Exclusion of Induced Bacteriophage from Cells of a Lysogenic Bacillus megaterium Committed to Sporulation

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Spontaneous release of the temperate bacteriophage T (\(\phi T\)), carried by Bacillus megaterium 899a, occurred during early growth of the host cells. Rejuvenated cells (accomplished by a \(5\times\) dilution in fresh medium) and unrejuvenated cells were induced by mitomycin C during the course of sporulation and subsequent phage \(\phi T\) production measured by burst size. Induction of sequential samples of unrejuvenated cells resulted in burst sizes that fell to zero as \(T_0\) sporulation time in the main culture was approached. This drop in burst size was not considered a sporulation event, as it also occurred during analogous stages of growth in an asporogenous mutant. Rejuvenated, induced portions of the culture of sporulating cells of B. megaterium 899a gave large burst sizes until \(T_{1,4}\), when the burst sizes fell to zero. The stage I asporogenous mutant, treated in a similar manner, gave lower, but still substantial, burst sizes; thus, the sharp decline in burst size of induced rejuvenated sporulating cells appeared to be a sporulation event. Sporulating cells induced at times shortly after \(T_{1,5}\) formed spores in which the induced phage were trapped until germination of the spores, which formed infectious centers. This induced phage-trapping was maximal when the sporulating cultures were induced at \(T_{1,2}\). Commitment to sporulation could be defined by our system as that point beyond which rejuvenated sporulating cells were unable to support the replication of the phage. This point also correlated with the increase in induced phage-trapping by spores. Two other methods gave a similar commitment time. Commitment to sporulation, in spite of added glucose or fresh complex medium, occurred at the same time. Electron micrography showed that the committed cell was still undergoing engulfment. The fate of induced phage \(\phi T\) was determined at different points during growth and sporulation. Induction at times prior to \(T_0\), which were longer than the eclipse period of the phage, resulted in a burst size of approximately 50. At times prior to \(T_0\), shorter than the phage eclipse period, induction led to lysis with low burst sizes, approaching zero. The pattern of spontaneous phage release during growth was similar. From \(T_0\) up to the point of commitment to sporulation, induction resulted in the blocking of spore formation without lysis. At the commitment point, induced phage were trapped and carried into spores which germinated to give infectious centers. The spontaneous derepression of phage at a time which blocked spore formation led to \(7 \times 10^4\) infectious centers per ml and would not normally be noticed. Derepression at the time of phage entrapment was not observed to occur without induction with mitomycin C.

Sporulating cells are committed if they cannot revert to vegetative growth and division when fresh medium replaces the exhausted medium. Failure to rejuvenate occurred first in the forespore and then in the mother cell in both Bacillus subtilis and Bacillus cereus. However, B. subtilis was committed to sporulation slightly earlier than B. cereus (3, 6). Rejuvenation of blocked stage II mutants supported this earlier B. subtilis commitment time (6, 17). It is possible that the above temporal differences may be accounted for by differences in the quantity of fresh nutrients added and the duration of incubation before examination of the rejuvenated cells.

A system for the study of bacterial sporulation through the use of a lytic bacteriophage which grew in log-phase cells, but not in sporu-
lating cells, has been reported (15). Consequently, in this study, an inducible phage carried by the sporulating strain *B. megaterium* 899a was used to analyze commitment by examination of pre-Tₐ and rejuvenated sporulating cells. *B. megaterium* 899a sporulates synchronously and carries a temperate phage with a known host (12).

Commitment to sporulation of *B. megaterium* 899a in the presence of added fresh nutrients was examined with the growth of a vegetative-specific prophage in induced cells used as a measure of the recovery of vegetative characteristics in rejuvenated cells. This allowed the cells diluted in fresh medium to be given a more immediate challenge to their status. The results are discussed in light of what may occur to other prophage carried in sporulating lysogenic bacteria.

**MATERIALS AND METHODS**

**Bacterial strains and cultivation.** *B. megaterium* 899a was sent to us by J. T. Wachsmann along with *B. megaterium* KM (T Tr U)⁻, which was used as a phage Tₐ (φT) indicator strain (12). An asporogenous strain, *B. megaterium* 899a Sp⁻1, which was blocked early in sporulation, was readily selected. Complete spore septum formation was not observed in the mutant examined by the phase-contrast microscope. *B. megaterium* KM Sp⁺ is a strain isolated previously from Northrop's strain of *B. megaterium* KM Sp⁻ (2).

The growth and sporulation medium used was a BBM-salts mixture (19), in which the original salts were modified so that 1 liter contained 0.1 M KNO₃, 1.2 x 10⁻⁴ M KH₂PO₄, 1.0 x 10⁻⁴ M K₃HPO₄, 1.0 x 10⁻⁴ M K₂SO₄, 2.5 x 10⁻⁴ M MgSO₄·7H₂O, 1.3 x 10⁻⁴ M MnSO₄·H₂O, 3.3 x 10⁻⁴ M FeSO₄·7H₂O, and 5.0 x 10⁻⁴ M ZnSO₄·7H₂O.

The pH was adjusted to 6.9 with a KOH solution, and the salts were then autoclaved. The final medium was prepared by mixing a sterile solution of 1:4 (wt/wt) blood base medium (BBM)-salts and then adding sterile CaCl₂ to give 4.0 x 10⁻⁴ M in the final medium.

**Buffers.** Spores were stabilized in a buffer containing 0.1 M NaCl, 0.01 M K₂HPO₄ at pH 7.2, and 10⁻³ M CaCl₂. Phage Tₐ was found to be stable at 4°C in a buffer containing 0.1 M NaCl, 0.01 tris(hydroxymethyl)aminomethane-hydrochloride at pH 7.2, 5 x 10⁻⁴ M MgCl₂, and 100 μg of peptone/ml (Difco).

**Phage assay.** Phage assay was similar to that described previously (12), except that the bottom layer of the medium was nutrient agar with 2 x 10⁻³ M MgCl₂ and the top layer was 4 ml of sloppy agar (one-third nutrient agar plus two-thirds nutrient broth with 2 x 10⁻⁴ M MgCl₂), 1 ml of phage suspension, and 0.2 ml of a suspension (10⁵ bacteria/ml) of the indicator strain. The indicator bacteria were grown overnight on nutrient agar. The plates were incubated for 36 h at 29°C.

**Phage induction.** The method used has been described elsewhere (12). Full lysis of the culture was produced by a minimum of 0.35 μg of mitomycin C (Nutritional Biochemicals) per ml, and 175 μl of a solution of 20 μg/ml was added to a 10-ml portion of the culture.

**Determination of average burst size.** At various times during sporulation a portion of the culture was induced. The cell concentration was determined in a Petroff-Hauser bacterial counting chamber viewed with a phase-contrast microscope, and φT was assayed 30 min before and 60 min after the beginning of phage lysis, which occurred 90 min after induction. It has been reported (1), and we have also noted, that there was no increase in cell numbers after induction of φT. The average burst size was estimated to be the increase in φT titer divided by the number of lysed cells.

**Determination of percentage of φT trapped in spores.** After sporulation lysis, spores were harvested, washed, and stored in spore buffer. They were heated at 75°C for 15 min to remove extraneous phage and then titered for infectious centers by using the phage assay. Spores of the untreated strain 899a gave rise to small colonies surrounded by a halo of phage lysis, and these are known as centered plaques. Spores containing induced, trapped φT lysed after germination with the release of φT, so giving rise to plaques. This is analogous to φS trapping in *B. subtilis* (15).

**Sporulation.** Growth and synchronous sporulation take place in the same medium. Sporulation medium was inoculated from an overnight nutrient agar culture to give an optical density at 645 nm (OD₄₅₀) of 0.05 with the Bausch & Lamb Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.) in 1-cm cells. A 40-ml amount of medium in a 500-ml side arm flask was shaken at 29°C, and the OD was monitored. Tₐ is the point at which division in the culture ceases (14) and it is recognized by a change in the slope of the OD time curve. Predetermined OD values were used for sporulation times prior to Tₐ and may be determined from Fig. 1. Samples of the sporulating culture were examined frequently in a phase-contrast microscope. Spore whitening began at Tₐ+1. Synchrony of sporulation was good, because 50% of the developing spores observed by phase-contrast microscopy turned white in 1 h.

**Rejuvenation.** The culture was rejuvenated by a 5x dilution in fresh medium at 29°C. The effect of commitment on sporulating cultures was seen by rejuvenating at different times during sporulation and comparing OD₄₅₀ or white spore count to the unrejuvenated control (6, 8). Ten minutes after rejuvenation, cultures were induced by addition of mitomycin C. In one set of tests, mitomycin C was added 1.5 h after rejuvenation, and this was called extended rejuvenation. In a separate series, glucose was added (final concentration of 10⁻³ M), 100 μl/10 ml of a solution of 9 mg/ml to 5-ml samples, and the white spore count was compared to the untreated control. This was the minimum concentration of added glucose to give 90% reduction in white spore formation.

**Electron microscopy.** Samples of the sporulating culture were prepared for electron microscopy by the method of Kellenberger et al. (9) with some modifications described recently (13).
Light microscopy. Sporulating cultures were routinely observed by phase-contrast microscopy. Spores undergoing whitening when observed by this method are referred to as white spores.

RESULTS

Phage produced without induction. Phage ϕT was produced during growth of B. megaterium 899a in the sporulation medium. The number of phage released showed a marked rise during mid-log-phase of growth of the culture (Fig. 1). The number of cells undergoing lysis in the culture is small and normally not detected. A small number of ϕT may have been released during sporulation, but the ϕT titer declined after T −1.5. Because of this, harvested spores were contaminated by free phage. In a separate publication, it was shown that ϕT is rapidly inactivated by heat and that it may be destroyed alone or in mixtures of spores by heating to 75 C for 15 min.

Because it has been reported that infection with a lytic phage of B. subtilis at early times in sporulation resulted in blocking of formation of spores (15), it was considered possible that derepression of ϕT at an early time during sporulation might block normal spore formation and may account for the fact that B. megaterium 899a sporulated with a frequency of approximately 95%.

To test this, estimates of infectious centers in the culture, without induction at two times early in sporulation, were determined (Table 1). These figures were obtained by rejuvenating the culture at the indicated times and proceeding as if the phage had been induced. The increase in phage titers was divided by the burst size obtained from rejuvenated, induced cells (Fig. 2). The incidence of infectious centers was very small and did not account for the relatively large number of cells which did not produce a spore.

Effect of phage induction on sporulating cultures prior to T₀. Induction of ϕT 90 min prior to T₀, followed by growth in the same medium, resulted 90 min later in lysis with an average burst size of approximately 50 phage/cell. When induction of ϕT is carried out at times closer to T₀, there is a very rapid reduction in burst size, accompanied by a lesser reduction in the degree of lysis of the culture.

Table 1. Incidence of infectious centers at initiation of sporulation

<table>
<thead>
<tr>
<th>Time relative to T₀ (h)</th>
<th>Infective centers/ml</th>
<th>Total cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀ ± 0.25</td>
<td>9.5 × 10⁴</td>
<td>0.0380</td>
</tr>
<tr>
<td>T₀ ± 1.25</td>
<td>6.0 × 10⁴</td>
<td>0.0240</td>
</tr>
</tbody>
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Fig. 1. Growth of and uninoculated phage production by B. megaterium 899a in sporulation medium. ○, OD₆₅₅; ♦, log₁₀ phage/ml.

Fig. 2. Burst size and percentage of lysis in rejuvenated and unrejuvenated sporulating cells of B. megaterium 899a Sp⁺ induced with mitomycin C. ■, Burst size and ○, percentage of lysis, in unrejuvenated sporulating cells; ♦, burst size and ▲, percentage of lysis, in rejuvenated sporulating cells. Time scale indicates when the treatment began on portions of the sporulating culture.
until at T₀ both percentage of lysis and burst size approach zero (Fig. 2). Readings at times more advanced than T₀ cannot be made because of lack of lysis of the culture. However, induction of the phage occurred at these late times. If the culture is induced and rejuvenated at T₀, for example, the former high burst size and degree of lysis is restored.

Induction of φT prior to T₀ in the sporulation medium of an asporogenous mutant likewise resulted in a rapid drop in average burst size as T₀ was approached (Fig. 3). The evidence from this mutant did not allow linking the rapid drop in burst size of induced pre-T₀ cells to sporulation. It is possible that absence of rapid cell growth at T₀ alone prevented production of many phage from cells induced at T₀ for example. Normally, the phage eclipse period is 90 min after induction. The pattern of phage production without induction supported these observations (Fig. 1). Thus, the phage were released in an early burst at a time greater than the eclipse period of φT prior to T₀.

**Effect of phage induction on rejuvenated post-T₀ sporulating cells.** Rejuvenation for 10 min prior to induction allowed the recovery of the previous higher burst sizes and percentage of lysis until T₂, after which the low percentage of lysis prevented further calculations being made (Fig. 2). After T₂, mitomycin treatment did not block sporulation (see below), but φT were carried into heat-resistant infectious centers, confirming that induction did take place (Fig. 5). Rejuvenation of the asporogenous mutant, followed by induction, resulted in 100% lysis of the culture at times past T₂ (Fig. 3). The burst size, although it declined at T₂, never approached zero as it did in the sporulating strain. Thus, the rapid drop to zero in burst size of rejuvenated, induced, sporulating cells at T₂ appears to be related to the sporulation process.

**Extended rejuvenation of sporulating cells.** Rejuvenation for 90 min prior to induction resulted in lysis of a number of the sporulating cells, releasing white spores. Untreated control cultures did not contain free spores at this time. This operation was successful at T₁.₇₅ for only a short period of time during sporulation (Fig. 4). Assuming that this lysis was due to phage activity, it can be concluded that extended rejuvenation does not allow the recovery of phage activity at sporulation times further advanced than T₁.₇₅. Phage activity in cells rejuvenated for 90 min and induced caused lysis in only the mother cell; the developing spore did not appear to be affected. Rejuvenation of sporulating cells at T₁.₇₅ caused approximately 50% of them to revert to growth, and these cells released φT on induction. Forty percent of those remaining, which proceeded in a committed manner to produce white spores, lysed after induction, but one could not tell if phage were released from these cells. The culture at that time was a mixture of vegetative and committed cells.

**Induced phage trapping.** Sporulating cultures induced with mitomycin C formed heat-resistant spores which lysed, releasing φT on germination. The highest percentage of heat-resistant spores containing such induced φT was obtained by inducing the culture at T₁.₇₅ (Fig. 5). This also blocked spore formation in 90% of the potential sporeforming cells. The maximum yield of spores containing induced phage occurred at T₂.₅ to T₃, and so the rapid reduction in burst size in rejuvenated, induced, sporulating cells can be correlated with the rise in the induced phage-trapping capacity of the culture (Fig. 6). Induction of φT in sporulating cells prior to commitment to sporulation resulted in the failure of potential sporeformers to produce a spore. After commitment to sporulation, however, induction of φT did not block sporulation, and many of the

![Fig. 3. Burst size and percentage of lysis in rejuvenated and unjured cells of B. megarum 899a Sp grown in sporulation medium and induced by mitomycin C.](http://jb.asm.org/Downloaded from http://jb.asm.org)
heat-resistant spores carried induced phage which replicated when the germinating spores returned to vegetative growth. Induced \( \phi T \) is excluded from full expression in cells of \( B. \ megaterium \) 899a committed to sporulation. Heat-resistant infectious centers were not observed without treatment with mitomycin C.

**The inhibitory effect of mitomycin C on sporulating cells.** As has been stated here, mitomycin C added to sporulating cells before commitment induced \( \phi T \) and blocked sporulation. Mitomycin C added after \( T +1 \) induced the phage (Fig. 5), and the number of cells which formed spores increased and was equal to the untreated control at \( T +2 \). Many of these mitomycin C-treated cells formed spores which were infectious centers. The increase in the number of induced, trapped phage in spores correlates with the increase in the number of mitomycin C-treated sporulating cells which completed spore formation (Fig. 5, 6). Thus, mitomycin C induced \( \phi T \), but did not block spore formation in those cells committed to sporulation.

The possibility that mitomycin C may have a detectable effect other than the induction of phage in precommitment, sporulating cells was tested by using \( B. \ megaterium \) KM sp\(^+\), which does not carry an inducible phage, but is closely related to \( B. \ megaterium \) 899a. Figure 5 indicated that the concentration of mitomycin C used does inhibit formation of white spores of \( B. \ megaterium \) KM sp\(^+\), but to a lesser extent than in \( B. \ megaterium \) 899a. The greater inhibition of white spore formation observed in \( B. \ megaterium \) 899a was possibly due to the induction of \( \phi T \).

**Effect of rejuvenation on sporulating cells.** Cultures rejuvenated by dilution with fresh medium prior to commitment to spore formation increased in OD (Fig. 7). Rejuvenation after commitment resulted in a much smaller change in OD. A curve of OD versus sporulation times in rejuvenated cultures showed the expected sharp drop in OD at \( T +2 \), the commitment point.

The incidence of cells which produced spores in a rejuvenated, sporulating culture has been used as a measure of commitment (8). Commitment to sporulation, determined by this method, in the presence of added glucose or
Fig. 6. Time of maximum production of spores as infective centers formed by induction of sporulating cultures by mitomycin C at various times: A, percentage of cells which proceed to form a spore which is an infectious center. (This is calculated from two curves on Fig. 5, the percentage of cells forming spores and percentage of spores carrying induced phage in mitomycin C-treated cultures.) At T +3, this curve merges with the curve; B, percentage of heat-resistant spores which germinate to give an infectious center; C, heat-resistant infectious centers per milliliter of culture; D, burst size of rejuvenated cells calculated as percentage of the T 0 value.

Fig. 7. Rejuvenation of a sporulating culture of B. megaterium 899a measured by OD; ○, OD 650nm at T +4 of a sporulating culture after rejuvenation at the times indicated; O, control culture measured at the time of rejuvenation.

again in the presence of added fresh complex medium, occurred at times indistinguishable from one another (Fig. 8). These commitment times were T +1.75 to T +2 and so agreed with the time of T +2, which was the time that rejuvenated, induced cells failed to allow replication of ϕT.

Morphology of the committed cell. Sporulating cells of B. megaterium 899a are committed as early as T +3. Samples of T +3 cells, viewed in the electron microscope, contained early stage III cells, none of which had completed engulfment of the forespore. A typical cell showed that engulfment was in progress, but was not completed (Fig. 9). The developing membrane is indicated by an arrow.

DISCUSSION

A system is described for the study of commitment to sporulation by using a temperate phage, carried by a sporforming bacillus, which, when induced, replicated in rejuvenated uncommitted cells. When the cells were committed to sporulation, the induced phage did not replicate but were carried into heat-resist-

ant spores which became infective centers. This appears to be the first report of a prophage being induced and carried in the replicative form into heat-resistant spores, although it has been reported that PBS-1, a pseudolysogenic noninducible phage of B. subtilis, can be carried in spores (16). This phage method of studying commitment to sporulation allows an immediate challenge to be made to the sporulating cell treated with fresh medium. That challenge is whether the cell has recovered those vegetative functions which allow the replication of the induced phage. This does not require extensive rejuvenation of the cell and so allows a more precise assessment of the commitment time.

It has been reported that ϕe, a lytic phage of B. subtilis, was excluded from sporulating cells (15). Infection by ϕe of sporulating cells at early sporulation times resulted in blocking of spore formation in potential sporforming cells; later in sporulation, the phage became trapped in heat-resistant spores. These data seem similar to the results described here with induced ϕT. However, these studies have been expanded to include pre-T 0 and rejuvenated sporulating cells and have shown that phage trapping and lack of full expression of induced phage T in sporulating cells of B. megaterium 899a occurred at the time of commitment to sporulation. Later ϕe studies have shown that a loss of
vegetative template activity of deoxyribonucleic acid-dependent ribonucleic acid (RNA) polymerase occurred prior to maximum entrapment of φe in B. subtilis spores (11). Assuming that the regulation of sporulation in B. subtilis and B. megaterium occurs in a similar manner, it is quite possible that the time of phage-trapping is separate from the loss of vegetative template specificity of deoxyribonucleic acid-dependent RNA polymerase and is the time of commitment to sporulation. Further work on

Fig. 8. Rejuvenation of a sporulating culture of B. megaterium 899a measured by the percentage of white spores formed in spite of rejuvenation compared to the control at T+7; ○, percentage of white spore formation in the culture rejuvenated with fresh medium; ■, percentage of white spore formation in glucose-treated cultures; ▲, control expressed as a percentage of the T+7 control values. Time scale indicates when the treatment of the culture began.

Fig. 9. Electron micrograph of a typical cell of a sporulating culture of B. megaterium 899a at T+2, at which time the culture was committed to sporulation in the presence of complex nutrients. The process of engulfment of the forespore was not yet complete. Extent to which the forespore was engulfed is indicated by the arrows. ×67,000.
RNA polymerase changes during sporulation has been reviewed recently (10).

This method of determining commitment to sporulation was tested against two other methods previously reported in the literature. The time of commitment to sporulation in the presence of glucose was found to be indistinguishable from the sporulation commitment time in the presence of fresh nutrients and agreed closely with the time of exclusion of replication of a phage from induced, rejuvenated, sporulating cells. More than one time of commitment to sporulation has been reported for other sporulating bacteria. B. subtilis was committed in the presence of glucose at T₀ (5) and in the presence of fresh medium at stage II (6). It has been suggested that commitment to sporulation is the result of formation of the double membrane around the forespore after engulfment (4). Our results indicated that B. megaterium 899a was committed to sporulation prior to completion of the double-engulfing membrane. Also, it has recently been reported that B. megaterium ATCC 19213 was committed to sporulation at stage II (7). It appears, therefore, that B. megaterium is committed to sporulation before the engulfing membrane is completed.

The studies reported here appear to be the first observations of the fate of a temperate phage carried by a lysogenic sporulating bacillus during growth and sporulation.

Different effects were seen when ϕT was induced at different times in the sporulating medium. The results are summarized in Fig. 10. Prior to TᵦE, the phage was produced at high burst size (approximately 50), but between TᵦE and T₀, induction produced lysis with low burst sizes which approached zero. From T₀ to Tᵦ, induction caused blocking of spore formation without lysis of the culture. At approximately Tᵦ, there was a period when induction led to trapping of ϕT in heat-resistant infective centers, but after that mitomycin C had no observable effect on the sporulating cultures.

Lysis of sporulating cultures of Bacillus species at times close to T₀ is not uncommon, e.g., B. cereus (19) and B. subtilis ATCC 6051 (15), and this lysis may be due to phage activity, although interference of spore formation by phage in these bacteria has not been investigated. Regulation of ϕT seemed to avoid interference with spore formation in B. megaterium 899a, for spontaneous expression of the effects seen on the induction of ϕT during sporulation were of very low incidence.

ACKNOWLEDGMENTS

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LITERATURE CITED


FIG. 10. Fate of induced ϕT during growth and sporulation. Tᵦ, Time when growth and division ceased and sporulation began; Tₓ, time prior to T₀ by the length of eclipse period of ϕT; Tᵦ, time of commitment to sporulation.