JOURNAL OF BACTERIOLOGY, Oct. 1968, p. 981-986
Vol. 96, No. 4
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Deoxyribonucleic Acid Synthesis During Microcyst Germination in *Myxococcus xanthus*

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Received for publication 27 May 1968

Deoxyribonucleic acid (DNA) synthesis was measured during microcyst germination in *Myxococcus xanthus* by radioactive thymidine incorporation, autoradiography, and chemical analysis. Microcysts contained an average of 6.6 conserved units of DNA, corresponding to 3 to 4 chromosomes per cell. Correlation of the DNA content and chromosome number of microcysts indicated that the molecular weight of the nonreplicating *M. xanthus* chromosome is 4.9×10^9 daltons. DNA synthesis was initiated 3.5 to 4 hr after induction of germination. From 4 to 6 hr, the rate of synthesis was constant and the accumulation was linear. After a lag period (6 to 6.5 hr), the rate of DNA synthesis increased, reaching a second plateau at 9 hr. From 9 to 11 hr, the rate was again constant and the accumulation was linear. Cellular division during germination showed an unusual kind of synchrony. A model is presented that accounts for chromosomal replication and cell division during microcyst germination.

The bacterium *Myxococcus xanthus* represents an interesting biological system for the study of the regulation of deoxyribonucleic acid (DNA) synthesis. Rosenberg, Katarski, and Gottlieb (13) presented a detailed description of DNA synthesis during the intracellular differentiation of motile vegetative rods to nonmotile spherical microcysts. Autoradiographic and DNA analysis studies indicated that chromosomes are completed during microcyst formation, but that they do not initiate the next round of replication. The net increase in DNA during morphogenesis is about 20%. These findings predict that microcyst germination should be accompanied by synchronous chromosome initiation and replication.

In this study, we investigated the kinetics of synthesis during microcyst germination and analyzed the microcyst DNA pattern of segregation into daughter cells. These experiments define chromosomal replication during a natural cycle of great physiological and morphological change.

MATERIALS AND METHODS

Bacterial growth and microcyst formation. M. xanthus strain FB was grown in NZ-Case medium (Sheffield Chemical Co., Norwich, N.Y.) and induced to form microcysts as described previously (1); the doubling time under these conditions is 270 min. An exponentially growing culture, with an optical density of 0.590 at 560 nm, was induced to form microcysts by the addition of glycerol to a concentration of 0.5 M. At 4.5 hr after induction, samples were dispensed into sterile tubes and frozen rapidly in a dry ice-acetone bath. The microcysts were stored at −40 C and thawed when needed. Viability remained high for at least 3 months.

Induction of microcyst germination. Germination was induced by diluting the thawed microcysts 10-fold into a fresh, prewarmed medium. Cultures were incubated at 30 C under conditions of vigorous gyratory shaking. Samples were removed with an automatic syringe, which eliminated having to uncover the flask and stop the shaking.

Kinetics of thymidine incorporation. DNA synthesis was measured by incorporation of both thymidine-methyl-^{3}H and thymidine-2-^{14}C (Schwarz Bio Research, Inc., Van Nuys, Calif.) into a cold trichloroacetic acid-insoluble fraction. The complex growth medium contained approximately 5 μg of thymidine per ml, as measured by isotope dilution experiments. Germination was performed in a medium that contained ^{14}C-thymidine. Samples (2-ml) from the labeled culture were transferred into prewarmed test tubes that contained ^{3}H-thymidine and were incubated at 30 C with gyratory shaking. Incorporation was terminated by the addition of 2 ml of 10% iced trichloroacetic acid. The samples were transferred to membrane filters and washed as described previously (1). Radioactivity was measured in a liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.) by use of 10 ml of toluene that contained 40 mg of 2,5-diphenyloxazole and 0.5 mg of *p*-bis-(2,5-phenyloxazolyl)-benzene per sample as scintillation fluid.

Chemical analysis for DNA. DNA was estimated

chemically after fractionation by a modification of the Schmidt-Thannhauser procedure (11). The culture (200 ml) was centrifuged, washed with buffer, and resuspended in iced 10% perchloric acid (PCA). After 1 hr, the precipitate was collected by centrifuging, washed with 5% PCA, resuspended in 0.5 M NaOH, and incubated at 37 C for 24 hr. The alkali hydrolysate was chilled to 0 C, and the precipitate was removed by centrifugation. The supernatant fraction was reprecipitated with 5% PCA. After the entire pellet was suspended in 0.3 ml of water, 0.6 ml of diphenylamine reagent was added (3).

Cell growth. The turbidity of germinating cells was measured at 560 nm by use of a 1-cm light path (Beckman DU spectrophotometer). Cell counts were performed on samples fixed in 2% formaldehyde by use of a Petroff-Hausser counting chamber.

Autoradiography of cells labeled with thymidine-methyl-^3H. Cells in 1 ml of culture were labeled with 500 μc of thymidine-methyl-^3H. Radioautographs were prepared as described previously (13), except that the Ilford K-5 photographic emulsion (Ilford, Ltd., Ilford, Essex, England) was used. Exposure times were adjusted so that 4 to 6 grains per labeled cell were present. The slides were developed as suggested by Caro et al. (2).

RESULTS

Kinetics of DNA synthesis during microcyst germination. Germination of microcysts of *M. xanthus* was initiated by dilution into fresh medium that contained ^{14}C-thymidine. At 15-min intervals, samples were removed and incubated for 10 min in ^3H-thymidine (Fig. 1). For 3.5 hr, there was no detectable DNA synthesis, as determined by ^3H and ^{14}C incorporation and chemical analyses (Table 1). Between 3.5 and 4 hr, the rate of DNA synthesis rose sharply and remained constant for 2 hr (Fig. 1, level A). During this period, DNA accumulated at a linear rate (^{14}C radioactivity).

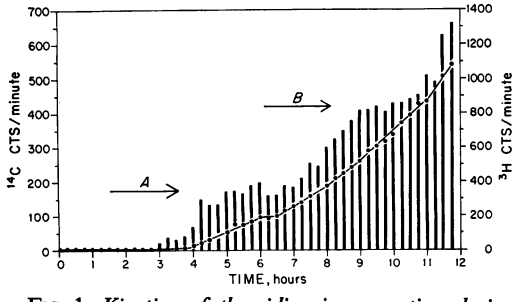

FIG. 1. *Kinetics of thymidine incorporation during microcyst germination. The microcysts were prepared by the glycerol induction technique and stored at −40 C. Germination was initiated by a 1:10 dilution in fresh media that contained 0.017 μc of ^{14}C-thymidine per ml. At 15-min intervals, duplicate 2-ml samples were removed and pulsed for 10 min with ^3H-thymidine (5 μc/ml). Incubation was terminated by addition of 2 ml of 10% trichloroacetic acid that contained 1 mg of thymidine per ml. The cumulative incorporation of thymidine into an acid-insoluble form (●) is represented on the left ordinate; the vertical bars represent the ^3H radioactivity incorporated during each 10-min interval. The arrows (A and B) represent levels of constant rate of synthesis.*

At 6 hr, there was a significant decrease in the rate of DNA synthesis that lasted for a period of 30 min. More frequent and shorter ^3H pulses (Fig. 2) confirmed the decreased rate of DNA synthesis during this interval from 6 to 6.5 hr. This lag occurred when the DNA had increased 80% (Table 1).

At 6.5 hr, there was a resumption of DNA synthesis. The rate of synthesis was similar to that found during the period from 4 to 6 hr. From 7.5 to 9 hr, the rate increased to a second plateau

TABLE 1. *DNA content of M. xanthus at specific times during germination*[a]

Time (hr)	DNA (μg/ml of culture)	Relative amount
0	0.17	1.0
3	0.17	1.0
6.5	0.31	1.8
12	1.13	6.5

[a] Microcysts were germinated by the dilution technique described in Fig. 1. Samples of 200 ml each were removed at timed intervals, and the DNA content was determined by the Dische reaction (3) after a modified Schmidt-Thannhauser procedure, which was described in Materials and Methods.

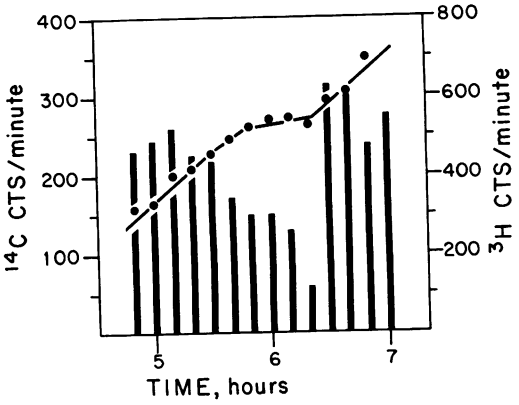

FIG. 2. *Kinetics of thymidine incorporation during microcyst germination; detail of 4- to 7-hr period. The experiment was performed as described in Fig. 1, except that the specific activities were 0.012 μc of ^{14}C-thymidine and 10 μc of ^3H-thymidine per ml of media, and duplicate 1-ml samples were withdrawn at 10-min intervals for 6-min pulses. The ordinate and abscissa are as in Fig. 1.*

period (Fig. 1, level B). Level B, like level A, lasted approximately 2 hr. These two periods of constant rate of ^3H incorporation, 4 to 6 hr and 9 to 11 hr, are an indication of the synchrony of chromosomal replication.

These kinetic experiments were repeated several times with different batches of microcysts. Similar data were obtained in all cases, although the initial lag period was sometimes shifted by 15 min.

Cell growth during microcyst germination. Figure 3 describes the changes in cell number and turbidity during germination. The turbidity measurements are difficult to interpret because of changing refractility and cell geometry. Microscopically, the microcysts lost their refractility in about 1 hr, and appeared as nodular rods by 2 hr. Cell division began at 4.5 hr and continued exponentially until 9 hr. Surprisingly, the cells quadrupled during this 4.5-hr period. Normally, this strain of *M. xanthus* has an optimal doubling time of 4.5 hr under these growth conditions (1). At 9 hr, the cells appeared to be abnormally small; there was no further increase in cell number for 2 hr. These results indicate a rather unusual synchrony of cell division; the relationship between DNA synchrony and division is not understood, nor is it known whether similar types of synchrony occur during the germination of naturally found microcysts.

Segregation of conserved units of DNA during microcyst germination. The number of conserved DNA units present in the microcyst was determined by labeling exponentially growing *M. xanthus* for several generations with ^3H-thymidine, microcysting the cells with 0.5 M glycerol, and then germinating the cells in a fresh medium that contained 100 μg of thymidine per ml. Samples were removed at intervals and prepared for autoradiography (10). Table 2 presents the data obtained from cells that were removed after 3, 12.5, 22.5, and 26 hr of germination. Exposure times were selected so that the mean observed grain count was about 4 to 6 per cell. Analysis of the corrected data is presented in Table 3. The

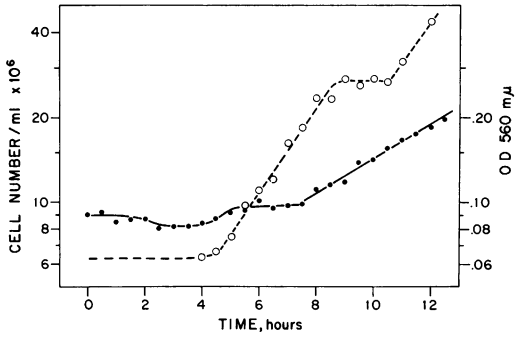

FIG. 3. *Kinetics of growth during microcyst germination. Duplicate samples of germinating microcysts were withdrawn at 30-min intervals for measurement of turbidity (●) and cell number (○). The optical density was measured at 560 nm by use of a 1-cm light path. Cell counts were performed on samples fixed in 2% formaldehyde by use of a Petroff-Hausser counting chamber. Over 1,000 cells were counted at each time interval.*

TABLE 2. *Distribution of radioactive DNA in daughter cells during germination and subsequent multiplication in nonradioactive medium*[a]

Grains per cell	No. of cells examined at hr indicated			
	3[b]	12.5	22.5	26
0	1	94	269	501
1	3	36	43	128
2	4	38	28	40
3	11	31	24	36
4	11	26	20	27
5	7	16	17	23
6	3	9	14	17
7	1	7	12	12
8	2	8	10	10
9	2	5	8	8
10	0	5	6	5
11	0	2	2	4
12	0	0	2	4

[a] Cells were grown for 6 generations and induced to form microcysts in a medium that contained 500 μc of ^3H-thymidine per ml. Microcysts were germinated by dilution in fresh medium that contained 100 μg of thymidine per ml. After dilution, the specific activity was 5% of the original level. The expected Poisson distribution for this residual radioactivity was calculated and appropriate values were subtracted.

[b] Cells removed from the medium at 3, 12.5, 22.5, and 26 hr had been exposed to ^3H-thymidine for 18, 72, 144, and 144 hr, respectively.

TABLE 3. *Analysis of data from Table 2*[a]

Time (hr)	No. of generations[b]	No. of cells examined	Avg no. grains per cell	Per cent without grains	Avg no. grains per labeled cell[c]
3.0	0	45	32.4	<2	32.4
12.5	2.4	274	5.6	54	9.4
22.5	4.6	460	2.6	68	4.9
26.0	5.4	826	2.2	76	4.9

[a] All values are standardized for 144 hr of exposure.

[b] Data obtained in part from Fig. 3.

[c] The cells that conserved some radioactivity gave rise to a Poisson distribution of grains. The mean was obtained from the slope of the frequency distribution, as described by Hanawalt et al. (7).

3-hr data are equivalent to microcysts, since no DNA synthesis or cell division took place at this time. The average number of grains per cell decreased, as expected, during successive generations in the nonradioactive medium. The average number of grains per labeled cell decreased from an initial level of 32.4 to a limit of 4.9; the percentage without grains increased continuously. This indicated that the microcysts contained 6.6 conserved units, or 3 to 4 chromosomes per microcyst.

Autoradiographic measurements of germinating microcysts. Chromosomal completion of two sequentially replicating chromosomes (13) during microcyst formation suggests heterogeneity in the DNA content of the microcysts. This heterogeneity would be expected to be maintained during microcyst germination.

An experiment was performed to determine whether all cells participated in the 4- to 6-hour period of DNA synthesis after microcyst germination. Microcysts were germinated in a medium that contained 500 μc of ^{3}H-thymidine per ml, and samples were removed for autoradiography at 5 and 6.5 hr. At both times, a 25% zero-class was found. This demonstrates that 25% of the cells did not synthesize DNA during the first 6.5 hr.

DISCUSSION

A working hypothesis has been constructed to describe chromosomal replication during the germination of *M. xanthus* microcysts to vegetative rods (Table 4).

Chromosomal content of microcysts. We have presented data (13) to indicate that (i) exponentially growing cells contain two to four chromosomes, (ii) the chromosomes are sequentially replicated during 80% of the division cycle, and (iii) chromosomes are completed but not initiated after microcyst induction. This model predicts a mixed population of microcysts that contain 2, 3, and 4 chromosomes. The fraction of cells in each class would depend on how the rest period in DNA synthesis coincides with the division cycle.

TABLE 4. *Postulated chromosomal content of M. xanthus at various times during germination*

Time (hr)	Chromosomal model[a]	Relative no. of growing points
0 to 3.5	$(III)_3$ $(IIII)_1$	0
4 to 6	$(YYY)_3$ $(IIII)_1$	9
6 to 6.5	$(II)_9$ $(YI)_2$	2
6.5 to 9	$(YI)_9$ $(YI)_2$	11-22
9 to 11	$(YI)_{18}$ $(YI)_4$	22

[a] Subscripts represent the relative number of cells in each class. For example, from 0 to 3.5 hr, 75% of the cells contain three chromosomes and 25% contain four. Completed chromosomes are represented by I, replicating chromosomes by Y.

Combining the data in Table 1 and Fig. 3, each microcyst contains an average of 27×10^{-9} μg of DNA. Since there are 6.6 conserved units of DNA (Table 3) or 3.3 chromosomes per cyst, it follows that the nonreplicating chromosome contains 8.2×10^{-9} μg of DNA or 4.9×10^{-9} daltons. For comparison, the *Bacillus subtilis* W23 chromosome has a molecular weight of 3.8×10^9 to 4.2×10^9 daltons (5).

Initiation of DNA synthesis. The data from Fig. 1 demonstrate that synchronous initiation of chromosomal replication occurred between 3.5 and 4 hr of germination. The rate of synthesis rose rapidly to a level that remained constant for 2 hr. This would be the case if a group of chromosomes began synthesis synchronously and no additional growing points were initiated for 2 hr. Hence, the rate of synthesis remained constant between 4 and 6 hr, although the number of cells almost doubled.

The model in Table 4 describes two classes of microcysts, 75% of which contain three chromosomes, and the remainder four. DNA initiation is postulated to occur simultaneously in all chromosomes of the former, but in none of the latter until after the completion of cell division. These aspects of the models are based on the following data: (i) autoradiography showed a mixed population of cells at 6.5 hr in which 25% of the cells had not made any DNA; (ii) the amount of DNA increased 75 to 80% by 6.5 hr (Table 1), at which time the ^{14}C accumulation experiments indicated a lag in synthesis (Fig. 2). Thus, the time required for 75% of the cells to double their DNA content was only 2 hr. The most reasonable way that these cells can double their DNA content in less than half of their generation time is for the initial synchronous replication to involve simultaneous initiation on all chromosomes, in contrast to the normal sequential pattern. The 25% class that does not synthesize any DNA until 6 hr is postulated to be the microcysts that already contain a full complement of four chromosomes.

Completion of the first round of replication. A large decrease in DNA synthesis was found in the accumulation and pulse experiments between 6 and 6.5 hr (Fig. 2). This is consistent with the 25- to 50-min rest period previously determined from autoradiographic analysis of pulse-labeled exponentially growing cells. Thus, the lag period described here is a measure of the synchrony of DNA synthesis during germination. The small amount of synthesis observed between 6 and 6.5 hr may be due to some initiation of the four-chromosome class.

Initiation of a second round of replication. At 6.5

hr, chromosomal replication returned to the sequential pattern. This accounts for the fact that the rate of DNA synthesis remained at the 4- to 6-hr level when synthesis resumed at 6.5 hr (Fig. 1) despite the doubling observed in cell number (Fig. 3). The linear increase in the rate of synthesis observed from 7 to 9 hr paralleled the change in cell number. This suggests that, after 7 hr, all cells have a constant number of growing points. The change from three cells with three replicating chromosomes each to nine cells with one replicating chromosome could take place by unequal distribution of cell material, followed by a rapid second division of the larger cell; this problem is under cytological investigation. When cell division ceases (9 to 11 hr), there is no further increase in the rate of DNA synthesis (Fig. 1, level B). The rate of synthesis at level B was 2.34 that of level A; the model predicts a ratio of 22:9, or 2.44.

Quantity of DNA per cell during microcyst germination. Correlation of the data from Fig. 1 and 3 and from the chemical analysis of DNA in Table 1 yields a profile of the DNA content per cell during microcyst germination (Fig. 4). The changes in slope that occur are indicative of synchrony at both the DNA and cellular levels. The maximum amount of DNA per cell observed is 20.4% above that of the microcyst; the theoretical increase that would be expected if cells increased their chromosomal content from 3.3 to a maximum of 4 is 21.2%. Similarly, the minimal amount of DNA per cell observed is 31.6% below that of the microcyst; the theoretical decrease that would be expected if all cells were born with two chromosomes is 39.4%. In both cases, there is good agreement between the observed data and the values predicted from the model. Furthermore, the decline in synchrony should be accompanied by decreased maxima and increased minima, reaching a level which is 20% below that of the microcyst, 22.4 $\times$ 10^{-9} μg/cell.

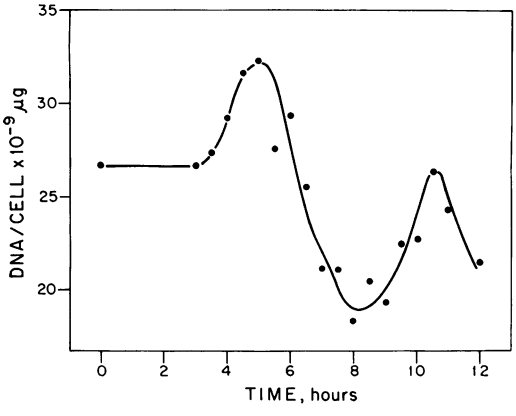

FIG. 4. *Quantity of DNA per cell during microcyst germination. Data from Table 1, Fig. 1, and Fig.3 are correlated.*

Comparison of DNA synthesis during endospore and microcyst germination. Microcyst and spore formation both represent intracellular differentiation of vegetative cells to more resistant resting cells. In both cases, these resting cells contain completed chromosomes (12), which suggests that the formation of resting cells is coupled to a block in chromosome initiation. This is a natural extension of the Replicon model (8).

The multiple chromosomal content of microcysts is in sharp contrast to the single chromosome that is present in the endospore (6). This fundamental difference exists because, unlike endospore formation, all of the contents of the vegetative cells of the myxobacteria are enclosed in microcysts (4). Consequently, microcysts are a mixed population with respect to chromosomal content, and they reflect the age differences of cells at the time of induction.

Chromosome initiation in germinating endospores was preceded by a lag period in which there was no net DNA synthesis; after this period, multiple growing points were observed (14, 15). The presence of multiple growing points in the germinating spore leads directly to the restoration of the chromosomal complement of the exponential cell; the microcyst, which has an average of 20% more DNA than the exponential cell, undergoes two rapid cell divisions upon germination. The rapid division of the DNA-rich germinating cells suggests a control mechanism that links DNA content and cell division.

Finally, after completing this manuscript, we discovered an early paper by E. Klieneberger-Nobel that described cytological changes during morphogenesis of the myxobacteria (9). In *Myxococcus virescens* and *Myxococcus fulvis*, multiple nuclear structures were observed during various stages of growth. On the basis of data obtained by utilization of chromosome strains, a general model for "nuclear" division during morphogenesis was proposed in 1946. To our amazement, this early model, based entirely on cytological evidence, is remarkably similar to the model proposed here.

ACKNOWLEDGMENTS

This investigation was supported by National Science Foundation grant GB-6155. David Zusman is a recipient of a National Institutes of Health predoctoral traineeship (GM-01297).

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