Tertiary, the last of which is especially well established (*2, 5*). It has been supposed that the sudden explosions and extinctions of species might be related to reversals of the geomagnetic field (*16, 18*), for during the polarity transition the shielding effect of the geomagnetic field is removed and the cosmic ray flux consequently is increased. The effect will obviously be enhanced when reversals occur frequently. A comparison of Fig. 2 with Simpson's (*16*) curves of the rate of organic evolution shows that there is no significant correlation with apparent reversal frequency. Accelerations in evolution seem to occur at times when the reversal rate has either maximum or minimum values.

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Regulation of Chromosome Replication in Bacillus subtilis: Marker Frequency Analysis after Amino Acid Starvation

Abstract. *Marker frequency analysis of DNA isolated from amino acid–starved* Bacillus subtilis *cells shows that most chromosomes have not completed replication to the terminus. This finding agrees with earlier results concerning replication after amino acid starvation in this organism. The results are not compatible with regulation of chromosome replication at the initiation step only, and they suggest that a second regulatory circuit controls replication under conditions of amino acid starvation.*

Chromosome replication, a controlled process which occurs during a definite interval of the cell cycle for higher organisms (*1*), is regulated during the sequence of growth and division in bacterial cells (*2*). The available information on the control of replication of the bacterial chromosome as well as other self-replicating units led Jacob and Brenner (*3*) to propose the *replicon model* to explain the regulated relationship between replication, cell growth, and cell division (*4*). A replicon is a unit of replication that may be equivalent to a bacterial chromosome. This model proposes a positive mode of regulation which involves, in its simplest form, a protein whose synthesis is directed by a structural gene on the replicon. This protein, called the initiator, interacts specifically with another region of the replicon, the replicator, and triggers the beginning of the replication process. Once started, replication proceeds sequentially until the replicon has been duplicated. To begin another round of replication, a new initiator must trigger the replication before the process can start again.

If chromosome replication was regulated at the initiation step only, then blocking protein synthesis by amino acid starvation would stop new initiations but would allow replicons in the act of replication to continue until the replicon had been duplicated. Thus, a population of aligned replicons would be produced that were completed and ready to be initiated again to start another round of replication. This approach has been used by many investigators to show that the chromosome of *Escherichia coli* is equivalent to a single replicon and that it has a unique, hereditable site, the origin, from which replication is initiated (*5*). Some reports are incompatible with this conclusion, and they will be discussed later (*6*).

Yoshikawa and Sueoka (*7, 8*) have shown that the chromosome of *Bacillus subtilis* replicates from a unique origin in a sequential manner toward an identifiable terminus. My recent findings have led me to question whether control at the initiation step alone could account for the effect of amino acid starvation on chromosome replication in *B. subtilis* (*9, 10*). Chromosome replication following amino acid starvation was not synchronized from the known origin, as would be expected had they completed replication during the starvation period. This result prompted a closer examination of chromosome replication during amino acid starvation in *B. subtilis*. The marker frequency analysis developed by Sueoka and Yoshikawa is admirably suited to examine this situation (*7, 11*). If the initiation event serves as the only regulatory step in the control of chromosome replication, then blocking the initiator by blocking protein synthesis through starvation for a required amino acid would lead to a population of cells whose chromosomes are replicated to the terminus. The frequency of all genes would be the same, hence the ratio of any two genetic markers, as measured by marker frequency analysis, would be 1.0. Conversely, had chromosomes not completed their replication to the terminus, as is suggested by the earlier studies (*9, 10*), the ratio of a genetic marker located near the origin to another located near the terminus would exceed 1.0. Thus, marker frequency analysis provides an unambiguous choice between the two alternatives.

Table 1 lists the strains of *B. subtilis* used in this study. Conditions and media for growing cells have been

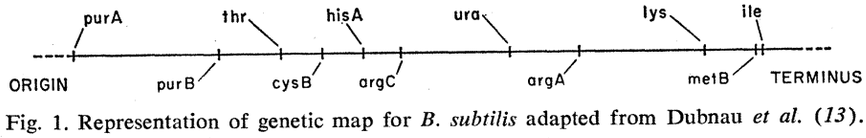

Fig. 1. Representation of genetic map for *B. subtilis* adapted from Dubnau *et al.* (*13*).

Table 1. List of *Bacillus subtilis* strains.

Strain	Other designation	Genotype*	Source
		W23	
BCW 503	SB 12	*thr his leu*	E. Nester
		168	
BC 26		*phe*-12 *arg*A3 *ery*-1	D. Dubnau
BC 34		*ura*-26	D. Dubnau
BC 35	Mu8u5u6	*pur*B6 *leu*-8 *met*B5	N. Sueoka
BC 36	Mu8u5u5	*thr*-5 *leu*-8 *met*B5	N. Sueoka
BC 39		*trp*-2 *leu*-2 *arg*C4	J. Marmur
BC 51		*pur*A16 *leu*-8 *met*B5 *lys*-21	
BC 53		*pur*A16 *leu*-8 *met*5 *ile*-64	
BC 64	168-MALT	*pur*A16 *leu*-8 *met*B5 *thy*A *thy*B	M. C. Wilson
BC 86		*pur*A16 *met*B5 *arg*A3	BC 26 × BC 100 Leu$^+$ Arg$^-$ transformant
BC 87		*pur*A16 *met*B5 *ura*-26	BC 34 × BC 100 Leu$^+$ Ura$^-$ transformant
BC 88		*leu*-8 *met*B5 *arg*C4	BC 39 × BC 100 Pur$^+$ Arg$^-$ transformant
BC 100	Mu8u5u16	*pur*A16 *leu*-8 *met*B5	N. Sueoka
BC 200		*leu*-8 *met*B5 *thy*A *thy*B	Spontaneous Pur$^+$ revertant from BC 64

* Gene symbols indicate the following: *arg* (arginine), *cys* (cysteine), *ery* (erythromycin), *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). Leu$^+$, Arg$^-$, Pur$^+$, Arg$^-$, Ura$^-$, indicate phenotypes (requiring or not requiring).

described (*9, 10*). Extensive marker frequency analysis was performed on DNA isolated from strain BCW 503 after leucine starvation. Cells were grown in a liquid minimal medium (in balanced growth), filtered, and washed free of required leucine. They were then suspended in medium of the same composition but lacking leucine. After 150 minutes of amino acid starvation, DNA synthesis had stopped with a 30 to 35 percent increase (*10*). DNA was prepared from a sample of these cells as described before for gradient analysis (*9*). Spore DNA was isolated from 24-hour-old spores of this strain (*12*). After DNA was released, the spore coats were removed by centrifugation, and the supernatant DNA preparation was treated further as described for cell DNA. Concentration of DNA was determined by absorbance at 260 nm and corrected for residual protein concentration, as determined by absorbance at 280 nm. Methods for transformation have been described (*9*). The final concentration of DNA used in all transformations was set at about 0.1 μg/ml. All marker frequency ratios are represented relative to the *met*B marker, located near the terminus of the chromosome (Fig. 1), unless otherwise noted. All marker frequency ratios have been normalized for differences in individual marker transformation efficiencies by multiplying them by the reciprocal of that marker-pair ratio determined with spore DNA (*7, 11*). Spores contain completed chromosomes, and thus spore DNA contains equal frequencies of all genetic markers. The frequency ratios are the average of four or more experiments.

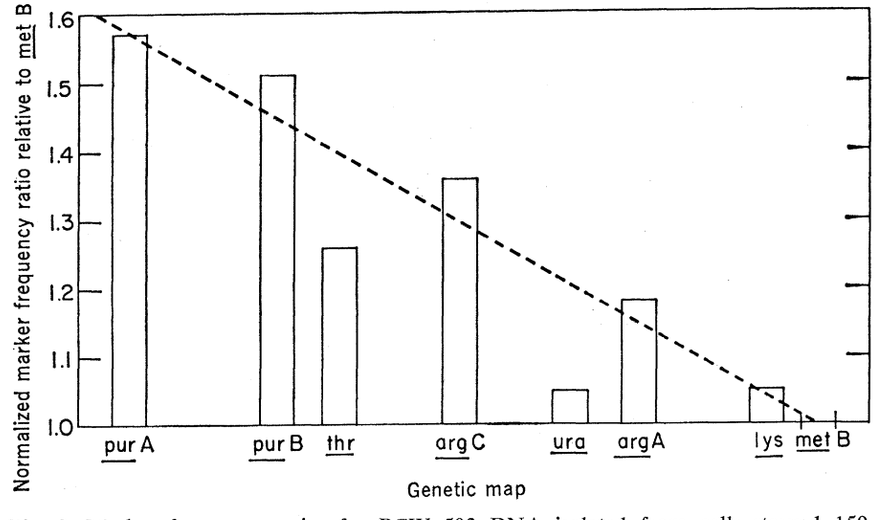

Fig. 2. Marker frequency ratios for BCW 503 DNA isolated from cells starved 150 minutes for leucine. All ratios are represented relative to the *met*B marked which is located near the terminus of the chromosome. All ratios have been normalized for differences in individual marker transformation efficiences by multiplying them by the reciprocal of that marker-pair ratio determined with spore DNA. The marker frequency ratios shown are the average of four or more experiments.

The results of marker frequency analysis on DNA isolated from amino acid–starved BCW 503 is shown in Fig. 2. Quite clearly, the marker frequency ratios for all seven markers examined exceed 1.0. The marker frequency ratio is highest for a marker closest to the origin and decreases in value as the position of the marker approaches the terminus. The straight line connecting *pur*A with the *met*B position illustrates the expected marker frequency distribution if those chromosomes that have not completed replication have stopped randomly at positions between those two markers. The observed values approximate this line. The maximum ratio obtained is 1.6 for *pur*A/*met*B, a point to which we will return later.

Marker frequency analysis was performed on DNA isolated from BC 200 cells after 150 minutes of leucine starvation. At this time BC 200, a derivative of strain 168, had synthesized 60 to 70 percent more DNA (*9*). For these cells the frequency ratio of *pur*A to *ile* marker was 1.4.

These results show that not all chromosomes of amino acid–starved *B. subtilis* replicate to completion at a terminus, as would be expected if replication were controlled only at the initiation step. This observation is consistent with and supported by earlier results on replication after amino acid starvation in *B. subtilis* (*9, 10*). For BCW 503 starved cells, the ratio of *pur*A to *met*B of 1.6 shows that at least 60 percent of the chromosomes have not completed replication to the *met*B marker. This value is a minimum estimate since the *pur*A and *met*B markers are not located at the origin and the terminus, respectively. To correct for the position of these markers, on the assumption that the sum of their distance from the origin and terminus is about 10 percent of the distance between them (*13*), the expected marker frequency ratio for *pur*A to *met*B for

a population of chromosomes all incompletely replicated becomes 1.8 rather than 2.0. Then the estimate of incomplete BCW 503 chromosomes increases to 89 percent. This estimate may further increase, since *B. subtilis* growing in the glucose-salts medium, with a generation time of 70 to 80 minutes, may have a resting period for DNA synthesis during its cell cycle (*14*). Another factor which would increase this estimate is that the *met*B marker may not lie at the terminus of all replicons in *B. subtilis*, a subject which will be dealt with in a subsequent report.

Among the 89 percent of chromosomes that were incomplete, replication appears to have stopped in a random fashion with regard to position on the chromosome. This viewpoint is supported by the decreasing gradient of marker frequency ratios from the origin to the terminus. The pattern of replication after amino acid starvation of BCW 503 also indicated a random distribution of replication activity along the chromosome (*10*). Cells in balanced growth have a random distribution of replication forks on the chromosome (*10*). These replication forks apparently stop with the same random distribution during amino acid starvation of BCW 503 cells. On restart of growth, replication merely resumes from sites where it had stopped.

Regulation at the initiation step alone cannot account for the effects of amino acid starvation on chromosome replication in *B. subtilis*. Recent findings with *E. coli* and *Salmonella typhimurium* may indicate that regulation only at the initiation step is not sufficient to account for the effects of amino acid starvation on chromosome replication in all of the strains of these organisms either (*6, 15*). My results along with others concerning the ratio of DNA to mass during and after amino acid starvation (*16*) suggest that a second regulatory circuit acts to control chromosome replication in *B. subtilis*. This second regulatory circuit appears to act independently of the initiation control circuit during amino acid starvation.

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Inhibition of Growth of Leukemia Cells by Enzymic Folate Depletion

Abstract. *A new bacterial enzyme, designated as carboxypeptidase G_1, inhibited growth of the L5178Y and L1210 murine leukemias, as well as the Walker carcinoma and the human lymphoblastoid line (RPMI 4265), propogated in vitro. This enzyme hydrolyzes the glutamate moiety from both oxidized and reduced folate forms, and thus it may prove to be of value in creating rapid folate depletion in vivo.*

The successful use of the enzyme L-asparaginase to produce a rapid depletion of the amino acid L-asparagine, necessary for the growth of certain human lymphoblastic leukemias (*1*), has stimulated the search for other enzymic therapies for neoplastic disease. Encouraged by the early results of Heinle and Welch (*2*) that demonstrated a beneficial result of dietary folate depletion on human leukemia, and the finding that an experimental tumor, the Walker 256 carcinoma, was markedly inhibited by dietary folate deficiency (*3*), we initiated the studies described below. Leucovorin (5-formyltetrahydrofolate), a close congener of the less readily available naturally occurring major folate coenzyme 5-methyltetrahydrofolate, was used as the sole carbon-nitrogen source to select an organism that would be capable of hydrolyzing reduced folate coenzymes. A pure culture of a Gram-negative rod, identified as a strain of *Pseudomonas stutzeri* was isolated (*4*). An enzyme, designated as carboxypeptidase G_1 (*5*), capable of cleaving terminal glutamate or aspartate residues from folate coenzymes or peptides was purified some 600-fold by ammonium sulfate fractionation, gel filtration on Sephadex G-100, and chromatography on diethylaminoethyl (DEAE)-cellulose. This enzyme differs from the previously described carboxypeptidase G (*6*) in several respects, the most important of which is the ability of the G_1 enzyme to hydrolyze reduced folate coenzymes (*7*).

The highly purified enzyme was tested for its ability to inhibit the growth of murine L5178Y cells, propogated in vitro (Fig. 1). An I_{50} (inhibition of 50 percent of the cells) was obtained with 0.012 unit of enzyme activity (*8*). Inhibition of the growth

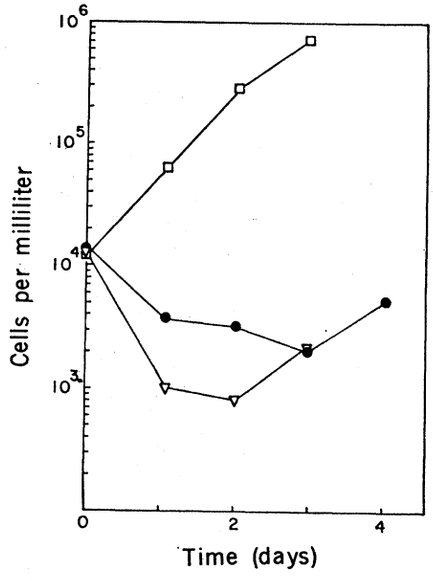

Fig. 1. Growth curve of 5178Y cells in the presence or absence of carboxypeptidase G_1. Cells were grown at 37°C and sampling for cell counting was performed each day on a model B Coulter counter. □, Tris buffer control; ●, 0.025 unit of enzyme per milliliter; ▽, 0.25 unit of enzyme per milliliter.