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Regulation of Chromosome Replication in *Bacillus subtilis*: Effects of Amino Acid Starvation in Strain 168

JAMES C. COPELAND

Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439

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Regulation of chromosome replication in *Bacillus subtilis* strain 168, in response to starvation for an essential amino acid, was found to differ from that reported for *Escherichia coli*. Not all replication points stop at the terminus during amino acid starvation. There is some evidence, however, to indicate that preferred stopping sites might exist. Initiation at the origin can occur in the absence of total protein synthesis as well as when the deoxyribonucleic acid (DNA)- mass ratio is unbalanced. DNA synthesis appears to be controlled independently of the initiation event by a second regulatory circuit, that may utilize the DNA-mass ratio. Once initiated, chromosome replication does not always go to completion in an uninterrupted sequence.

A review of the literature on regulation of chromosome replication in bacteria indicates that the initiation process consists of at least two events (12–15, 17, 18). It has been proposed (12) that a protein initiator and membrane attachment are involved in regulating the replication of replicons. In *Escherichia coli*, the withdrawal of a required amino acid allows "rounds" of replication in progress to complete (10, 15, 16, 18), but new rounds of replication, after withdrawal of the amino acid, are blocked (4, 26). In other words, once initiated a round of replication goes to completion. Regulation of replication would therefore be an all or none event determined by the occurrence or non-occurrence of the initiation event (16). This form of replication regulation has been proposed to exist for *Bacillus subtilis* strain 168 (1). The work of Yoshikawa, O'Sullivan, and Sueoka (27, 28, 29), however, suggests that a less rigidly regulated replication control system might exist in this strain of *B. subtilis*. It is the purpose of this report to show that regulation of chromosome replication, in response to starvation for a required amino acid in the 168 strain of *B. subtilis*, differs from that in *E. coli*. This difference allows examination of another regulatory circuit that affects DNA synthesis and may involve the ratio of deoxyribonucleic acid (DNA) to cell mass. (16, 17).

MATERIALS AND METHODS

Strains. Table 1 lists all strains used in this study, with their genotype and source. BC200, used in the amino acid starvation experiments, was confirmed as a 168 strain by its sensitivity to the defective phage PBSZ and by its resistance to defective phage PBSX. These defective phages were obtained by inducing to lyse strains W23 and 168, respectively (22).

Media and growth conditions. The composition of minimal medium has been described (5). Liquid minimal medium contained 0.5% glucose and only the required growth factors. Solid minimal medium, in addition to 2% agar, also contained L-glutamic acid and L-asparagine. All additions were made at 50 μg/ml, except when noted.

To maintain cells in balanced growth the following protocol was used. A single colony, grown on solid minimal medium, was picked with a needle and inoculated into liquid minimal medium. This culture was incubated 20 to 24 hr. An appropriate dilution of this culture was made into fresh liquid minimal medium so that, after 16 to 17 hr of incubation, the cell density would be 1 to 5 $\times$ 10^7 cells/ml; this involved 10 to 12 generations. This second growth medium contained thymidine at a concentration of 1 μg/ml, rather than 50 μg/ml. Cell density was always maintained below 5 $\times$ 10^7 cells/ml by dilution, when necessary. Medium changes were made by filtration with membrane filters (0.47-μm pore diameter; Millipore Corp., Bedford, Mass.).

Complete medium, when used, was Brain Heart Infusion (Difco). All incubations were at 34 C. All optical density determinations were made with a Klett-Summerson colorimeter by using a blue filter (no. 42).

Density transfer experiment and transformation assay. Details of these procedures have been described previously (5). Cells starved for leucine were collected by filtration, washed with sterile D_2O, and suspended in enriched D_2O medium. At appropriate intervals,

TABLE 1. *List of strains of Bacillus subtilis 168*

Strain	Other designation	Genotype[a]	Source
BC34		*ura-26*	
BC35	Mu8u5u6	*purB6 leu-8 metB5*	N. Sueoka
BC36	Mu8u5u5	*thr-5 leu-8 metB5*	N. Sueoka
BC37	BD73	*purA16 metB5 lys-21 phe-12 argA3*	D. Dubnau
BC38		*hisA trp-2 argC4*	J. Marmur
BC39		*trp-2 leu-2 argC4*	J. Marmur
BC50		*purA16 leu-8 metB5 nia-38*	
BC51		*purA16 leu-8 metB5 lys-21*	
BC53		*purA16 leu-8 metB5 ile-64*	
BC64	168-MALT	*purA16 leu-8 metB5 thyA thyB*	M. C. Wilson
BC67	SB3	*hisA trp-2 cysB*	E. Nester
BC100	Mu8u5u16	*purA16 leu-8 metB5*	N. Sueoka
BC200		*leu-8 metB5 thyA thyB* (spontaneous Pur+ revertant of BC64)	

[a] Gene symbols indicate mutations in cistrons leading to requirements for the following: *arg*(arginine), *cys*(cysteine), *his*(histidine), *ile*(isoleucine), *leu*(leucine), *lys*(lysine), *met*(methionine), *nia*(niacin), *phe*(phenylalanine), *pur*(adenine or guanine), *thr*(threonine), *thy*(thymine), *trp*(tryptophan), and *ura*(uracil).

samples were taken and prepared for CsCl density centrifugation.

Chemical determination of DNA and ribonucleic acid (RNA). Samples (2 ml) were quick-frozen in a dry ice-ethyl alcohol bath and stored at −96 C until used. RNA was separated from the DNA by a modified Schmidt-Thannhauser procedure (23). Thawed samples were centrifuged and suspended in an equal volume of 10% trichloroacetic acid. The samples were incubated in an ice bath for 30 min, centrifuged, and washed with an equal volume of cold 10% trichloroacetic acid. The pellets were then suspended with 0.2 ml of 1 M KOH and incubated 15 to 18 hr at 34 C. Each sample was precipitated with 0.8 ml of an acid mixture (0.28 ml 1 N HCl, 0.62 ml of 10% trichloroacetic acid, and 0.10 ml of water per ml) and incubated in an ice bath for 1 hr. The pellets and supernatant fluid were separated by centrifugation, and each sample was dried completely under vacuum. DNA determinations were made on the pellets by adding 0.1 ml of 10% perchloric acid and 0.1 ml of 5% diphenylamine (Fisher Scientific Co., Pittsburgh, Pa.) in glacial acetic acid containing 80 μg of acetaldehyde per ml. Tubes were sealed with Parafilm (Marathon) and incubated for 12 to 16 hr at room temperature (23 to 24 C) in the dark. Absorbance was measured at 595 nm and corrected for light scatter by subtracting absorbance measured at 700 nm (2, 11)

RNA determinations were made on the dried supernatant fluid. The samples were first dissolved with 0.2 ml of distilled water. To each sample was added 0.2 ml of orcinol reagent [1.0% orcinol (Fisher Scientific Co.) and 0.5% $FeCl_3$ made up in concentrated HCl]. The samples were capped and heated in a boiling water bath for 10 min. After cooling, absorbance was measured at 660 nm (20).

Standard curves were obtained with ribose for RNA and deoxyadenosine for DNA determinations. All pipettings that required accuracy for critical quantitation were performed with Eppendorf automatic pipettors. Orcinol and diphenylamine reagents were made just before they were used.

RESULTS

Cell growth and amino acid starvation. Cells grown in the minimal medium at 34 C have a mass-doubling time of 60 to 70 min. Figure 1 shows that cells in balanced growth are not disturbed appreciably by filtering and washing. That part of the culture in which the amino acid was replaced continued to grow with the expected mass-doubling time. The cells deprived of an essential amino acid stopped growing almost immediately after it was removed. There is, on the average, a 5% increase in optical density before growth completely stops. Soon after removal of the amino acid, there is a 30

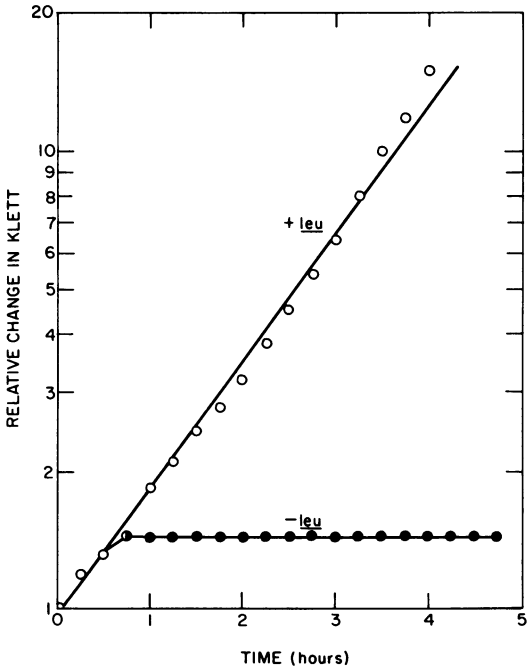

FIG. 1. *Effect of starvation of BC200 for leucine. Cells in balanced growth filtered and washed with minimal medium minus leucine. The amino acid was added back to the control* (○), *and it was kept below 1 × 10⁸ cells/ml by periodic dilution.* (●) *Cells starved for leucine.*

to 40% increase in viable cell number. Thereafter, the viable number of cells remains constant.

Kinetics of macromolecular synthesis during amino acid starvation. In Fig. 2 are shown the kinetics of total DNA and RNA during starvation for an essential amino acid. In this analysis, the change in optical density is equated with change in total protein. The control determinations show that RNA, probably ribosomal RNA, is affected for a short time (Fig. 2) by the filtering and washing process. Yet, protein and DNA made after filtering are unaffected. This suggests that ribosomes in *B. subtilis* are particularly sensitive organelles (5). After removal of the amino acid, leucine, approximately 5% more protein is made and, thereafter, the total amount does not change. RNA synthesis stops soon after removal of the amino acid. Then, its total amount slowly decreases during the starvation period. The kinetics of DNA synthesis during amino acid starvation are unlike the kinetics expected when only rounds of replication in progress are allowed to complete, and subsequent rounds are blocked. DNA synthesis continues during amino acid starvation until 35 to 40% more is made after the amino acid was withdrawn. There follows a temporary plateau, after which there is a secondary rise in the total amount of DNA by an average increase of 70% to a second and permanent plateau (Fig. 2).

Restart kinetics. After 150 min of amino acid starvation, when all of the DNA to be made is completed, the readdition of the amino acid leads to an immediate increase in cell growth, paralleled by a similar increase in viable cell number. In Fig. 3 are shown the kinetics for DNA, RNA, and protein synthesis. Protein and RNA synthesis begin immediately upon addition of the amino acid. During the first doubling, after the starvation period, the rates of increase are less than those for cells in balanced growth. But after doubling, the rates of synthesis are like those for cells in balanced growth. No DNA is made for the first 40 to 50 min after the amino acid was replaced. Then, DNA is synthesized, but discontinuously. A plateau occurs when 20 to 25% more DNA is made

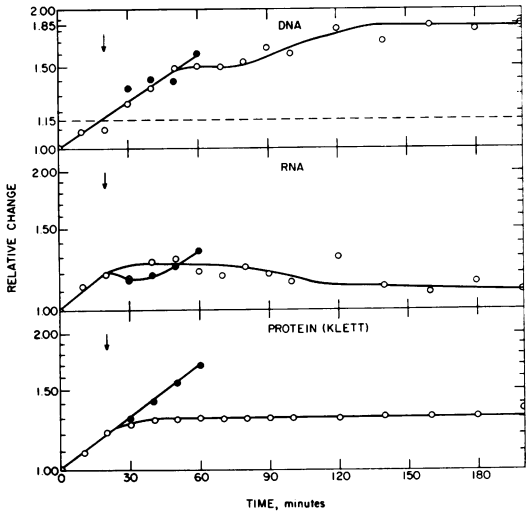

FIG. 2. *Total DNA, RNA, and protein measurements on BC200 during starvation for leucine. Arrows indicate when cells were filtered and washed. (○) Cells without leucine. (●) Cells with leucine added back after filtration. Broken line in DNA curve is the average base line at the time of filtering. The average amount of DNA at time zero is 0.34 μg/ml, RNA is 1.98 μg/ml, and optical density for protein is 0.044.*

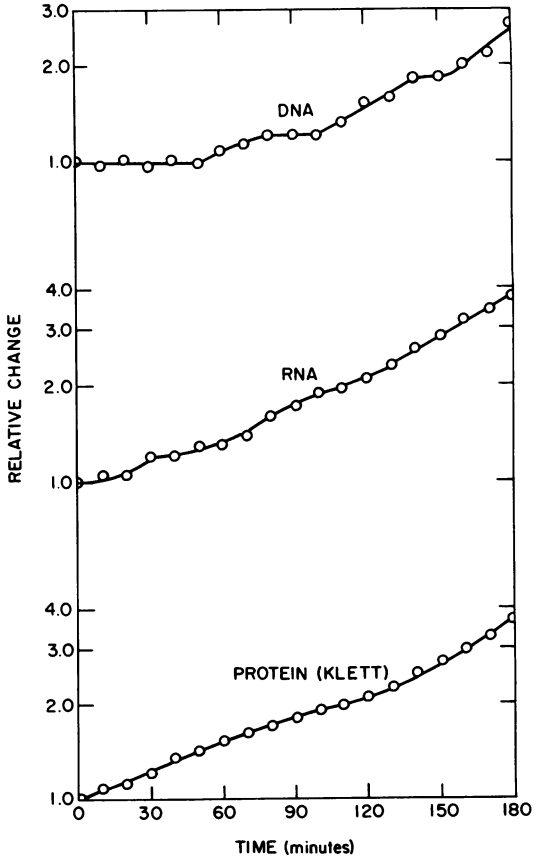

FIG. 3. *Restart kinetics for DNA, RNA, and protein. BC200 starved for leucine for 150 min, as indicated in Fig. 2, at which time leucine was added back to a final concentration of 50 μg/ml. The average amount of DNA at time zero is 0.63 μg/ml, RNA is 2.20 μg/ml, and optical density for protein is 0.053.*

after the amino acid was replaced. This amount added to the amount of DNA made during amino acid starvation is equivalent to a doubling, relative to the amount present at the onset of amino acid starvation. DNA synthesis begins again at about that time when RNA and protein have doubled after restart. Thereafter, DNA synthesis is continuous, but with a characteristic inflection when 80 to 90% is made.

Genetic analysis of DNA made during restart. Restart after amino acid starvation can be accomplished in a nutritionally rich D_2O medium. The kinetics for DNA synthesis are like those in Fig. 3. Other workers have shown that the use of D_2O as a density label causes no significant pathological effects. For example, deuterated spores, germinated in water medium, produce the same gene replication order as light spores germinated in a D_2O medium (5, 21, 24, 27). Both agree with the replication order of vegetative cells of *B. subtilis* W23 grown to stationary phase in a D_2O medium and subsequently shifted to a light water medium (9, 29). The DNA made after restart in D_2O was separated from DNA made during amino acid starvation by centrifugation in a CsCl density gradient (19). The fractionated gradient was analyzed genetically by transformation to identify the replicating parts of the chromosome (29). In this analysis, twelve different markers (*see* Fig. 4) were assayed in each of four different gradients. The gradients were derived from samples taken when DNA increased 10, 13, 22, and 55% after restart.

In Fig. 5 is represented the per cent replication per gradient for each genetic marker versus the amount of DNA synthesized when the sample was taken. It is readily apparent that upon restart all of the markers assayed were actively replicating. The amount of replicating activity for each marker differs quantitatively in a particular way, which will be discussed later. Another characteristic property of all markers is that their replicating activity falls toward zero after about 20% more DNA has been made. This observation is consistent with the kinetic results obtained on restart of DNA synthesis, i.e., there is a plateau in DNA synthesis when 20 to 25% DNA is made. This agreement further indicates that restart in the D_2O medium produces no effects peculiar to the medium. The kinetic and the genetic analyses of the restart condition supported by the observation on DNA made during amino acid starvation show that chromosome replication need not stop at a unique terminus in *B. subtilis* 168.

Another observation made from these data, but better illustrated in Fig. 6, is that markers

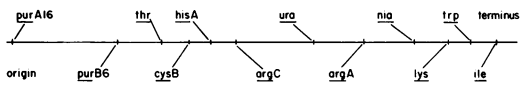

FIG. 4. *Representation of genetic map for B. subtilis adapted from Dubnau et al.* (9).

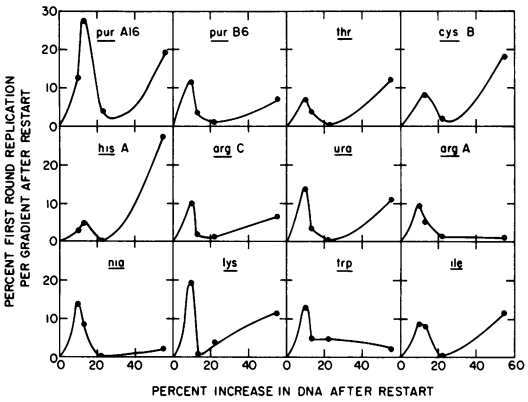

FIG. 5. *Replication activity expressed as per cent of total first round replication per genetic marker per gradient after addition of leucine to amino acid-starved BC200. This is computed from gradients assayed by transformation as illustrated in Fig. 6. The first round after restart is defined as transfer of transforming activity to the hybrid region. When the sum of activity under the curve equals 100 per cent, it would indicate that all copies of a given genetic marker present at restart have replicated once. The relationship is expressed simply as per cent replicating activity* $= (LH/2)/[LL + (LH - HH)/2 + HH/2] \times 100$. *Where LL is equivalent to the transforming activity in the light fraction, LH with that in the hybrid fraction, and HH with that in the heavy fraction, each expressed as per cent of total transforming activity in the gradient.*

appear to be replicating not in the sequence expected from their map position. For example, the *hisA* marker has been replicated more than the *purB* marker. The known map order is *purA*, *purB*, then *hisA* (*see* Fig. 4), but the apparent order of replication after amino acid starvation is *purA*, *hisA*, then *purB*. Also, the map order is *purA*, *argC*, then *lys*, but the apparent order of replication after amino acid starvation is *purA*, *lys*, then *argC*. Other misordering can be seen in Fig. 5.

To demonstrate that replication is not proceeding in a random order after restart, replicating activity for each marker after 22% of the DNA has been synthesized is compared to that activity occurring between 22 and 55% of the DNA synthesized (Fig. 7). Initially, replicating activity is greatest on the last half of the chromosome. The one noticeable exception is at the *purA* marker, near the known origin. This ac-

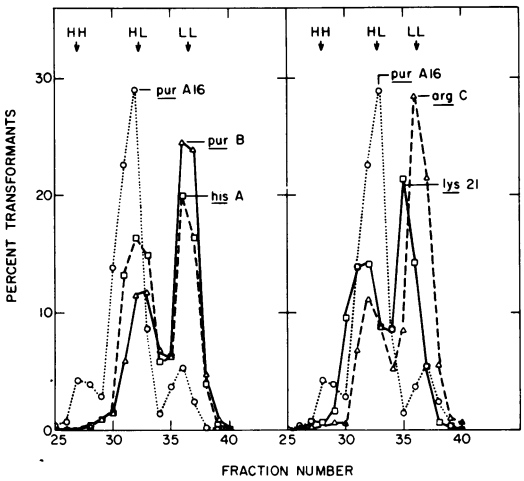

FIG. 6. *Transformation assay of gradient taken when 55% of the DNA was synthesized after restart. Cells of BC200 were previously starved of leucine for 150 min. LL indicates peak of light fraction, HL that for the hybrid fraction, and HH for the heavy fraction.*

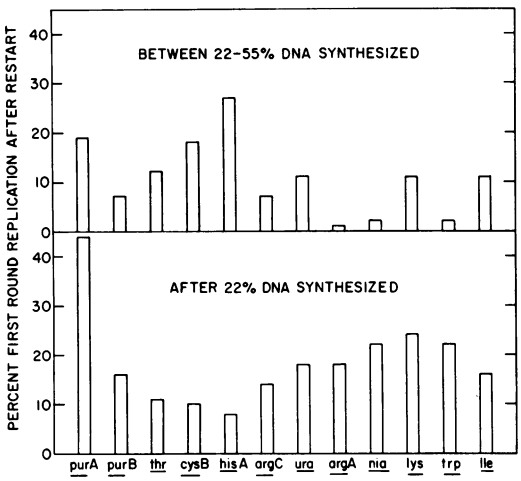

FIG. 7. *Bar graph illustrating the distribution of replication activity for genetic markers according to their order on the genetic map of B. subtilis. The lower section represents the distribution after 22% DNA synthesis, which is equivalent to the first plaetau upon restart. The upper section represents the distribution for DNA synthesis afterwards to 55%, i.e., replication activity at 55% DNA minus replication activity at 22% DNA synthesized.*

tivity represents newly initiated chromosomes. Later, replicating activity is lowest on the last half of the chromosome and, now, is highest on the first half. These data indicate that replication after amino acid starvation is ordered and reflect the state the chromosome comes to as a result of the amino acid starvation.

DISCUSSION

Regulation of chromosome replication in *B. subtilis* 168 in response to starvation for an essential amino acid is different from that in *Escherichia coli*. For this discussion, it will be assumed that *B. subtilis* contains either one chromosome or perhaps one functional unit of replication carrying all of the essential genetic information that comprises its genome. This is not proven, but most available information is consistent with this concept (6, 9, 25).

In *B. subtilis* 168, replicating chromosomes appear to be completed in the absence of the amino acid, if the near 40% more DNA made before synthesis stops temporarily is in fact the amount necessary to complete rounds in progress (18). It then appears that some of the completed chromosomes initiate a new round of replication that stops with a broad distribution of resting sites located mainly over the last half of the chromosome. This would account for the additional DNA made beyond that needed to just complete rounds in progress. This arrangement would best account for the pattern of gene replication found upon restart. Also, the observation that new initiations can occur in the absence of total protein synthesis strongly suggests that some of those protein elements used in the previous initiation event can be used again in the next.

The observed misorder of markers, when arranged according to their replicating activity compared to their map order, might be accounted for in at least two ways. It could indicate that there are preferred stopping sites for replication forks not able to proceed to the normal terminus. This would result in some markers that exhibit high replicating activity upon restart, whereas others, in between preferred stopping sites, would show relatively lower activity. This would give the appearance of markers replicating out of order, compared to their position on the chromosome. Another possibility to explain these data is that new rounds of replication can be initiated at sites other than the normal origin of the chromosome, as was demonstrated recently in *E. coli* (26).

One striking feature of these data stems from consideration of the ratios of DNA to RNA and protein before, during, and after amino acid starvation. During amino acid starvation, the amount of RNA drops slightly and protein changes little, or not at all. But DNA is made until there is about 70% more, creating an

imbalance in the ratios of DNA to RNA and protein, relative to the amount present in cells during balanced growth. Upon restart, the complementary situation results with RNA and protein being made in the absence of DNA synthesis. As more RNA and protein are made, and normal ratios are approached, DNA synthesis is switched on again. Genetic analysis indicates that those rounds that were in progress continue at the same time as new rounds are begun. Then DNA synthesis and replicating activity shut off when exactly a doubling in amount of DNA is reached, relative to that present at the time amino acid starvation began. It does not begin again until the normal ratios are obtained by continued RNA and protein synthesis. DNA synthesis, thereafter, appears to be continuous and, together with RNA and protein synthesis, the cells appear to be back in balanced growth. These observations are taken as support for a concept first suggested by Maaløe (16, 17) and more recently extended by Donachie (7) and Donachie et al. (8) that DNA synthesis is regulated by its relationship to RNA, protein, cell mass, or other factors that contain some or all of the former. (For convenience this relationship will be referred to as the DNA-mass ratio.) One particular proposal relates the initiation event to a definite DNA-mass ratio or a multiple of that mass. (7)

It is evident from these results that the regulatory circuit involving the DNA-mass ratio can operate independently of the initiation event which controls the onset of replication. New rounds of replication, started by addition of the amino acid, are stopped, as shown by the plateau in amount of DNA made and the concurrent sharp decrease in replicating activity, particularly for the *purA* marker. DNA synthesis does not begin again until the normal DNA-mass ratio is attained.

It should be emphasized that chromosome replication, once begun, need not go to completion in an uninterrupted sequence. This is clearly shown during amino acid starvation, when newly initiated rounds eventually stop before reaching the terminus. Also, upon restart, newly initiated rounds stop replicating activity until a balanced ratio of DNA-mass is reached. Furthermore, initiation events occur when cells are in an unbalanced condition. This happens during amino acid starvation after rounds in progress come to completion, during which time total RNA and protein has changed only slightly. Again, new initiations occur upon restart before the balanced ratio of DNA to mass is reached. It may be this uncoupling of the initiation event from the control circuit, involving the ratio of DNA to mass, that makes *B. subtilis* 168 different in its response to amino acid starvation from *E. coli*.

Preliminary evidence indicates that this effect is peculiar to the 168 strain of *B. subtilis*, because starvation of strain W23 for the same amino acid, leucine, produces kinetics for DNA synthesis like that obtained in *E. coli* (*in preparation*). This seems to confirm the original suggestion of Yoshikawa et al. (27–29) that strain 168 differs from W23 by its looser control of chromosome replication. Caro and Berg (3) recently found anomalous replication patterns for *E. coli* K-12 cell lines after starvation for an essential amino acid. For some, the pattern of replication is similar to that obtained for *B. subtilis* 168 after amino acid starvation as presented in this paper. This may indicate that among *E. coli* strains too, there are different regulatory responses to amino acid starvation as it affects chromosome replication.

Recently, Anraku and Landman (1) reported that amino acid starvation in *B. subtilis* 168 results in completion of rounds of replication analogous to that of *E. coli*. Experimentally, their procedures differed in many important ways from those used to obtain the results reported here. Their cultures were started by scraping growth from the surface of an agar plate and suspending these cells at a density of 10^8 cells per ml in an enriched medium that contained acid-hydrolyzed casein. The cell density was allowed to increase to about 4×10^8 cells/ml before they were centrifuged, washed, and then suspended in a completely minimal medium that lacked both the acid-hydrolyzed casein and an essential amino acid. It is doubtful that cells prepared this way were in balanced growth, both because of the method of inoculation and the high cell density employed. The growth of strain BC200 becomes unbalanced when it reaches about 1×10^8 cells/ml, during which time DNA synthesis is declining. Because of the procedure used by Anraku and Landman, it is difficult to ascribe the shut off of DNA synthesis to cells in an unbalanced growth condition, to the shift down and removal of the essential amino acid, or both. Other differences, such as cell line or amino acid starved for, may contribute to the different result obtained. In any event, the results obtained with BC200 cells carefully maintained in balanced growth show that in *B. subtilis* strain 168 new rounds of chromosome replication can be initiated in the absence of an essential amino acid. They reveal

another regulatory circuit that controls DNA synthesis independently of chromosome initiation.

The results of this study suggest there are at least two regulatory circuits that affect chromosome replication, and affect it differently. Initiation of replication is an all or none event, it acts only once during a round of replication. Once initiated, a given round of replication tends toward completion to its terminus. The second circuit is operative during chromosome replication and modulates replication activity by turning it on and off relative to other cellular factors that involve cell mass or correlate with it. This second circuit could serve to entrain chromosome replication to other cellular events, such as cell growth and division.

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