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

G. S. HENDRY AND P. C. FITZ-JAMES
Department of Bacteriology and Immunology and Department of Biochemistry, University of Western Ontario, London, Ontario, Canada

Received for publication 4 January 1974

Spontaneous release of the temperate bacteriophage T (φT), carried by Bacillus megaterium 899a, occurred during early growth of the host cells. Rejuvenated cells (accomplished by a 5× dilution in fresh medium) and unrejuvenated cells were induced by mitomycin C during the course of sporulation and subsequent phage φT production measured by burst size. Induction of sequential samples of unrejuvenated cells resulted in burst sizes that fell to zero as T₀ 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 T₁/₂, 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 T₁/₂ 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 T₁/₂₀. 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 φT was determined at different points during growth and sporulation. Induction at times prior to T₀, which were longer than the eclipse period of the phage, resulted in a burst size of approximately 50. At times prior to T₀, 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 T₀ 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 × 10⁴ 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. Wachsman along with B. megaterium KM (T Tr U) ‐, which was used as a phage T (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 × 10⁻³ M K₂HPO₄, 1.0 × 10⁻³ M K₃HPO₄, 1.0 × 10⁻³ M K₂SO₄, 2.5 × 10⁻⁴ M MgSO₄·7H₂O, 1.3 × 10⁻⁴ M MnSO₄·H₂O, 3.3 × 10⁻⁴ M FeSO₄, and 5.0 × 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 × 10⁻¹ 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 10⁻¹ M CaCl₂. Phage T 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 × 10⁻⁸ 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 × 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 × 10⁻³ M MgCl₂), 1 ml of phage suspension, and 0.2 ml of a suspension (10⁶ 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 φT 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 (OD₆₄₅) of 0.05 with the Bausch & Lomb 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. T₀ 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 T₀ and may be determined from Fig. 1. Samples of the sporulating culture were examined frequently in a phase-contrast microscope. Spore whitening began at T₀++. 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 5× 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 OD₆₄₅ or white spore count to the un-rejuvenated 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 10⁻² M), 100 μl 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 \( \phi T \) 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 \( \phi T \) may have been released during sporulation, but the \( \phi T \) titer declined after \( T_{-1.5} \). Because of this, harvested spores were contaminated by free phage. In a separate publication, it was shown that \( \phi T \) 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 \( \phi T \) 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 \( T_e \). Induction of \( \phi T \) 90 min prior to \( T_e \), 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 \( \phi T \) is carried out at times closer to \( T_e \), 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 ( T_e ) (h)</th>
<th>Infective centers/ml</th>
<th>Total cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( T_{+0.25} )</td>
<td>( 9.5 \times 10^4 )</td>
<td>0.0380</td>
</tr>
<tr>
<td>( T_{+1.25} )</td>
<td>( 6.0 \times 10^4 )</td>
<td>0.0240</td>
</tr>
</tbody>
</table>

![Fig. 1. Growth of and uninduced phage production by \( B. megaterium \) 899a in sporulation medium. \( \bullet \), OD\(_{64.5\text{mm}} \); \( \Delta \), log\(_6 \) phage/ml.](http://jb.asm.org/)

![Fig. 2. Burst size and percentage of lysis in rejuvenated and unrejuvenated sporulating cells of \( B. megaterium \) 899a Sp\(^+\) induced with mitomycin C. \( \bullet \), Burst size and \( \% \), percentage of lysis, in unrejuvenated sporulating cells; \( \Delta \), burst size and \( \% \), percentage of lysis, in rejuvenated sporulating cells. Time scale indicates when the treatment began on portions of the sporulating culture.](http://jb.asm.org/)
until at $T_0$, both percentage of lysis and burst size approach zero (Fig. 2). Readings at times more advanced than $T_0$ 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 $T_{+1}$, for example, the former high burst size and degree of lysis is restored.

Induction of $\phi T$ prior to $T_0$ in the sporulation medium of an asporogenous mutant likewise resulted in a rapid drop in average burst size as $T_0$ was approached (Fig. 3). The evidence from this mutant did not allow linking the rapid drop in burst size of induced pre-$T_0$ cells to sporulation. It is possible that absence of rapid cell growth at $T_0$ alone prevented production of many phage from cells induced at $T_{-1.0}$, 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 $\phi T$ prior to $T_0$.

**Effect of phage induction on rejuvenated post-$T_0$ sporulating cells.** Rejuvenation for 10 min prior to induction allowed the recovery of the previous higher burst sizes and percentage of lysis until $T_{+2}$, after which the low percentage of lysis prevented further calculations being made (Fig. 2). After $T_{+2}$, mitomycin treatment did not block sporulation (see below), but $\phi T$ 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 $T_{+2}$ (Fig. 3). The burst size, although it declined at $T_{+2}$, 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 $T_{+2}$ 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 $T_{+1.75}$ 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 $T_{+2}$. 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 $T_{+1.75}$ caused approximately 50% of them to revert to growth, and these cells released $\phi T$ 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 $\phi T$ on germination. The highest percentage of heat-resistant spores containing such induced $\phi T$ was obtained by inducing the culture at $T_{+1.0}$ (Fig. 5). This also blocked spore formation in 90% of the potential sporeforming cells. The maximum yield of spores containing induced phage occurred at $T_{+2.0}$ to $T_{+2.5}$, 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 $\phi T$ in sporulating cells prior to commitment to sporulation resulted in the failure of potential spoformers to produce a spore. After commitment to sporulation, however, induction of $\phi T$ did not block sporulation, and many of the
heat-resistant spores carried induced phage which replicated when the germinating spores returned to vegetative growth. Induced φT 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 φT 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+3. 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 φT, 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 φT.

**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. 4.** Freed spores as percentage of total white spores at T+1 in a culture treated by extended rejuvenation followed by induction with mitomycin C. Time scale indicates when such treatment began on portions of a sporulating culture of B. megaterium 899a.

**Fig. 5.** Effect of induction with mitomycin C on a sporulating culture of B. megaterium 899a. ○, Percentage of heat-resistant spores which carry induced phage; ▲, percentage of white spores observed at T+1 after treatment of the culture with mitomycin C; O, white spore formation in the untreated control; ■, percentage of white spores observed at T+4 after mitomycin C treatment of B. megaterium KM Sp+ sporulating cultures at the times indicated.
again in the presence of added fresh complex medium, occurred at times indistinguishable
from one another (Fig. 8). These commitment times were T_{+1} 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
\phi T.

**Morphology of the committed cell.** Sporulating cells of *B. megaterium* 899a are committed
as early as T_{+}. Samples of T_{+} 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 sporulating 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 \phi e, a lytic phage of
*B. subtilis*, was excluded from sporulating cells
(15). Infection by \phi e 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 \phi T.
However, these studies have been expanded to
include pre-T_{S} 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 \phi e studies have shown that a loss of

![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_{+}, 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_{S} value.](http://jb.asm.org/)

![Fig. 7. Rejuvenation of a sporulating culture of B. megaterium 899a measured by OD; A, OD_{650} at T_{+} of a sporulating culture after rejuvenation at the times indicated; B, control culture measured at the time of rejuvenation.](http://jb.asm.org/)
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_{+7}, 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. x67,000.
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 Tₙ (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 indicate 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 Tₜ, the phage was produced at high burst size (approximately 50), but between Tₜ and Tₙ, induction produced lysogeny at low burst sizes which approached zero. From Tₙ to Tₜ, induction caused blocking of sporulation, without lysis of the culture. At approximately Tₜ, 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 Tₜ 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**

This work was supported by a fellowship to G. S. H. from Labatt's Breweries, London, Ont., and by a grant to P. C. F.-J. from the Medical Research Council of Canada.

**LITERATURE CITED**


