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Cell-Cycle-Specific Inhibition by Chloramphenicol of Septum Formation and Cell Division in Synchronized Cells of *Bacillus subtilis*

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The relationship between protein synthesis and processes of cell division was studied by using synchronized cells of *Bacillus subtilis* 168. The addition of chloramphenicol at the beginning of synchronous growth prevented septum formation and cell division, suggesting the requirement of protein synthesis for the processes of cell division. Experiments in which the drug was added to the cells at different cell ages showed that the protein synthesis required for the initiation of septum formation was completed at about 15 min and that the protein synthesis required for cell division was completed at about 45 min. By interpreting the result from the concept of the transition point for protein synthesis, it was suggested that the processes of cell division in *B. subtilis* require at least two kinds of protein molecules which are synthesized at distinct stages in the cell cycle. This was supported by the result of an experiment in which starvation and the readdition of a required amino acid to exponentially growing cells induced two steps of synchronous cell division. Further, the two transition points are in agreement with the estimations obtained by residual division after the inhibition of protein synthesis in asynchronous cells. The relationship of the timing between the completion of chromosome replication and the two transition points was also studied.

In their studies on the control of bacterial cell division, Jones and Donachie (13, 14) proposed a model in which cell division of *Escherichia coli* requires the completion of two parallel sequences of events consisting of protein synthesis and chromosome replication. A similar model was presented independently by Shockman et al. (24) for cell division in *Streptococcus faecalis*, which includes, in addition, a separate series of timed events called the W cycle, leading to the synthesis of a complete new unit of cell surface. In either model, the completion of both chromosome replication and protein synthesis are required for cell division. The requirements of specific proteins for cell division have been suggested in *Bacillus subtilis* cells by the findings that the recovery of septum formation (1, 3) or cell division (18) in temperature-sensitive divisionless mutants is blocked by inhibitors of protein synthesis added during, but not after, a short period of recovery at the permissive temperature. However, cell division in *B. subtilis* does not seem to be coupled with the completion of chromosome replication of the ongoing cell cycle, because it occurs in the absence of DNA synthesis (1, 5).

It was of special concern to us to know whether the cells in which septum formation has been initiated once are able to divide without further protein synthesis. If the division of cells in the septated state requires additional protein synthesis, the inhibition of protein synthesis during septum formation should prevent the occurrence of subsequent cell division. In this paper, using synchronized cells of *B. subtilis*, we studied the effects of chloramphenicol on both septum formation and cell division and found that the effects were dependent largely on the cell age at which the drug was added. For interpreting the age dependency of the effects, we applied the concept of the transition point of protein synthesis (8, 12, 17, 19, 25) to the cell cycle of this bacterium. Further, changes in cell morphology were studied in connection with the termination of chromosome replication and the timings of the transition of protein syntheses.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *B. subtilis* 168 (*thyA thyB hisB31*) was obtained from H. Saito of the Institute of Applied Microbiology, University of Tokyo. The cells were grown with shaking at 37°C in a medium described previously (7). Thymine and L-histidine were supplemented at final concentrations of 0.1 and 0.2 mM, respectively. Glucose was added at a final concentration of 4 or 28 mM.

Synchronization by cyclic glucose starvation. A synchronized culture was obtained by repeating 8 to 10 cycles of glucose starvation in a chemostat-synchronizing fermentor (7, 15). The synchronized cells were then grown after a fourfold dilution with fresh medium containing 28 mM glucose and subjected to the subsequent experiments. The total cell number was counted with a counting chamber.

Observation of cell morphology. Cell wall was stained with crystal violet after mordanting with tannic acid (20) and observed with a light microscope. A cell which had a clearly stained cross wall was defined as a septated cell, and one in which the septal site had developed to fully hemispherical adjoining ends (23) was defined as a double cell. Frequencies of septated or double cells were determined by scoring 250 to 500 total cells. In some samples, cells were negatively stained with 1% ammonium molybdate (pH 7.0) (6) and observed with a JEOL JEM 100U electron microscope for a confirmation of the results of the light microscopic method. In this case, cells having electron-dense bands at the equatorial area were counted for a determination of the frequencies of septated cells.

Inhibitors of DNA or protein synthesis. Cells were inhibited by the addition of nalidixic acid (20 μg/ml) or chloramphenicol (200 μg/ml). At this concentration of chloramphenicol, the pulse incorporation rate of thymidine or uridine by cells in an asynchronous population was suppressed to the level of 55 or 58% respectively, after 30 min of treatment, whereas that of leucine was suppressed selectively to the level of 4%.

In experiments to examine the effects of the inhibition of protein synthesis on septum formation and cell division in synchronized cells, chloramphenicol (200 μg/ml) was added to the cultures at different cell ages, and the changes in the frequencies of septated cells and in the total cell number were then determined.

Starvation for thymine or histidine. In an experiment of thymine starvation, exponentially growing cells were rapidly collected on an HA membrane filter (Millipore Corp.) and washed with and suspended in a prewarmed medium deprived of thymine and histidine. The cells were incubated with shaking at 37°C after the immediate addition of histidine at the normal concentration of 0.2 mM. In an experiment of histidine starvation and readdition, cells were starved for histidine. After incubation, histidine was added to the culture. The total cell number and the frequency of septated cells were subsequently followed.

RESULTS

Period of septum formation. Morphological changes during the synchronous growth of *B. subtilis* 168 were observed by light microscopy after cell wall staining or by electron microscopy after negative staining (Fig. 1). The frequency of septated cells changed markedly, exhibiting maximum and minimum frequencies at 35 and 75 min of cell age, respectively. The oscillation was repeated in the second cycle. As shown in Fig. 1, an estimate of the period of the septated-cell state was 20 to 55 min. During a short period after the completion of septum formation until cell separation (division), a small number of cells was present in a double-cell state. The average generation time, as determined by the increase in cell count, was 65 min for the first and the second cycles.

Requirements of protein synthesis for the processes of cell division. When chloramphenicol was added during the very early period (0 and 10 min) of synchronous growth, only slight increases in the frequency of septated cells were observed (Fig. 2). By contrast, when the time of the drug addition was delayed to 20 min, the frequency increased markedly and reached 70% of the control, although these cells continued to maintain a high frequency of the septated-cell state in contrast to the control cells. This result indicates that the addition of chloramphenicol does not prevent septation in cells aged for more than 20 min. An estimate of the critical cell age is about 15 min (0.23 of the generation time). At this time the addition of the drug blocked the forthcoming septum formation by 50%. According to the definition by Mitchison (19), the "transition point" is a point in the cell cycle before which an agent will delay or prevent the forthcoming cell cycle events and after which it will not. The chloramphenicol transition point for septum formation may be called TP(Sept).

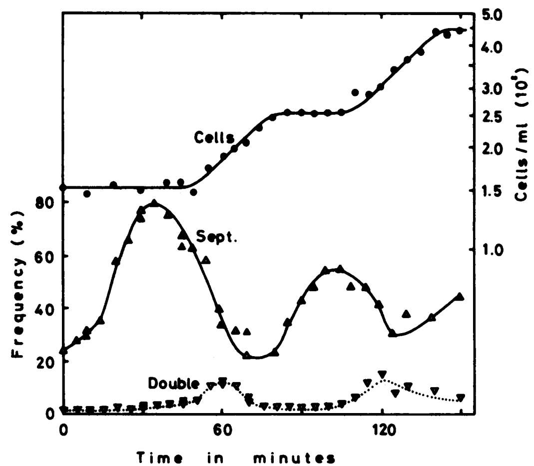

FIG. 1. *Changes in the frequency of septated cells and double cells during the cell cycle of B. subtilis 168. Synchronized cells were grown in medium containing 28 mM glucose. Symbols:* ●, *total cell number;* △ *and* ▲, *frequency of septated cells (Sept.) determined by light microscopy after cell wall staining and by electron microscopy after negative staining, respectively;* ▽ *and* ▼, *frequency of double cells by light microscopy and by electron microscopy, respectively.*

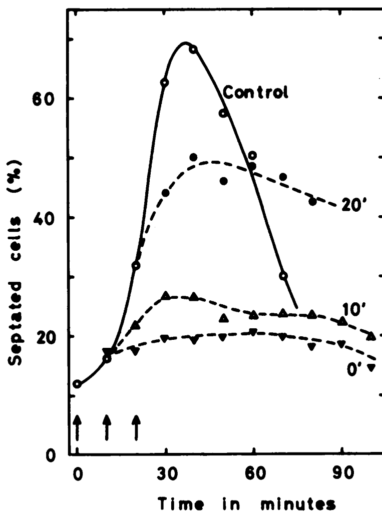

FIG. 2. *Effect of the inhibition of protein synthesis on morphological changes during the cell cycle of B. subtilis 168. The frequency of septated cells was determined after the addition of chloramphenicol (200 μg/ml) to synchronized cultures at 0, 10, and 20 min. A control culture is also shown. Arrows indicate times of the addition.*

As shown in Fig. 3, protein synthesis is also required for cell division as described previously (1, 3, 10, 11, 13, 14, 18). When chloramphenicol was added to synchronized cells during the early period (0 to 20 min), no increase in the total cell number was observed. The final yields of cell number, shown by the broken line in Fig. 3, was increased as the time of the drug addition was delayed and reached the control level when the drug was added just before cell division (60 min). The timing of protein synthesis required in this case, TP(Div), was deduced from this experiment and was estimated to be about 45 min (0.64 of the generation time).

Relationship between cell cycle events and the termination of DNA replication. The results shown in Fig. 2 and 3 led us to assume that the processes of cell division in *B. subtilis* require at least two kinds of protein molecules which are synthesized at distinct periods in the cell cycle. Three sets of separate experiments were performed to determine whether this assumption holds true in normally growing cells, especially in connection with the termination of DNA replication.

Exponentially growing cells were inhibited by the addition of chloramphenicol (200 μg/ml) in experiment 1 and, in experiment 2, by the addition of nalidixic acid (20 μg/ml) and by thymine starvation; residual cell divisions (10) were determined (Table 1). In experiment 1, the calculated value for TP(Div) was close to that of synchronized cells. The value for the difference between TP(Div) and TP(Sept) was estimated theoretically from the frequency of septated cells after completion of the residual cell division. The value was comparable to that (0.41) obtained in synchronized cells.

In experiment 2 (Table 1), it was shown that the numbers of the residual division in the absence of DNA replication amounted to 2.07 and 2.10, respectively, with a slight difference be-

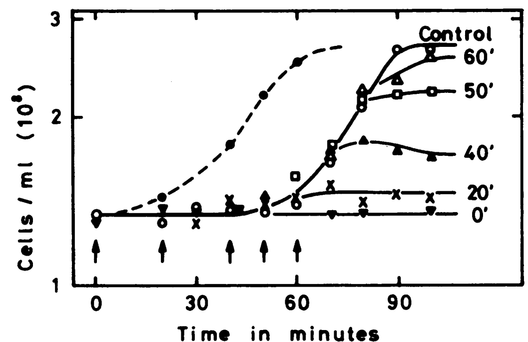

FIG. 3. *Effect of the inhibition of protein synthesis on cell division during the cell cycle of B. subtilis 168. Total cell numbers were determined after the addition of chloramphenicol (200 μg/ml) to synchronized cultures at 0, 20, 40, 50, and 60 min. A control culture is also shown. Arrows indicate times of the addition. A plateau of the cell number reached after the incubation with the drug is plotted versus the time of the addition (●).*

TABLE 1. *Transition points estimated by the residual cell division experiment*

Treatment	Cell no. after residual division/N_0[a] (r)	TP(Div)[b]	Age at termination of DNA replication (a_T/τ)[c]	Septated-cell frequency after residual division (s)	ΔTP[d]
+ Chloramphenicol	1.36	0.56		0.34	0.42
+ Nalidixic acid	2.07		0.95		
− Thymine	2.10		0.93		

[a] For N_0, see the legend to Fig. 4.
[b] $TP(Div) = 1 - \ln r/\ln 2$.
[c] $a_T/\tau = i - \ln r/\ln 2$, where i is the smallest integer so that $i \geq \ln r/\ln 2$, as derived from reference 9.
[d] $\Delta TP = TP(Div) - TP(Sept) = \ln (1 + s)/\ln 2$.

tween the two methods. This result suggests that a block in the termination of DNA replication does not affect the occurrence of cell division in the ongoing cell cycle, but does in the next rounds of cell cycles. The cell age at which DNA replication is terminated was estimated theoretically as 0.95 and 0.93.

In experiment 3, exponentially growing cells were subjected to histidine starvation for a period of about one generation time to insure that they were arrested at the two transition points. Protein synthesis was then reinitiated by the addition of the required amino acid (Fig. 4).

During starvation, a 31% increase in the cell number was observed with a concurrent decrease in the frequency of septated cells, whereas optical density reflecting cell mass was nearly unchanged, consistent with earlier observations (2). These facts indicate that at the beginning of starvation, 31% of the population in the exponential phase had passed the step which requires an extensive amount of protein synthesis in the cell cycle. The two transition points estimated from the residual increase in cell number and the final frequency of septated cells attained at the end of histidine starvation were comparable to those of synchronized cells (Table 2).

The readdition of the required amino acid to the starved culture caused an approximate doubling in the cell number in two steps, the first step of synchronous division just after the readdition and the second step 90 min later. Similarly, doublings in the cell number were also observed in the third plateau (d in Fig. 4) as compared with the first one (b) and in the fourth (e) plateau as compared with the second one (c). Further, each burst in cell number was concurrently accompanied by a sharp decrease in the frequency of septated cells. These results suggest that during starvation, one part of the population was aligned at the stage just before cell division and the other part was aligned at the earlier stage of the cycle. By measuring cell length in the photographs of cell wall-stained cells at the end of starvation, we obtained a bimodal distribution differing from that of normal cells. Each of the modes coincided, respectively, with that of septated and nonseptated cells. This result supports the above suggestion (data not shown). Such alignments of cells could be interpreted by assuming that in the cell cycle there exist two distinct periods of protein synthesis required for the processes of cell division.

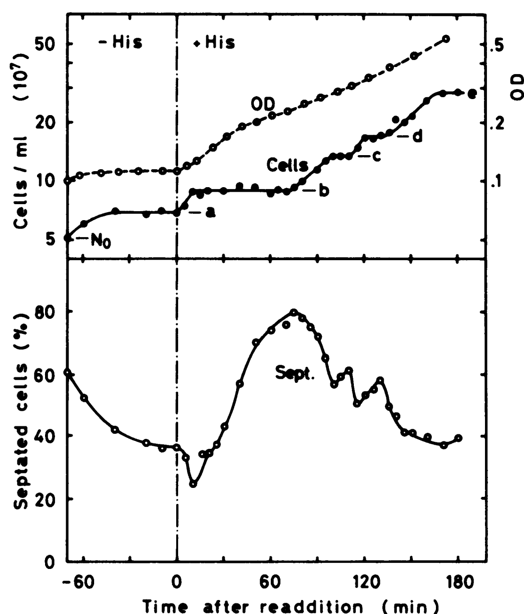

FIG. 4. *Two steps of synchronous cell division induced by amino acid starvation in B. subtilis 168. A required amino acid (histidine) was removed from the exponentially growing culture at −70 min. At 0 min, histidine was added to the culture. Optical density (OD) (○——○), total cell number (●), and the frequency of septated cells (○——○) were determined. N_0 (5.2 × 10^7 cells per ml) represents the cell number at the beginning of starvation, and a (6.8 × 10^7 cells per ml), b (9.0 × 10^7 cells per ml), c (13.5 × 10^7 cells per ml), d (17.0 × 10^7 cells per ml), and e (28 × 10^7 cells per ml) represent each plateau level of the cell number.*

TABLE 2. *Transition points estimated by the histidine starvation and readdition experiment*[a]

Plateau	Cell no. at plateau/N_0 (r)	TP(Div)[b]	TP(Sept)[c]	Frequency of septated cells (s)	ΔTP[d]
a	1.31	0.61		0.36	0.44
b	1.73		0.21		
c	2.60	0.62			
d	3.27		0.29		
e	5.38	0.57			

[a] For N_0, a, b, c, d, and e, see the legend to Fig. 4.
[b] TP(Div) = $i - \ln r/\ln 2$ for plateaus a, c, and e, where i is the smallest integer so that $i \geq \ln r/\ln 2$.
[c] TP(Sept) = $i - \ln r/\ln 2$ for plateaus b and d.
[d] Same as in Table 1.

DISCUSSION

It becomes evident from our results with *B. subtilis* cells that TP(Div) and TP(Sept), as revealed by chloramphenicol inhibition of synchronized cells, are, respectively, 0.64 and 0.23 (or 0.41 earlier than the former) of the cell age.

Results of residual cell division after the addition of chloramphenicol and after histidine starvation suggest that the transition points in normally growing cells are almost identical to

those in synchronized cells (Tables 1 and 2).

The suggestion of two transition points in a normal cell cycle is supported further by the results of the histidine starvation and readdition experiment (Fig. 4 and Table 2). The two steps of synchronous division were analyzed by assuming that the two transition points obtained by chloramphenicol inhibition hold true for those obtained by histidine starvation. The results may be described as follows.

During starvation, those cells which have passed TP(Div) at the beginning divide once and cause an increase in the cell number. The newborn cells then grow but are arrested at a stage before the initiation of septum formation in the next cycle, together with those which have started from the early stage of the cycle. The first increase just after the readdition of histidine is due to the fraction of cells which has been situated between TP(Sept) and TP(Div) and is arrested at a point just before cell division (Fig. 2). Multiple steps in increase occurring thereafter may be interpreted as the repetition of partial synchronous divisions.

Results of the experiment shown in Fig. 4 were summarized in Table 2. As shown in Table 2, theoretical estimations of transition points are comparable to those obtained by the chloramphenicol inhibition experiment of synchronized cells (Fig. 2 and 3). Thus, the results shown in Tables 1 and 2 confirm the assumption that the processes of cell division in *B. subtilis* require at least two kinds of protein molecules which are synthesized at distinct stages in the cell cycle.

By collating the estimated TP(Sept) and TP(Div) with the results of the observation on cell morphology, it was shown that TP(Sept) and TP(Div) are situated, respectively, at the early stage of the cell cycle and at the latest stage of the septated state. Again, by collating the results of DNA inhibition experiments (Table 1), it was shown that replication is terminated slightly after the completion of the septated state. However, inhibition of the termination does not affect the occurrence of cell division in the ongoing cell cycle, consistent with earlier reports (1, 5). The residual increase in the cell number of a little over two times during the inhibition of DNA synthesis suggests that the block of DNA termination prevents cell division in the forthcoming cell cycle. This suggestion is further supported by our results from separate studies in which newborn cells of *B. subtilis* 168 and the DNA elongation mutant were inhibited from DNA synthesis (manuscript in preparation).

A few results of our experiments on histidine starvation are difficult to interpret and await further studies. First, in the experiment of Fig. 4, the plateau (b) before the second-step increase was much longer (90 min) than what could be expected based on the generation time. It seems as though cell aging was delayed. We prefer to investigate this finding in relation to DNA replication. Second, the result of two-step synchrony induced by amino acid starvation is not consistent with earlier reports for *E. coli*. It has been shown that the synchronization of cell division was induced by amino acid starvation in strains K-12 and B (16, 21, 22), but was absent in strain B/r (22). It was assumed that in strain B, synchronization was due to a shift forward in the cell cycle during starvation (21). It is not clear at present whether in *B. subtilis* the discrepancy might come from the specific effect of histidine starvation on the synthesis of the transition protein or the diversity of the transition points.

At present, the characters of the two transition protein molecules are still unclear, and the identities of the functions of these proteins and those suggested in the models of *E. coli* (13, 14) or *S. faecalis* (4, 10, 24) have not been defined. However, studies with several mutants defective in septum formation at the nonpermissive temperature are in progress in our laboratory and may provide information on these points.

ADDENDUM IN PROOF

A detailed electron microscopic study of DNA replication, cell division, and cell separation in *Bacillus subtilis* Marburg growing with a doubling time of 65 min has been reported (N. Nanninga, L. J. H. Koppes, and F. C. de Vries-Tijssen, Arch. Microbiol. **123**:173–181, 1979). Their observations are in agreement with our results, i.e., that DNA replication is terminated at 0.93 to 0.95 of cell age and septum formation initiated at 0.31 (20 min).

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