JOURNAL OF BACTERIOLOGY, Feb. 1968, p. 304-309
Copyright © 1968 American Society for Microbiology

Vol. 95, No. 2
Printed in U.S.A.

# Initiation of Deoxyribonucleic Acid Synthesis After Thymine Starvation of *Bacillus subtilis*

NEVILLE R. KALLENBACH AND RONG-INE MA

*Department of Biology, Leidy Laboratories, University of Pennsylvania, Philadelphia, Pennsylvania 19104*

Received for publication 13 November 1967

Evidence for premature initiation of deoxyribonucleic acid (DNA) replication after thymine starvation of *Bacillus subtilis* W23T$^-$ is presented, based on (i) increase in the number of *ade*$^+$ relative to *met*$^+$ transformants yielded by the DNA isolated from cultures after starvation (the *ade*$^-$ marker being near the origin of replication, whereas *met*$^-$ is close to the terminus), and (ii) increase in both the initial rate and final level of tritiated thymine incorporation in the presence of chloramphenicol after release from starvation. The marker ratio data agree quantitatively with the hypothesis that the initiation is induced only on one arm of each chromosome which was replicating prior to starvation.

Starvation of bacteria for thymine has a number of physiological consequences which are of particular interest for their bearing on the processes which regulate the cellular deoxyribonucleic acid (DNA) replication cycle. Synthesis of DNA ceases rapidly on thymine deprivation of thymine auxotrophs (5) or inhibition of thymidylate biosynthesis by agents such as fluorouracil (6), whereas ribonucleic acid (RNA) and protein continue to be produced for some time. Loss of viability (thymineless death) ensues after an initial lag period of variable duration, depending on the organism and its conditions of growth, from less than 30 min (5) to several hours (9). Deprivation of thymine has been shown to lead to increased rates of mutation (12) and recombination (10), induction of prophage and bacteriocins (23), and a modification of the bacterial DNA associated with loss of specific transforming activity (22) and capacity to support in vitro RNA synthesis (16). Although the precise nature of the lethal event has not been established, the involvement of protein or RNA synthesis is evident in that thymineless death can be prevented by prior inhibition of protein or RNA synthesis (11, 17, 21).

A striking alteration in the replication cycle of the DNA of *Escherichia coli* has been observed to result from sublethal periods of thymine starvation (20). The normal sequence of DNA replication in bacteria is such that initiation of a new round of replication of the bacterial chromosome occurs only when the previously initiated round is complete (14, 17). This initiation, moreover, occurs at a unique site on the bacterial chromosome (14). After sublethal periods of thymine starvation of a thymine auxotroph of *E. coli* 15, premature initiation of replication at the origin occurs, resulting in the presence of an additional growth point per chromosome. This condition persists for about one generation time following release from starvation, after which replication with only one growth point per chromosome (*see* 4) resumes. Cell division has been reported to be synchronous for several generations after sublethal periods of thymine deprivation (2).

The premature initiation which is induced is remarkable in that the induction is apparently asymmetric, taking place on only one of the two arms of the original replicating chromosome, as shown both by density gradient centrifugation studies of the DNA (20) and by tritium autoradiography of whole cells (15). This asymmetry has important implications for current models of the control of replication and segregation of the DNA in bacteria, which require the two complementary strands of a chromosome to differ in the manner of their attachment to the elements of the cell postulated to regulate DNA synthesis (14).

Initiation of DNA synthesis can be investigated directly, in terms of specific genetic loci, by the transformation marker system in *Bacillus subtilis* described by Yoshikawa and Sueoka (26). In the experiments reported here, the levels of transformants yielded by the DNA of a thymine auxotroph of *B. subtilis* W 23 (8) have been measured both under normal growth conditions and after release from thymine starvation. The recipient markers used were a pair which have been previously shown to be widely separated on the chromosome with respect to the replication cycle in this organism: *ade*-16, located very near the

chromosomal site of origin of replication (19) and *met*, located near the site of termination (26). The ratios of $ade^+$ to $met^+$ transformants obtained by low concentrations of the donor DNA isolated from W23T⁻ strain in exponential growth (in glucose-minimal medium) were found to be close to twice those observed with similar concentrations of DNA isolated from stationary cultures of this strain. After release from a 30-min period of thymine starvation, the ratio of these transformants increased, within 20 min, to 3, and then fell to a level near 1.5. That this increase is not due to selective loss of certain fragments of the DNA during extraction is indicated by a control experiment in which the isolation procedure was varied. Moreover, the rate of DNA synthesis, as measured by incorporation of tritiated thymine into the cells, increases after thymine starvation, even when chloramphenicol is added at the time of thymine restoration. Chloramphenicol has been shown to inhibit initiation of DNA replication while permitting completion of already initiated chromosomes (*see*, for example, 25).

## MATERIALS AND METHODS

*Strains.* The *B. subtilis* strains used in this study were: W23T⁻, a thymine-requiring mutant obtained from F. Rothman (8); SB25, a histidine- and indole (tryptophan)-requiring mutant of 168, obtained from E. Nester (18); and Mu8u5u16, a leucine-, methionine-, and adenine-requiring mutant of 168 from N. Sueoka.

*Media and transformation procedure.* Recipient cultures were made competent by the procedure of Anagnostopoulos and Spizizen (1). The growth period in the minimal A medium could be varied from 4.5 to 7 hr without affecting the ratios reported. Frozen recipient cells which were stored at −70 C for up to 2 months after addition of 15% (v/v) glycerol to the culture and immersion in liquid nitrogen were also used.

Cultures of the donor strain were grown at 37 C on a rotatory shaker in glucose minimal medium (24) supplemented with 0.05% casein hydrolysate and 50 μg of thymine per ml; the generation time of W23T⁻ is routinely found to be 40 to 50 min in this medium. Medium transfers were accomplished by rapid filtration of the cells onto a large-size membrane filter (Schleicher and Schuell Co., Keene, N.H.).

Samples of cultures for DNA extraction (usually 100 to 200 ml) were withdrawn at desired times, heated at 60 C for 10 min, washed, and suspended in saline-EDTA (0.15 M NaCl, 0.1 M ethylenediaminetetraacetate, *p*H 8) made 20% in sucrose. After digestion with lysozyme, lysis was completed by addition of sodium dodecyl sulfate (1%). The lysate was shaken in cold buffer-saturated phenol for 20 min and centrifuged briefly; the aqueous phase was precipitated with two volumes of cold ethyl alcohol. The threads recovered were resuspended in phosphate-EDTA buffer (0.006 M $Na_2HPO_4$, 0.002 M $NaH_2PO_4$, 0.001 M disodium EDTA) and finally dialyzed against the same buffer supplemented with 0.18 M NaCl (7). DNA concentrations were determined by the procedure of Keck (13), with a preparation of calf thymus DNA (Worthington Biochemical Corp., Freehold, N.J.) as standard. The concentration of 1 optical density unit of the standard was taken to be 50 μg/ml. Unless otherwise specified, transformation experiments were performed with a final DNA concentration of 0.025 μg/ml, a concentration well below the saturation level, which was generally above 1 μg/ml. Transformants were scored as colonies on minimal-glucose-agar plates with appropriate supplements.

*Incorporation experiments.* Tritiated thymine (methyl) was obtained from New England Nuclear Corp., Boston, Mass. Samples (0.5 ml) of cultures at different times after addition of labeled thymine were mixed with an equal volume of cold 10% trichloroacetic acid, filtered onto glass-fiber discs after 1 hr, and were washed with cold 5% trichloroacetic acid and then with ethyl alcohol. The discs were dried in vacuo, liquid scintillator (Liquifluor, New England Nuclear Corp.) was added, and they were then counted in a scintillation spectrometer.

## RESULTS

*Marker frequency ratios during normal growth.* A culture of W23T⁻ was grown as described above at 37 C, samples were withdrawn at the times indicated by arrows in Fig. 1, and the DNA was extracted. The $ade^+$ and $met^+$ transformants resulting from transformation of the recipient $ade^-$ and $met^-$ strain by each sample were scored (Table 1). The results are in accord with previous data reported for *B. subtilis* W23 strains (26), the

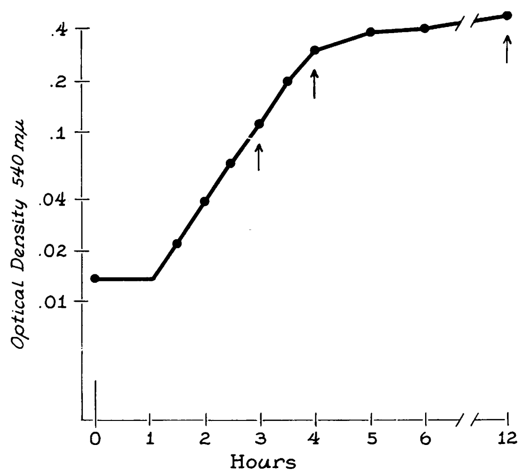


FIG. 1. *Growth of W23T⁻ in complete minimal medium at 37 C. The culture was aerated by rotatory shaking. Samples at the times indicated by arrows were withdrawn for DNA isolation and assay of transformant frequencies as described in the text. The relative optical density was monitored with a Klett colorimeter.*

ratio of transformants $ade^+/met^+$ obtained with the DNA from exponential-phase cells being twice that obtained with the DNA from the stationary phase.

*Thymineless death. B. subtilis* W23T⁻ exhibits a typical loss of viability in the absence of thymine (8), as recorded in Fig. 2. The initial culture was grown to exponential phase (3 hr, $10^8$/ml) in the medium supplemented with thymine, filtered, and transferred to a medium lacking thymine. The lag period before death was approximately 20 min in the minimal glucose medium. Extensive filament formation was observed in the starved culture, as well as an apparent increase in total mass.

*Frequency ratios following thymine starvation.* A similar exponential-phase culture (3 hr) was starved 30 min before restoration of thymine. Samples were then removed from the culture at intervals, DNA was extracted as described, and the number of $ade^+$ and $met^+$ transformants was assayed for each sample. Results of two experiments are summarized in Table 2. The ratio of $ade^+$ to $met^+$ transformants increased to 3.0 at 20 min after the restoration of thymine, from the initial value of 2.0, that of the DNA from the unstarved culture. After an additional 40 min, the ratio dropped to 1.5.

The viable-cell count during this period increased with no apparent lag, although in Fig. 3b a possible plateau from 30 to 50 min after restoration of thymine is indicated. Incorporation of tritiated thymine by the culture did not level off during this time interval, however (Fig. 3a), so that it is not clear whether the culture is synchronous after thymine starvation. It should be noted that *E. coli* 15T⁻ starved for thymine under

TABLE 1. *Frequencies of ade⁺ to met⁺ transformants from the DNA of exponential- and stationary-phase cultures*[a]

| Time of DNA samples (hr) | No. of transformants/0.1 ml | | Ratio, $ade^+/met^+$ |
|---|---|---|---|
| | $ade^+$ | $met^+$ | |
| 3 | 1,050 | 535 | 1.96 |
| | 1,600 | 800 | 2.00 |
| 4 | 1,533 | 953 | 1.61 |
| | 2,290 | 1,490 | 1.54 |
| 12 | 1,285 | 1,200 | 1.07 |
| | 1,230 | 1,170 | 1.05 |
| | 2,515 | 2,210 | 1.14 |

[a] DNA of the donor strain, W23T⁻, was isolated from the 3-, 4-, and 12-hr points of the growth curve shown in Fig. 1. The recipient strain was $leu^-$ $met^-$ $ade^-$. Each number represents an average of triplicate plate counts.

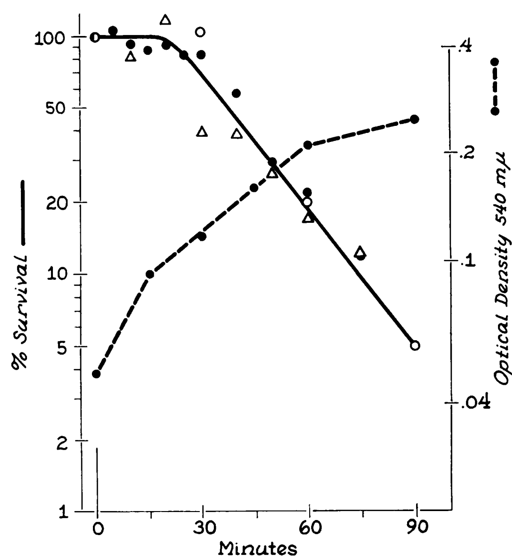


FIG. 2. *Thymineless death of W23T⁻. A culture grown for 3 hr in complete medium was filtered and transferred to minimal medium lacking thymine. Viable-cell counts were determined by plating. Results of three different experiments are summarized. The relative optical density was measured as in Fig. 1.*

similar conditions to those of our experiments shows little or no increase in cell count for 40 min after thymine restoration, and thereafter appears to divide synchronously for several generations (2). DNA synthesis proceeds in a stepwise manner during this period (2).

An experiment was performed to ascertain whether the ratios of transformants obtained with the DNA from the culture 20 min after thymine restoration could be changed by using a different extraction procedure. A culture at this time was divided into two parts: one was treated in the usual way to isolate the DNA; the second was subjected to a procedure including digestion of the lysate with Pronase (3). This step can lead to release of nucleic acids occluded by protein during the usual extraction. The two preparations gave essentially equal ratios of $ade^+$ to $met^+$ transformants, the normalized values being 3.08 for the standard and 2.98 for the Pronase-digested material.

As a routine control, the samples of DNA used in these experiments were tested for their ability to transform the recipient strain SB25 $his^-$ $try^-$, in which the two markers are linked (18). The ratios of $try^+$ to $his^+$ transformants which were obtained fell within a range 1.00 to 1.14, with a mean value of 1.05, although the specific transforming activities (transformants per unit con-

TABLE 2. *Frequencies of ade⁺ to met⁺ transformants after 30-min thymine starvation of the donor culture*[a]

| DNA samples (min) | Expt | No. of transformants/ 0.1 ml | | Ratio, *ade*⁺/ *met*⁺ | Normalized ratio, *ade*⁺/ *met*⁺ |
|---|---|---|---|---|---|
| | | *ade*⁺ | *met*⁺ | | |
| 0 | 1 | 4,840 | 2,350 | 2.06 | 2.00 |
| | 2 | 5,390 | 2,420 | 2.21 | 2.00 |
| 10 | 1 | 6,130 | 2,850 | 2.15 | 2.09 |
| | 2 | 9,030 | 3,680 | 2.45 | 2.22 |
| 20 | 1 | 6,650 | 2,140 | 3.10 | 3.01 |
| | 2 | 6,345 | 1,970 | 3.21 | 2.91 |
| 30 | 1 | 4,440 | 1,610 | 2.75 | 2.69 |
| | 2 | 6,565 | 2,835 | 2.30 | 2.09 |
| 60 | 1 | 2,970 | 2,130 | 1.40 | 1.36 |
| | 2 | 2,940 | 1,980 | 1.49 | 1.35 |
| 90 | 3 | 1,340 | 815 | 1.64 | |
| 120 | 3 | 2,530 | 1,810 | 1.40 | |

[a] W23T⁻ was grown for 3 hr in the medium described and was starved for 30 min for thymine. Samples of DNA were taken at times (indicated in the first column) measured in minutes from the time of thymine restoration. The recipient strain was *leu met ade*. Recipient cell counts were from $1.4 \times 10^7$ to $2.6 \times 10^7$ per 0.1 ml of the transforming medium in all experiments. The ratios were adjusted (normalized) to make the 0-min value equal to 2.0. Each number is a mean of triplicate plate counts. Experiments 1, 2, and 3 refer to an assay of the designated samples with a particular preparation of competent cells.

centration of DNA) varied considerably from sample to sample.

*DNA synthesis following thymine starvation.* To show that the data on frequency of transformants reflect initiation of new DNA synthesis as opposed to a change in physical state of the DNA, tritium-labeled thymine was restored to a culture after 0, 15, and 30 min of thymine starvation. At the time of restoring thymine, 100 μg of chloramphenicol per ml was added to suppress further initiation of DNA replication (25). As shown in Fig. 4, incorporation of label proceeded at a higher rate after thymine starvation, and reached a greater value after 140 min. The initial incorporation rate after 30 min of starvation was very close to twice that of the unstarved culture. However, the amount incorporated by the starved culture after 140 min was only 45% higher than that of the unstarved one. This is considerably less than the increase required for two growth

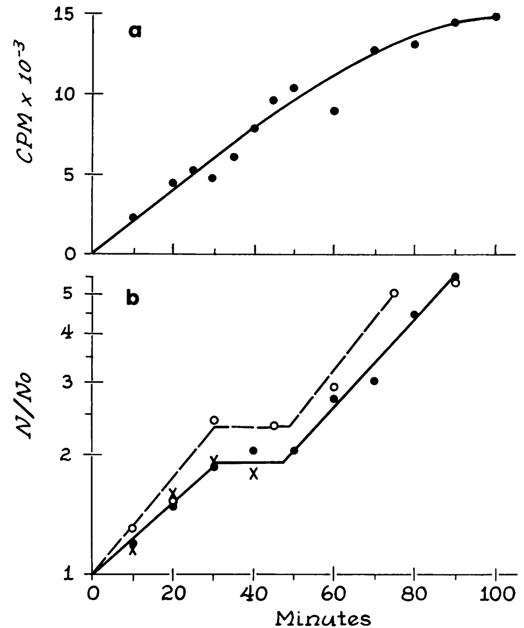


FIG. 3. *Growth and DNA synthesis after 30 min of thymine starvation. A 3-hr culture of W23T⁻ was transferred by filtration to medium lacking thymine for 30 min. (a) Tritiated thymine (10 μc/ml, 50 μg/ml) was added, and 0.5-ml samples of the culture at different times were counted as described in Materials and Methods. (b) Viable-cell counts were determined by plating; the curves shown represent three separate experiments, with different values of the initial cell concentration (No).*

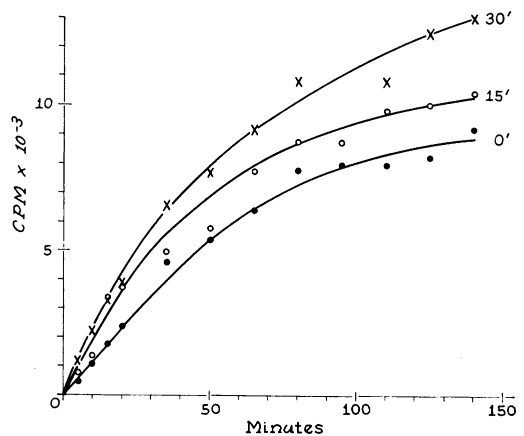


FIG. 4. *DNA synthesis after thymine restoration in the presence of chloramphenicol. A 3-hr culture of W23T⁻ was transferred to medium lacking thymine. Tritiated thymine (12 μc/ml, 50 μg/ml) and chloramphenicol (100 μg/ml) were added to portions of the culture at 0, 15, and 30 min after thymine deprivation, and the incorporation in 0.5-ml samples was measured at different times.*

points to complete replication in the absence of further initiation—nearly 170% (20). This disparity may be due to some residual initiation in the presence of chloramphenicol, or to the fact that the incorporation level at 140 min may not be the maximal value attainable. Furthermore, as is evident from Fig. 2, some loss of viability can be observed within 30 min of starvation.

## DISCUSSION

The finding that the DNA isolated from *B. subtilis* W23T⁻ after thymine starvation exhibits increased ratios of $ade^+$ relative to $met^+$ transformant frequencies is in accord with the hypothesis that premature initiation of replication, similar to that found in *E. coli* by Pritchard and Lark (20), is induced in this organism. While any process capable of selectively degrading or sequestering $met^+$ bearing chromosome fragments could be invoked to explain the transformation data, incorporation experiments with tritiated thymine clearly show both a higher initial rate and a larger total amount of DNA synthesised after starvation.

The quantitative value of 3.0 for the $ade^+/met^+$ transformant ratio 20 min after restoring thymine to the starved culture is precisely that expected if the premature initiation occurred on only one arm of each replicating chromosome present, as is the case in *E. coli* 15 (15, 20). Moreover, the rate of DNA synthesis after 30-min starvation is initially double that of the unstarved culture, again as anticipated if a single new growth point is induced per chromosome. The absolute value of the ratio observed may conceivably reflect some decrease in $met^+$ specific transforming activity, but any such effect would tend to increase the apparent ratio observed, so that the value 3.0 represents an upper limit in any case. Hence, the possibility can be excluded that initiation occurs dichotomously on *both* arms of all replicating chromosomes which were present before starvation, although the possibility that such a process takes place in a fraction of the cells present cannot be ruled out by any of our data.

It seems plausible that the value of 1.5 of the $ade^+$ to $met^+$ transformants of the DNA from the culture 60 min after restoring thymine relates to a condition of the cells in which the original growth point has reached the terminal site for replication, leaving only the newly induced growth point on the chromosome.

The kinetics of the induction process require some comment. The maximal $ade^+$ to $met^+$ transformant ratio is reached 20 min after thymine restoration, whereas there is clearly no lag in incorporation of tritiated thymine after restoration (Fig. 3a). This situation could arise in two distinct ways: (i) recovery from starvation is heterogeneous in time among the cells in the population, or (ii) DNA synthesis resumes at the original growth point without delay whereas induction of the second point is slower. No decision between these alternatives can be made from these experiments.

## ACKNOWLEDGMENT

This investigation was supported in part by National Science Foundation grant GB-4200.

## LITERATURE CITED


1. ANANGOSTOPOULOS, C., AND J. SPIZIZEN. 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:741–746.
2. BARNER, H. D., AND S. S. COHEN. 1956. Synchronization of a thymineless mutant of *Escherichia Coli*. J. Bacteriol. **72**:115–123.
3. BERNS, K. I., AND C. A. THOMAS, JR. 1965. Isolation of high molecular weight DNA from *Hemophilus influenzae*. J. Mol. Biol. **11**:476–490.
4. BONHOEFFER, F., AND A. GIERER. 1963. On the growth mechanism of the bacterial chromosome. J. Mol. Biol. **7**:534–540.
5. COHEN, S. S., AND H. D. BARNER, 1954. Studies on unbalanced growth in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S. **40**:885–893.
6. COHEN, S. S., J. G. FLAKS, H. D. BARNER, M. R LOEB, AND J. LICHTENSTEIN. 1958. The mode of action of 5-fluorouracil and its derivatives. Proc. Natl. Acad. Sci. U.S. **44**:1004–1012.
7. CROTHERS, D. M. 1964. The kinetics of DNA denaturation. J. Mol. Biol. **9**:712–733.
8. FARMER, J. L., AND F. ROTHMAN. 1965. Transformable thymine-requiring mutant of *Bacillus subtilis*. J. Bacteriol. **89**:262–263.
9. GALLANT, J. A. 1962. Sublethal thymineless damage in *Escherichia coli* B 3. Biochim. Biophys. Acta. **61**:302–304.
10. GALLANT, J., AND T. SPOTTSWOOD. 1965. The recombinogenic effect of thymidylate starvation in *Escherichia coli* merodiploids. Genetics **52**:107–118.
11. HANAWALT, P. C. 1963. Involvement of synthesis of RNA in thymineless death. Nature **198**:286.
12. KANAZIR, D. 1958. The apparent mutagenicity of thymine deficiency. Biochim. Biophys. Acta **30**:20–23.
13. KECK, K. 1956. An ultramicro technique for the determination of deoxypentose nucleic acid. Arch. Biochem. Biophys. **63**:446–451.
14. LARK, K. G. 1966. Regulation of chromosome replication and segregation in bacteria. Bacteriol. Rev. **30**:3–32.
15. LARK, K. G., AND R. BIRD. 1965. Premature chromosome replication induced by thymine starvation: restriction of replication to one of the two partially completed replicas. J. Mol. Biol. **13**:607–610.
16. LUZZATI, D. 1966. Effect of thymine starvation

on messenger ribonucleic acid synthesis in *Escherichia coli*. J. Bacteriol. **92**:1435–1446.

17. MAALØE, O., AND P. C. HANAWALT. 1961. Thymine starvation and the normal DNA replication cycle I. J. Mol. Biol. **3**:144–155.
18. NESTER, E. W., M. SCHAEFER, AND J. LEDERBERG. 1963. Gene linkage in DNA transfer: a cluster of genes concerned with aromatic biosynthesis in *Bacillus subtilis*. Genetics **48**:529–551.
19. O'SULLIVAN, A., AND N. SUEOKA. 1967. Sequential replication of the *Bacillus subtilis*: chromosome IV. Genetic mapping by density transfer experiment J. Mol. Biol. **27**:349–368.
20. PRITCHARD, R. H., AND K. G. LARK. 1964. Induction of replication by thymine starvation at the chromosome origin in *Escherichia coli*. J. Mol. Biol. **9**:288–307.
21. ROLFE, R. 1966. On the mechanism of thymineless death in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S. **57**:114–121.
22. SICARD, N., AND C. ANAGNOSTOPOULOS. 1964. Activite transformante de 1 acide de-soxyribonucleique de *Bacillus subtilis* lors de la carence en thymine. Compt. Rend. **259**:4173–4176.
23. SICARD, N., AND R. DEVORET. 1962. Effets de la carence en thymine sur des souches d'*Escherichia coli* lysogenes K 12 T, et coliconogenes 15 T. Compt. Rend. **255**:1417–1419.
24. SPIZIZEN, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. U.S. **44**:1072–1078.
25. YOSHIKAWA, H. 1965. DNA synthesis during germination of *Bacillus subtilis* spores. Proc. Natl. Acad. Sci. U.S. **53**:1476–1483.
26. YOSHIKAWA, H., AND N. SUEOKA. 1963. Sequential replication of *Bacillus subtilis* chromosome, I. Comparison of marker frequencies in exponential and stationary growth phases. Proc. Natl. Acad. Sci. U.S. **49**:559–566.