<td>+</td>
<td>1.0</td>
<td>&gt;99</td>
</tr>
<tr>
<td>231</td>
<td>-</td>
<td>+</td>
<td>1.0</td>
<td>&gt;99</td>
</tr>
<tr>
<td>168 ind- and 168*</td>
<td>+</td>
<td>-</td>
<td>NSb</td>
<td>90</td>
</tr>
<tr>
<td>Marburg (Yale)</td>
<td>+</td>
<td>-</td>
<td>NS</td>
<td>80</td>
</tr>
<tr>
<td>Aterrimus 6460</td>
<td>+</td>
<td>-</td>
<td>NS</td>
<td>-</td>
</tr>
<tr>
<td>μ-resistant 32</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
<td>52</td>
</tr>
</tbody>
</table>

* Efficiency of plating (EOP) was determined by assay in PA agar with a lawn of 10⁷ spores. The EOP on W-23-S' was arbitrarily set at 1.0. Adsorption was determined by adding phage (MOI = 0.5 to 1.0) to 3 × 10⁸ to 1 × 10⁹ cells per ml in TV broth. After 15 min at 37°C, cells were removed by centrifugation at 3,000 × g. Unadsorbed phage were assayed in PA agar with W-23-S' as indicator. In columns 1 and 2, activity was scored as +; no activity as -.

* Not sensitive.
Adsorption for the points shown varied from 92 to 99%. At the same time that infected cells were assayed, samples were tested for free phage that might have escaped antiserum treatment. All such controls were negative. Phage were also added to noninduced cells at the same multiplicities used above. Assays made after antiserum treatment revealed no plaques associated with these control cells.

Multiplicity dependence. One could argue that the observed infective centers represent reversibly adsorbed phage that escape antiserum inactivation by virtue of their transitory association with the cell surface. Typically, adsorption was reduced by as much as 50% immediately after UV irradiation, although no significant effect on adsorption was noted after MC treatment. The fact that UV light had even a slight effect on adsorption made conceivable a more subtle effect in which the initial reversible step of the adsorptive process could take place, but the second irreversible step was prevented. Such incompletely adsorbed phage could then come off and infect the indicator cells when the sample was plated. If the infective centers that were observed were the result of surviving input phage, then one would expect the phenomenon to exhibit complete multiplicity dependence; i.e., the higher the MOI, the higher the number of infective centers would be without a plateau being reached until all phage adsorption sites were saturated. The data in Fig. 2 show that this is not the case. Multiplicities were based on viable-cell counts made immediately prior to induction and are therefore only approximate. An MOI of 14 was required to obtain the maximum (10<sup>6</sup> to 3 x 10<sup>8</sup> PFU/ml) number of infective centers after UV induction. With MC-induced cells, the number of infective centers (10<sup>4</sup> to 4 x 10<sup>6</sup> PFU/ml) peaked at an MOI of close to 1.0. As noted under Materials and Methods, MC and UV were used at doses resulting in an approximately equivalent loss in colony-forming ability. Thus, the magnitude of the difference in the MOI required to achieve the maximal number of infective centers suggested that MC was more efficient than UV light in sensitizing cells to SP-10(C).

Use of streptomycin to arrest intracellular phage...

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**Fig. 1.** Kinetics of induction of B. subtilis 168 ind and susceptibility of the induced cells to SP-10(C). Samples (0.9 ml), removed at various times during post-induction incubation, were added to tubes containing 0.1 ml of phage (MOI = approximately 20). After 15-min adsorption at 37°C without shaking, antiserum was added to inactivate free phage. Infective centers were assayed in minimal 10 agar seeded with B. licheniformis 9945A-S'. Changes in turbidity were followed by means of a Klett-Summerson colorimeter (no. 54 filter). Symbols: O, infective centers after UV; Δ, optical density after UV; O, infective centers after MC; Δ, optical density after MC.

**Fig. 2.** Effect of multiplicity of infection (MOI) on the number of infective centers produced by SP-10(C) in induced cells. Cells (2.5 hr) were concentrated to a density of 5 x 10<sup>9</sup>/ml. Separate cultures were induced with UV or MC, and postinduction incubation was continued for 120 and 140 min, respectively. Phage were added at the multiplicities indicated, and adsorption was allowed to proceed statically for 15 min (97 to 99% adsorption). Samples were assayed in minimal 10 agar seeded with B. licheniformis 9945A-S' spores after 5-min exposure to antiserum to inactivate free phage. Free phage assays run at each multiplicity were negative. Symbols: O, MC; O, UV light.
development. Complete multiplicity dependence would have constituted strong evidence for an artifact resulting from the survival of input phage. However, this is not necessarily true. There may have been only a limited number of receptor sites affected by the inducing agents. Once all of these sites were tied up by reversibly adsorbed phage, the remaining particles would be either irreversibly bound or would remain free and therefore antiserum-sensitive. A more direct method for differentiating between infected cells and phage particles depends on the use of streptomycin to kill cells and thereby stop further phage maturation without affecting mature phase particles. The effect of streptomycin on SP-10(C) maturation within UV-induced cells is shown in Fig. 3. Phage were adsorbed to the induced cells at the peak of their sensitivity. Antiserum was added in sufficient quantity to inactivate free phage completely and was subsequently removed. Duplicate samples were assayed in the presence and absence of streptomycin on a streptomycin-resistant indicator. By the time of the first sampling, 40-fold fewer infective centers were present in the presence of streptomycin than in its absence. The number of streptomycin-insensitive infective centers increased with time until, by 75 min, they represented more than half the number produced in the absence of the antibiotic. We interpreted the experiment to mean that SP-10(C) was multiplying intracellularly for at least the first 75 min. Upon exposure to streptomycin, the infected cells were killed, thus preventing further phage maturation. Only those cells that contained mature particles at the time of contact with the streptomycin produced plaques. As a control, a streptomycin-resistant strain of B. subtilis 168 ind was induced, and infective centers were assayed both in the presence and absence of streptomycin (Fig. 4). As expected, streptomycin had only a slight effect on the number of infective centers produced. This control served to eliminate the possibility of a significant streptomycin effect on the injection of phage DNA. In another control (not shown), SP-10(C) plated in the presence and absence of streptomycin exhibited no difference in EOP on B. licheniformis 9945A-S'. Also, the EOP values of SP-10(C) on the streptomycin-resistant mutants of 168 and 9945A were identical to those obtained on the streptomycin-sensitive strains. Essentially the same results as those obtained with UV-induced cells (Fig. 3 and 4) were obtained with MC-induced cells.

In the curves obtained in the absence of streptomycin, as well as in the control curve in its presence, an increase in the number of infective centers was observed after 60 min. This rise, together with the approximately 75-min intracellular multiplication period exhibited by the streptomycin-sensitive host in the presence of the antibiotic, allows a crude estimate of a latent period of 60 to 75 min. Bott and Strauss (6) showed that SP-10 has a latent period of about 55 min in the permissive host W-23. The curve in Fig. 3 obtained in the absence of streptomycin and both of the curves in Fig. 4 show that an increase in infective centers occurred between 15 and 30 min. We believe this increase to be an artifact resulting from clumps that persisted after resuspension of the infected cells. The drop in infective centers after the peak was probably due to readsorption of progeny phage to cellular debris.

No evidence for recombination or phenoletic mixing between SP-10 and PBSX-like particles.
by Anderson and Felix (3) and Lederberg (15, 16), in which the susceptibility to heterologous phage was in part determined by the presence of prophage. However, in our system the non-permissive cells became sensitive to phage upon exposure to UV or MC, and the appearance of sensitivity to SP-10(C) was correlated with the induction of a defective phage.

Two, not necessarily mutually exclusive, hypotheses have been considered. (i) SP-10(C) replication is prevented in the noninduced cells by an immunity substance or repressor (14) that prevents multiplication of the defective particles. Upon exposure of the cells to UV or MC, the repressor would be inactivated, resulting in induction and concurrent sensitization to SP-10(C). (ii) UV and MC serve to inhibit a DNA restriction system (4) that would otherwise inactivate the incoming SP-10(C) DNA. Such a restriction system could be under prophage or host control, or both. Bertani and Weigle (5) and Luria (17) have reported that UV irradiation of host cells allows the growth of certain restricted phages. Epstein (10) demonstrated that UV treatment of competent B. subtilis 168 enhanced transfection, presumably as a result of the saturation of degradative enzymes active on DNA. Sarkar (24) reported in vitro studies in which MC-treated Escherichia coli DNA was degraded only 10% by deoxyribonuclease I and II, presumably as the result of cross-links introduced in the DNA by the MC (29). However, deoxyribonuclease activity has been shown to increase on MC treatment of B. subtilis (19). Results obtained by Gwinn and Thorne (12) with a helper phage system revealed that SP-10 was produced via transfection of competent cells only when helper was introduced before or soon after the addition of the SP-10 DNA. We have looked for evidence of DNA restriction by adsorbing SP-10(C) labeled with 32P to B. subtilis strains 168 ind- and W-23-S5 and determining the degree of acid solubilization of the phage DNA (unpublished data). Incomplete degradation occurred in 168 ind- (20 to 30% of the adsorbed label within 15 min); less than 10% of the injected DNA became acid-soluble in the permissive host, W-23-S5. This preliminary evidence implied that the situation may be similar to that described by Lederberg (15, 16) and Dussoix and Arber (9) in which restriction of γ was controlled by prophage Pl. Although this possibility cannot be ruled out, it seems unlikely to us that induction of a prophage would cause the inactivation of a restriction mechanism controlled by that prophage. We have been unable to demonstrate particles which plaque on 168 after adsorption of SP-10 or SP-10(C) to the nonpermissive

![Graph](image-url)
strain. Therefore, if restriction is operating, there is probably no accompanying modification (4).

We favor the first hypothesis given above and suggest that the DNA degradation we observed was a secondary effect and that it was a repressor, probably associated with the defective prophage, that actually prevented SP-10(C) from multiplying in *B. subtilis* 168 ins-. This hypothesis is supported by the results of Gwinn and Lawton (personal communication) with intact SP-10(C). They found that, although the efficiency of rescue of the SP-10(C) genome by unrelated helper phage decreased as a function of time, whole SP-10(C) genome could survive for at least 90 min after injection into *B. subtilis* 168, well beyond the estimated latent period. Thus, it appears unlikely that DNA degradation is the primary reason for the inability of SP-10 to propagate in 168.

We are unable to explain completely either the need for prolonged postinduction incubation before the cells achieve maximal sensitivity to SP-10(C) or the low numbers of susceptible cells that are obtained after induction. It may be, however, that (i) "switch-off" of the repressor is initially only partial, and (ii) postinduction protein synthesis is required to complete the process.

Direct evidence for the participation of a defective prophage depends on the isolation of a strain that has been "cured" of prophage and comcomitantly rendered SP-10 sensitive. However, we were unable to isolate a phage SP-10(C)-sensitive strain of *B. subtilis* 168 ins- either by the "curing" procedure of Sarnoff et al. (25) or by mutagenesis with a variety of agents followed by replica-plating onto plates with and without SP-10(C).

We were also unsuccessful in attempts to transflect (11) cells of *B. subtilis* 168 ins- that had been exposed to UV or MC after the attainment of competence (2). Although we are unable to explain this failure, it is of some interest that cells are maximally inducible at a time when they are actively dividing (unpublished data), whereas competent cells are presumably not multiplying (20).

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LITERATURE CITED


