*DNA SYNTHESIS DURING GERMINATION OF BACILLUS SUBTILIS SPORES**

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Genetic and autoradiographic studies on the replication of the bacterial chromosome show that replication is sequential from a fixed starting point of the chromosome unidirectionally to the end of the chromosome, with one replication point.[1-4] An experiment on the heat-sensitive sex factor of *Escherichia coli* suggested the involvement of protein in the initiation of episome replication.[5] The initiator hypothesis was later extended to the replication of the *E. coli* chromosome itself.[6] The experiment by Lark gave evidence indicating that two distinct proteins regulate the initiation of chromosome replication.[7] These studies on the regulation of initiation and termination of chromosome replication led to the proposal of a model in which the starting point might have a unique structure associated with the cell membrane and the replication might be initiated by a protein called initiator acting on the starting point, which, by itself, was in a repressed state.[5-8] The nature and function of the initiator protein, however, is not yet known.

Synchronous germination of *B. subtilis* spores has been shown in various media,[9-11] and successive commencement of RNA, protein, and DNA synthesis was observed during germination.[9] Measurement of genetic marker frequency during germination showed that the replication of the chromosome is also highly synchronous.[11, 12]

This communication reports experimental results which show that the germination system provides a unique opportunity to study the regulation of the initiation of chromosome replication.

Materials and Methods.—For H^3-thymidine incorporation a thymine-requiring mutant of 168, thy$^-$, ind$^-$, leu$^-$ (MY2Y1U2), given us by Dr. Rothman, was used. The leucine requirement was added later by UV irradiation. A mutant of W168, leu$^-$, ade$^-$, met$^-$ (MU8U5U16) was used as recipient cell for frequency analysis.

All spores were isolated from Schaeffer's agar plates,[13] after having been grown for 24 hr. Schaeffer's agar contains 8 gm dehydrated nutrient broth (Difco), 0.25 gm $MgSO_4 \cdot 7H_2O$, 1 gm KCl, 1.25 mg $MnCl_2$, 15 gm agar (Bacto Agar Difco), 1000 ml H_2O. 10^{-6} *M* $FeSO_4$, and 10^{-3} *M* Ca $(NO_3)_2$ were separately autoclaved and added. The final pH was 7.0. A mixture of spores and cells was collected from the agar plates and lysed with 1 mg/ml lysozyme at 37°C for 1 hr. Lysis of cells was then completed by addition of 1% sodium lauryl sulfate and incubation at 37°C for 30 min. Lysed cells were washed by repeated centrifugation. The purified spores were suspended in distilled water and stored at 4°C. A Klett reading of 50 units of the suspension corresponds to 8×10^6 spores/ml. Spore DNA was isolated by first treating the spores with urea-mercaptoethanol[14] and then following Marmur's procedure.[15]

Germination of spores was carried out by shaking at 37°C in either enriched medium containing 8.75 gm antibiotic medium 3 (Difco), 100 mg L-glutamic acid in 1000 ml basal medium (medium C),[12] or a synthetic medium as described by Donnellan *et al.*[10]

DNA synthesis was followed by incorporation of H^3-thymidine during germination of thymine-requiring mutant spores. The spores were germinated in the presence of 20 μC/ml H^3-TdR (California Biochemicals, specific activity 3C/mmol) and 2 μg/ml TdR. Incorporation was stopped by 10% TCA and the precipitate was then collected on S&S membrane filters. Radioactivity was counted by Nuclear-Chicago scintillation counter.

Crude DNA polymerase was prepared by lysis of cells from germinated spores (8×10^8 cells/ml)

by 1 mg/ml lysozyme in 30% sucrose, 0.1 *M* Tris-HCl, 0.003 *M* $MgCl_2$, 0.001 *M* 2-mercaptoethanol, pH 8.2 at 37°C for 30 min. The resulting protoplasts were dialyzed against the same buffer without sucrose at 4°C. Lysates were then centrifuged at 6000 rpm for 15 min to separate supernatant from precipitate fraction. In some cases lipase or DNase were added at the same time as the lysozyme. This procedure was carried out throughout in a dialysis bag.

Both fractions were assayed for DNA polymerase activity by incorporation of H^3-dATP in acid-insoluble fraction by the method of Okazaki and Kornberg.[16] The reaction mixture of 0.5 ml contains 50 mμmoles Tris-HCl, pH 8.2, 4 μmoles $MgCl_2$, 1 μmole 2-mercaptoethanol, 14 mμmoles each of TTP, dCTP, dGTP, and H^3-dATP (2×10^7 cpm/μmole), 10 μg *B. subtilis* DNA, and 0.25 ml crude enzyme. When dAT copolymer was substituted for *B. subtilis* DNA, dCTP and dGTP were omitted from the reaction mixture. After 45 min incubation at 37°C the reaction was terminated by the addition of cold 10% TCA, and the acid-insoluble fraction was isolated by filtration.

Lipase from wheat germ (Worthington Biochemical Corp.) and beef pancreatic DNase (Worthington Biochemical Corp.) were used for treatment of the crude enzyme. The dAT copolymer was kindly donated by Dr. A. T. Ganesan.

Experimental Results.—Effect of chloramphenicol (CM) on DNA synthesis during germination: DNA synthesis during germination of spores in synthetic medium was measured by H^3-TdR incorporation by a thymine-requiring mutant. As shown in Figure 1, DNA synthesis takes place 2 hr after the commencement of germination but the increase in turbidity is not observed until later. It is noticed that DNA synthesis proceeds linearly during the first generation and also during the second, but the rate is twice that of the first generation. Generation time was determined by morphological changes of spores and the doubling of genetic markers. Addition of CM at zero time did not produce any effect on the primary steps of germination such as turbidity decrease or sensitization to both heat and lysozyme treatment. However, CM arrested succeeding development of germinated spores and markedly inhibited DNA synthesis. The remaining constant incorporation of H^3-TdR shown in Figure 1 will be discussed later. If CM was added at 3 hr, DNA

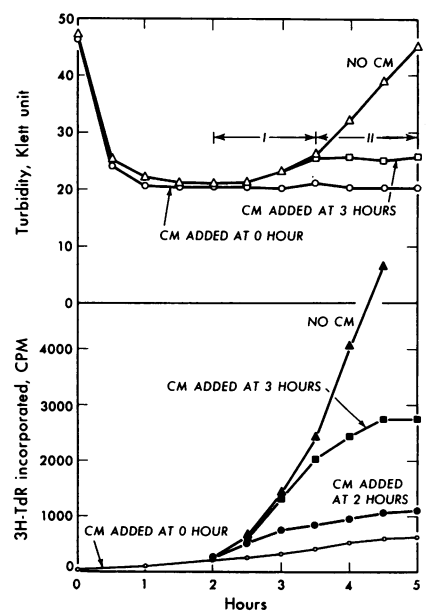

FIG. 1.—Effect of chloramphenicol (CM) on germination and DNA synthesis. Spores of a triple mutant MY2Y1U2 (thy^-, ind^-, leu^-) were germinated in 10 ml of synthetic medium to which had been added 20 μg/ml TdR, 50 μg/ml L-leucine, and 50 μg/ml L-tryptophan. The starting concentration of spores was 8×10^6 spore/ml; 20 μg/ml CM was added at the times indicated. The method of incorporation of H^3-TdR is described in the text. *I* and *II* in the figure represent the first and second generation, respectively. DNA's were isolated during germination, and the efficiency of transformation for adenine and methionine markers was measured using MU8U5U16 (leu^-, met^-, ade^-) as the recipient cell. The ratio of ade/met, normalized against that of spore DNA, changes from 1 to 2 periodically during germination. The time between the first and second doubling of adenine marker was taken as the first generation. The end of the second generation corresponds to the time when more than 80% of the spores had become four cells as seen under the microscope.

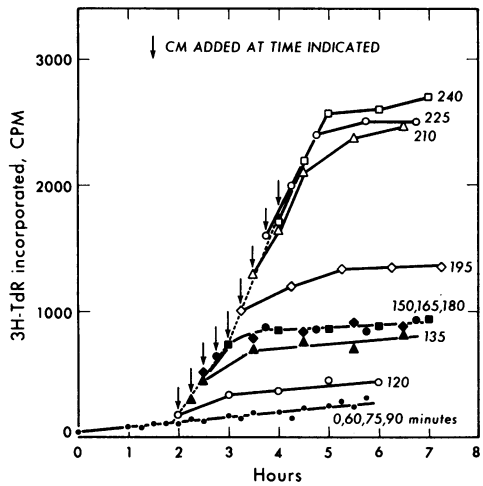

FIG. 2.—Effect of CM on DNA synthesis. The conditions of germination of spores and incorporation of H^3-TdR was the same as in Fig. 1. The numbers shown in the figure represent the times at which CM was added.

synthesis continued for approximately 90 min and eventually stopped. The amount of DNA synthesized in the presence of CM was approximately the amount of DNA synthesized in the first generation during germination without CM. This led to the assumption that the addition of CM at 3 hr allows the cells to complete chromosome synthesis which had started before CM addition and inhibits the initiation of the second cycle of replication. In order to test this assumption, CM was added at various times during the first and second generations and incorporation of H^3-TdR was followed. The result of such an experiment is shown in Figure 2. Two distinct levels of saturation of H^3-TdR incorporation were observed. Thus the addition of CM at any time between 135 and 180 min led to the incorporation of about 700 cpm, and the addition of CM at any time between 210 and 240 min led to the incorporation of about 2100 cpm in 0.1 ml of culture (basal level of incorporation shown as zero, 60, 75, 90-min line in Fig. 2 was subtracted). These two levels are comparable to the amount of DNA synthesized during the first generation and the first two generations, respectively. Taking the lower level as 100 per cent of the DNA synthesized during the first generation and the difference between the two levels as 100 per cent of the second generation, the final amount of DNA synthesized in the presence of CM (CM-DNA) was plotted against the time when CM was added (Fig. 3). The total amount of DNA synthesized before CM addition is also shown in Figure 3. If it is correct to assume that the cells which have initiated the

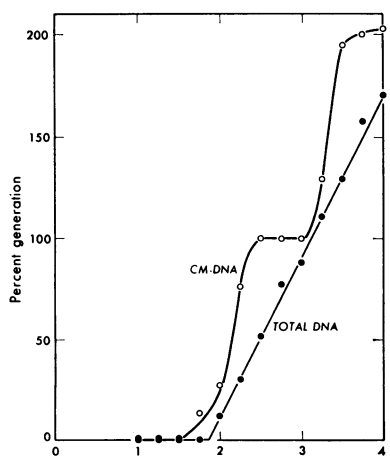

FIG. 3.—Initiation of chromosome replication and DNA synthesis during germination.

Zero, 60, 75, 90-min line of Fig. 2 was regarded as zero for normal replication (basal). The difference between 150, 165, 180-line and basal line (700 cpm) was regarded as 100% of DNA synthesized during the first generation. The difference between 210-min line and 150, 165, 180-min line (2100 cpm) was regarded as 100% of DNA synthesized during the second generation. The amount of H^3-TdR that had been incorporated before CM was added (total DNA), and the final amount of H^3-TdR incorporated in the presence of CM (CM-DNA) were calculated in relation to the above two levels of incorporation. Thus, when a given amount of incorporation (A) is <700 cpm, the per cent generation $(A) = (A/700) \times 100$, and when a given amount of incorporation (B) is > 700, the per cent generation $(B) = 100 + (B - 700)/(2100 - 700) \times 100$. These calculated values were plotted against the time when CM was added.

replication of the chromosome complete this cycle, the CM-DNA should represent the fraction of cells which have initiated replication at the times indicated. The stepwise increase of the fraction at the beginning of each generation indicates that the initiation of chromosome replication is markedly synchronous. This also indicates that a protein whose synthesis is inhibited by CM is synthesized at each generation in order to initiate the cycle of replication. The stability of this protein is shown in Figure 4. Spores of a triple mutant (leu$^-$, thy$^-$, ind$^-$) were germinated in the absence of TdR. In one case, H^3-TdR was added at various times later than 3 hr after the commencement of germination; in another, CM was added at 3 hr followed by H^3-TdR. In the absence of CM, DNA synthesis started immediately after addition of TdR and the rate of synthesis was identical regardless of the time of addition of TdR. In the presence of CM, DNA synthesis also started immediately after the addition of TdR but it stopped after an amount equivalent to that of one generation had been synthesized. This experiment indicates that the ability of the germinated spore to undergo one cycle of replication is established before CM addition and can be maintained without further protein synthesis.

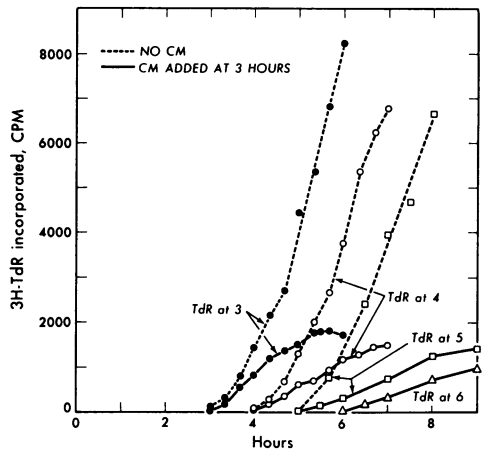

FIG. 4.—Initiation of DNA synthesis upon the addition of TdR to TdR-starved germinated spores.

Conditions of germination of H^3-TdR incorporation were the same as in Fig. 1, except that H^3-TdR was added at various times to TdR-starved germinated spores. In one case 20 μg/ml CM was added at 3 hr.

DNA synthesis in the presence of chloramphenicol: As is shown in Figure 1, there was a constant incorporation of H^3-TdR even when CM was added at the start of germination. The incorporation was independent of the concentration of CM over a range of 10 μg/ml to 100 μg/ml. Under the same conditions, no detectable increase of the relative frequency of adenine to methionine was observed. P^{32}-labeled phosphate and 5-BdU were incorporated by germinating spores in the presence of CM, and DNA was isolated by CsCl gradient centrifugation (Fig. 5*A*). As a comparison, 5-BdU was incorporated by P^{32}-labeled spores in the absence of CM (Fig. 5*B*). In the presence of CM most of P^{32} was incorporated into DNA of normal density, and only a small fraction of P^{32}-labeled DNA had a slightly heavier density. Both adenine and methionine markers were found in the heavier fraction in about the same proportion. This is a marked contrast to the normal replication shown in Figure 5*B* where only the adenine marker is found in the hybrid DNA. The details of this repairlike synthesis and its implications will be presented elsewhere. The result shown above clearly demonstrates that spores contain enzyme systems necessary for the incorporation of bases, nucleosides, and phosphate into DNA, and no novel enzymes are required for this purpose.

The presence of DNA polymerase in the spore: Although the isolation of enzymes directly from the spore is difficult, the enzymes isolated from germinated spores in

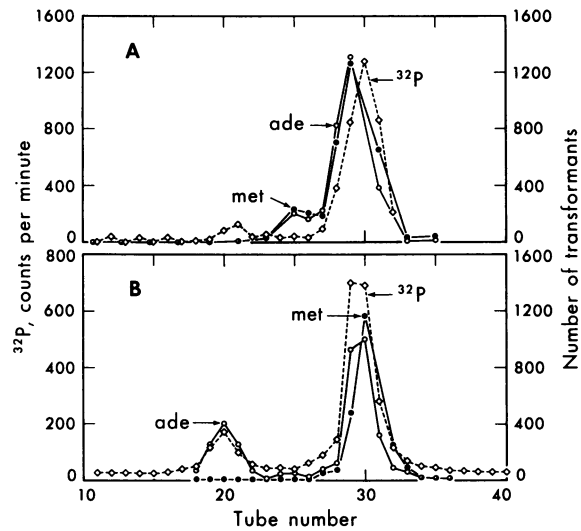

FIG. 5.—Centrifugation of DNA isolated from spores germinated in the presence of CM. (*A*) Spores of a triple mutant MY2Y1U2 (thy$^-$, ind$^-$, leu$^-$) were germinated in a synthetic medium with 50 μg/ml CM, 50 μg/ml L-try, 50 μg/ml L-leu, 50 μg/ml 5-BdU, 5 mC P^{32} for $5^1/_2$ hr. Total volume was 10 ml. After 6 hr incubation at 37°C with shaking, the culture was heated at 60°C for 10 min, centrifuged, and washed. Cells were then lysed with 1 mg/ml lysozyme at 37°C for 30 min, and lysis was completed by 1% sodium lauryl sulfate treatment. The lysate was placed in CsCl solution (0.01 *M* Tris-HCl, 0.001 *M* EDTA, pH 8.4) and centrifuged at 35,000 rpm for 40 hr at 25°C in Spinco model L-11. Samples were then fractionated by collecting dropwise from a hole made at the bottom of the tube. Each fraction was hydrolyzed by 0.1 *N* NaOH at 80°C for 30 min and precipitated by 10% TCA. The acid-insoluble count was measured. Transforming activity of each fraction was assayed for adenine and methionine markers. (*B*) P^{32}-labeled spores of MY2Y1U2 (thy$^-$, ind$^-$, leu$^-$) were germinated in a synthetic medium in the presence of 50 μg/ml 5BdU, 50 μg/ml L-leu, 50 μg/ml L-try for 4 hr. DNA was isolated and fractionated in the same way as in (*A*).

the presence of CM can be considered as being essentially those of the spore itself because protein synthesis is completely inhibited under these conditions. Crude enzymes were isolated from germinated spores and vegetative cells by the procedure described in *Materials and Methods*. In no case was a detectable amount of DNA released to the supernatant fraction. The DNA polymerase activity of the supernatant fraction is totally dependent on the addition of primer DNA. On the other hand, the enzyme activity of the precipitable fraction is not affected by the addition of primer DNA but is markedly activated by treatment with a low concentration of DNase or a relatively high concentration of lipase (Table 1). The enzymes from various spores and cells were assayed and the results are listed in Table 2. The supernatant fraction isolated from spores germinated in the presence of CM showed a slight enzyme activity. The precipitable fraction, however, was completely inactive unless it was treated by DNase or lipase. It is of considerable interest that the induction of supernatant enzyme and activation of precipitable fraction were observed during germination. The characteristics of these two enzyme fractions will be presented elsewhere. These experiments prove the existence of DNA polymerase in the spore although the implication of the activation of the precipitable fraction by DNase in relation to the initiation mechanism is not clear at the moment.

TABLE 1

EFFECT OF VARIOUS PRIMERS AND DNASE TREATMENT ON THE SUPERNATANT AND PRECIPITABLE ENZYMES

	Enzyme Fraction[a]	
	Supernatant	Precipitable
−DNA	2	34
+DNA[b]	308	30
dAT Copolymer[c]	5063	256
Denatured DNA[d]	243	63
DNase pretreated DNA[e]	754	27
DNase treatment[f]	7	326

[a] The preparation of the enzyme fractions was described in *Materials and Methods*. The supernatant enzyme used was obtained from exponentially growing cells, whereas the precipitable enzyme was obtained from spores germinated in the presence of CM. The enzyme activity was shown by H^3-dATP incorporation into 0.2 ml of reaction mixture during 30 min incubation at 37°C.

[b] DNA was isolated and purified by Marmur's method from *B. subtilis* wild type (W23). 10 μg was added to 0.5 ml reaction mixture.

[c] 10 mμmole of dAT copolymer was added to 0.5 ml reaction mixture.

[d] DNA was heated at 95°C in 0.15 *M* NaCl, 0.015 *M* Na_3 citrate, pH 7.0 for 10 min, then quickly cooled in ice. 10 μg was added to 0.5 ml reaction mixture.

[e] DNA was incubated at 37°C for 30 min with 4×10^{-2} μg/ml pancreatic DNase in 0.1 *M* Tris-HCl, 3×10^{-3} *M* Mg^{++}, 10^{-3} *M* mercaptoethanol. It was then reprecipitated in 3 vol of 95% ethanol.

[f] The precipitable fraction was incubated at 37°C for 45 min with 4×10^{-3} μg/ml pancreatic DNase in 0.1 *M* Tris-HCl, 3×10^{-3} *M* Mg^{++}, 10^{-3} *M* mercaptoethanol. The reaction was stopped by adding 20 vol of ice-cold buffer and immediate centrifugation at 4°C. The precipitates were resuspended in the original volume in the same buffer.

Discussion.—Studies on the effect of CM on DNA synthesis during the germination of spores confirms Maaløe's early suggestion.[8] Thus, the cell which has initiated replication at the time of CM addition can continue to replicate and complete the cycle in the presence of CM, but cannot initiate a new cycle of replication. In *E. coli*, however, DNA synthesis, in the presence of CM, exceeds the completion of one cycle. From this fact Lark proposed a model in which two proteins, a structural and an initiator protein, regulate the initiation of replication.[7] Of these, the synthesis of the structural protein is less sensitive to CM. The different effect of CM on the initiation of chromosome replication in *E. coli* and *B. subtilis* might be due to the difference in their cell wall structures.

As is shown in Figure 4, the spores germinated in the absence of TdR and the presence of CM, added at 3 hr, start DNA synthesis immediately after the addition of TdR. This observation can be explained by one of the following two mechanisms. One alternative is that the initiator protein is stable and exerts its function when TdR becomes available. A second possibility is that initiation took place

TABLE 2

DNA POLYMERASE FROM VARIOUS SPORES AND CELLS

Conditions of germination[a]	Incubation time (hr)	Enzyme Activity[b]			
		Supernatant		Precipitate	
		−Lipase	+Lipase[c]	−Lipase	+Lipase
Complete + CM[d]	2.5	97	102	9	489
Complete − TdR	3.5	2146	2101	21	908
Complete 1	3.5	2416	2448	124	766
2	5.5	656	765	286	950
Exponential[e]		325	389	433	798

[a] Spores of MY1Y2U2 (thy⁻, ind⁻, leu⁻), 4×10^7 spores/ml, were germinated in synthetic medium. DNA synthesis started at 3.5 hr in this medium which contains 20 μg/ml TdR, 50 μg/ml leu, and 50 μg/ml try (complete medium).

[b] The method of enzyme assay was described in *Materials and Methods*, and expressed the same as in Table 1. The enzyme was isolated from the same number of cells in each case so that the activities shown in the table are comparable.

[c] 2.5 mg/ml lipase was added with lysozyme during the lysis process. 4×10^{-2} μg/ml DNase showed about the same effect.

[d] 50 μg/ml CM was added.

[e] The same mutant was grown in basal medium enriched with 0.05% casamino-acids, 50 μg/ml L-leu, 5 μg/ml L-try, and 20 μg/ml TdR. The enzyme was isolated during the exponential phase.

without TdR, and addition of TdR simply allows the commencement of the replication of the chromosome. The latter possibility is the more likely, if initiation involves the formation of a single-stranded region at the origin of the chromosome. This initiation mechanism will be discussed later.

The incorporation of H^3-TdR, 5-BdU, and P^{32} into DNA by spores germinated in the presence of CM is demonstrated. This proves the existence, in the spore, of an enzyme system which is necessary for the incorporation of bases and phosphate into the DNA molecule. This DNA synthesis is, however, different from normal chromosome replication, the sequential semiconservative replication. Most of the P^{32} was incorporated into DNA of normal density and only a small fraction of P^{32}-labeled DNA had a slightly heavier density. Both adenine and methionine markers were found in the heavier fraction in about the same proportion. This is a marked contrast to the normal replication where only the adenine marker is found in the hybrid DNA (Fig. 5*B*). This result suggests that the incorporation in the presence of CM is random along the chromosome and each incorporated piece is so small that 5BdU in the piece does not exert a significant effect on the density of DNA of approximately 2×10^7 mol. wt. This incorporation resembles the repair synthesis which is found as a consequence of the excision of nucleotides by UV treatment.[17]

Careful disruption of cells made it possible to separate DNA polymerase activity from exponentially growing cells into three fractions (Table 2). However, in the spore only one of these is predominant and is found to be precipitable with DNA in the cell membrane fraction and is incapable of incorporating deoxynucleoside-triphosphates into DNA unless it is first subjected to DNase treatment. The effect of a relatively high concentration of lipase is easily explained by the contamination of 0.01 per cent w/w DNase. The fact that a relatively constant amount of enzyme in the precipitable fraction was found from various spores and cells suggests that the precipitable enzyme is the one responsible for the replication of the chromosome. It is also assumed that the supernatant enzyme might be involved in repair synthesis. The final decision as to the role of the two enzyme fractions has to be made by direct demonstration of replication and repair of chromosome *in vitro*. Activation of the precipitable fraction by DNase suggests that the chromosome in the fraction is intact and does not serve as a primer for DNA polymerase. However, DNA polymerase can utilize this chromosome as a primer upon formation of a few breaks by DNase.

Summary.—The effect of chloramphenicol on DNA synthesis during germination of *B. subtilis* spores was studied. Analysis of DNA synthesized after the addition of chloramphenicol at various times showed that a protein is synthesized before each cycle of generation in order to initiate chromosome replication. Repair-type synthesis was found in spores germinating in the presence of chloramphenicol and under these conditions there was no indication of normal replication. The presence of DNA polymerase activity was demonstrated in the spore. The nature of this DNA polymerase was discussed in relation to the initiation mechanism of chromosome replication.

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Abbreviations: thy$^-$, ind$^-$, leu$^-$, ade$^-$, met$^-$, were used for mutants requiring thymine, indole,

leucine, adenine, and methionine, respectively; TdR, thymidine; dAT copolymer, copolymer of deoxyadenylate and deoxythymidylate; CM, chloramphenicol; 5BdU, 5-bromodeoxyuridine.

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[1] Yoshikawa, H., and N. Sueoka, these PROCEEDINGS, **49**, 559 (1963).
[2] *Ibid.*, 806.
[3] Nagata, T., these PROCEEDINGS, **49**, 551 (1963).
[4] Cairns, J., *J. Mol. Biol.*, **7**, 534 (1963).
[5] Jacob, F., S. Brenner, and F. Cuzin, in *Synthesis and Structure of Macromolecules*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 28 (1963), p. 329.
[6] Kohiyama, M., H. Lanfrom, S. Brenner, and F. Jacob, *Compt. Rend.*, **257**, 1979 (1963).
[7] Lark, C., and K. G. Lark, *J. Mol. Biol.*, **10**, 120 (1964).
[8] Maaløe, O., and P. C. Hanawalt, *J. Mol. Biol.*, **3**, 44 (1961).
[9] Woese, C. R., and J. R. Foro, *J. Bacteriol.*, **80**, 811 (1960).
[10] Donnellan, J. E., Jr., E. H. Nags, and H. S. Levinson, *J. Bacteriol.*, **87**, 332 (1964).
[11] Oishi, M., H. Yoshikawa, and N. Sueoka, *Nature*, **204**, 1069 (1964).
[12] Yoshikawa, H., A. O'Sullivan, and N. Sueoka, these PROCEEDINGS, **52**, 973 (1964).
[13] Personal communication from Dr. I. Takahashi.
[14] Takahashi, I., *J. Bacteriol.*, **87**, 1499 (1964).
[15] Marmur, J., *J. Mol. Biol.*, **3**, 208 (1961).
[16] Okazaki, T., and A. Kornberg, *J. Biol. Chem.*, **239**, 259 (1964).
[17] Pettijohn, D., and P. Hanawalt, *J. Mol. Biol.*, **9**, 395 (1964).

REGULATION OF PYRIMIDINE BIOSYNTHESIS IN SERRATIA MARCESCENS

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Recent work by Beckwith *et al.* (1962) on the regulation of pyrimidine biosynthesis in *Escherichia coli* has prompted an investigation of this biosynthetic pathway in *Serratia marcescens*. It was observed that the pathway for biosynthesis of pyrimidines is identical in *S. marinorubra* (W. L. Belser, 1961) and in *E. coli* (J. R. Beckwith *et al.*, 1962). The isolation of a number of uracil auxotrophs in *S. marcescens* made it possible to examine the pyrimidine pathway in this organism, and in addition to analyze the mode of regulation of enzyme synthesis.

All six of the enzymes in the pyrimidine pathway of *E. coli* were found by Beckwith *et al.* (1962) to be subject to regulation. Four of the six were coordinately repressed by additions of uracil or orotic acid to the media. The structural genes specifying the four coordinately regulated enzymes appeared to map in a cluster near the galactose locus. It was assumed that these four genes were part of a single operon. It was later demonstrated that one of the four genes (viz., the structural gene specifying orotidylic pyrophosphorylase) was located at some distance from the other three, near the mannitol locus, and thus could not belong to the same operon (A. L. Taylor *et al.*, 1964). The other two genes, specifying the noncoordinately regulated enzymes, mapped independently on the chromosome, and therefore do not belong to the same operon. Although no direct proof was available, it was