CHROMOSOME REPLICATION IN BACILLUS SUBTILIS CULTURES GROWING AT DIFFERENT RATES

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The chromosome of *Escherichia coli* is a circular structure[1] in which circular permutations of the genetic map are observed.[2] In *Bacillus subtilis*, on the other hand, no circular permutations of the genetic map have been observed[3] nor has it been possible to demonstrate the circularity of the chromosome by autoradiographic techniques.[4]

Although sequential replication of chromosomes has been described for exponentially growing *E. coli*[1, 5, 6] and for *B. subtilis*,[3, 7, 8] the systems have not hitherto been compared in other aspects of chromosome replication which could be related to chromosome structure.

In *E. coli* the replication rate decreases and the chromosome content may drop as the growth rate decreases.[9] In the present paper we describe a similar situation for *B. subtilis*.

Materials and Methods.—A variant of a thymine and indole-requiring strain of *Bacillus subtilis* which forms long chains on nutrient agar and fails to form spores on TY agar[10] was used in all experiments. The original strain has been described.[11]

The minimal medium of Spizizen[12] was used. The growth rate was varied in this minimal medium by using the following different carbon sources in addition to citrate present in the Spizizen medium: glucose, 0.5 per cent; sodium succinate, 0.5 per cent; sodium acetate, 0.5 per cent; proline, 0.3 per cent; sodium aspartate, 0.3 per cent. Although citrate was present in all cases, these media will be referred to as glucose, succinate, acetate, proline, or aspartate media, respectively. For routine growth, 40 μg/ml thymine and 50 μg/ml l-tryptophan were added.

Microcolonies were prepared for autoradiography by a modification of the method described previously.[13] The cells were labeled with methyl-labeled H^3-thymine (obtained from New England Nuclear Corp.). In pulse-label experiments 200 μc of H^3-thymine (1 mc per ml, 0.0157 mg per ml) were contained in 0.5 ml of the growth medium. The culture was incubated in the presence of H^3-thymine for approximately 10 per cent of the generation time. In continuous-label experiments the cells were grown for three generations in the presence of 200 μc of H^3-thymine in 1 ml of growth medium containing 40 μg per ml nonradioactive thymine.

In both pulse-label and continuous-label experiments the cells were removed from the radioactive medium by collection and washing on membrane filters,[6] then transferred immediately to nutrient agar slides (0.6% agar, 0.8% nutrient broth) and incubated at 37° in a wet chamber for 120–150 minutes, after which time the cells had formed chains of 16 to 32 cells. The slides were then air-dried and dipped in Kodak NTB-2 emulsion. After an exposure sufficient to give clusters, each containing between five and ten grains, the number of grain clusters per colony was scored by examination under 250× magnification in a Wild phase-contrast microscope.

Autoradiographs of individual *B. subtilis* cells were carried out as described previously.[13]

The DNA content of cells was determined by the Burton modification of the diphenylanine reaction.[14] The total cell count of *B. subtilis* preparations was obtained microscopically using a Petroff-Hausser bacterial-counting chamber. Cells to be counted were fixed in 0.5 per cent formalin.

The number of nuclear areas per cell at different growth rates was determined by microscopic examination of *B. subtilis* cells suspended in PVP (polyvinylpyrrolidon (Fluka)) (E. Kellenberger, personal communication). The PVP preparation contained: 6 gm PVP, 0.05 gm agar, and 8 ml of Spizizen's medium. It was kept at 45° to prevent solidification. Approximately 4–5 $\times$ 10^8 cells per ml were prepared by filtration and concentration of an exponentially growing culture. Varying amounts of these cells, usually 0.1–0.3 ml, were added to 0.5 ml of PVP to give a medium of the correct refractive index.

Results.—In all experiments cultures were used when in a state of balanced growth. To achieve this they were inoculated from TY agar into the medium of choice and grown for at least ten generations during which the growth rate was measured in the Coulter counter and a low culture density was maintained by dilution. This rate was constant in any given experiment (sometimes lasting several days).

Problems were encountered with cells growing in succinate or acetate media. A large proportion of such cells were found as doubles, counting as one in the Coulter counter. Moreover, although we observed a constant growth rate throughout any individual experiment, this rate differed between experiments, varying between 260 and 360 minutes per generation in succinate media and between 350 and 600 minutes per generation in acetate medium. The growth rates for the individual experiments in Table 1 are reported.

The DNA content of *E. coli* and *S. typhimurium* cells has been shown to vary with the growth rate.[15, 9] By using a complete medium or by changing the carbon source in minimal medium, the growth rate of *B. subtilis* has been changed and the DNA content of cells growing at slower and faster rates also determined. Values for the DNA content of these cells are given in Table 1. The amount of DNA per cell was found to be related to the growth rate. At doubling times of greater than 250 minutes (succinate and acetate) there is approximately one half the DNA content per cell as compared with cells growing with doubling times of 70–160 minutes (glucose, aspartate, and proline). Cells grown in Penassay medium, with a generation time of 25–30 minutes, appear to have about 60 per cent more DNA than those cells grown in Spizizen's medium with glucose as a carbon source.

These findings are consistent with the hypothesis that there are twice as many chromosomes per cell in glucose, aspartate, and proline cultures as there are in succinate and acetate cultures. The observation of 60 per cent more DNA in the Penassay cells than in the glucose cells is consistent with a dichotomous manner of replication described by Oishi, Yoshikawa, and Sueoka.[16]

In a situation where there are two genomes per cell with one replication point per genome, one would expect to find four conserved units of radioactive DNA per cell when cells incorporate radioactivity for a tenth of a generation and eight conserved units of radioactive DNA when the chromosomes are completely labeled.

The number of conserved units of DNA per cell was determined by the micro-

colony technique (see *Materials and Methods*). Cells were grown for a tenth of a generation or for three generations in the presence of H^3-thymine. Microcolonies were made and the number of grain clusters per microcolony was counted. The number of grain clusters per colony in glucose and acetate media is shown in Figure 1.

Whereas more than 90 per cent of the cells appear as single individuals in glucose cultures, a large proportion (70%) of the cells exist as pairs when grown in acetate medium. These pairs which are easily distinguished microscopically in the counting chamber will give rise to colonies with twice the expected number of grain clusters (thus 50% of the microcolonies arise from pairs of cells). As a result, pulse-labeled glucose-grown cells yield for the most part microcolonies with four grain clusters per colony, but pulse-labeled acetate-grown cells yield microcolonies containing two or four grain clusters per colony. When these data are corrected for the number of pairs present (Table 1), the average number of grain clusters per parent cell approaches two for acetate- or succinate-grown cells (in 100 microcolonies, 50 arise from single cells, 50 from pairs; the number of grain clusters observed in the population thus may be attributed to 150 parent cells).

The presence of microcolonies arising from two cells is also seen in the number of grain clusters found in colonies grown from completely labeled acetate cells in which many colonies with eight grain clusters are observed. When these data are corrected for the number of microcolonies arising from two cells, an average value of between four and five grain clusters per parent cell is obtained. Glucose colonies from completely labeled cells contain, on the average, about seven grain clusters (Table 1). It is interesting to note that as with *E. coli*[9, 17] a bimodal distribution of grain clusters per microcolony is obtained in colonies arising from fully labeled glucose cells. The cause of this is unknown.

TABLE 1

DNA CONTENT OF *B. subtilis* AT DIFFERENT GROWTH RATES

Carbon source of growth medium	Doubling time (min)	Amount of DNA/cell $\times 10^{-14}$ gm	Average Number Grain Clusters/Microcolony		Average number nuclei/ cell	Mol wt of chromosome (daltons $\times 10^9$)	Unlabeled cells in radioactive pulse* (%)
			Pulse	Fully labeled			
Succinate	260	0.84					
	360		2.24		1	3.76	25
Acetate	580	0.92			1	4.08	20
	535		2.2	4.64			
Aspartate	160	1.77			2	3.96	
Proline	160	1.71			2	3.78	
Glucose	80	2.05			2	4.55	0.5
	80		4.8	6.8			
Penassay	27	3.35			2–4		
Penassay stationary†		0.64–0.69				3.8–4.2 (per cell)	

The amount of DNA per cell was determined on 5 aliquots of 5 ml each from cultures at a cell density of approximately 10^8 cells/ml. The values for succinate, proline, and Penassay cells are an average of five such determinations on two separate cultures.

The average number of grain clusters per microcolony as well as the per cent of unlabeled cells following a radioactive pulse was determined as described in Fig. 1. These values have been corrected for the frequency of colonies arising from cell pairs.

The average number of nuclei per cell was found by phase microscopy of polyvinylpyrrolidon preparations.

The molecular weight of the chromosome was calculated from the amount of DNA present per chromosome. This value for the replicating chromosome was then divided by a factor of 1.4 to convert it to that for a nonreplicating chromosome.

* $x^2m + xn = r$, where r = observed fraction of unlabeled microcolonies, m = fraction of cells occurring as doubles, n = fraction of cells occurring as singles, and x = actual fraction of unlabeled cells.

† Taken from Massie and Zimm[19] for stationary cultures of strain W23; such cultures have between 1 and 2 nuclei per cell.

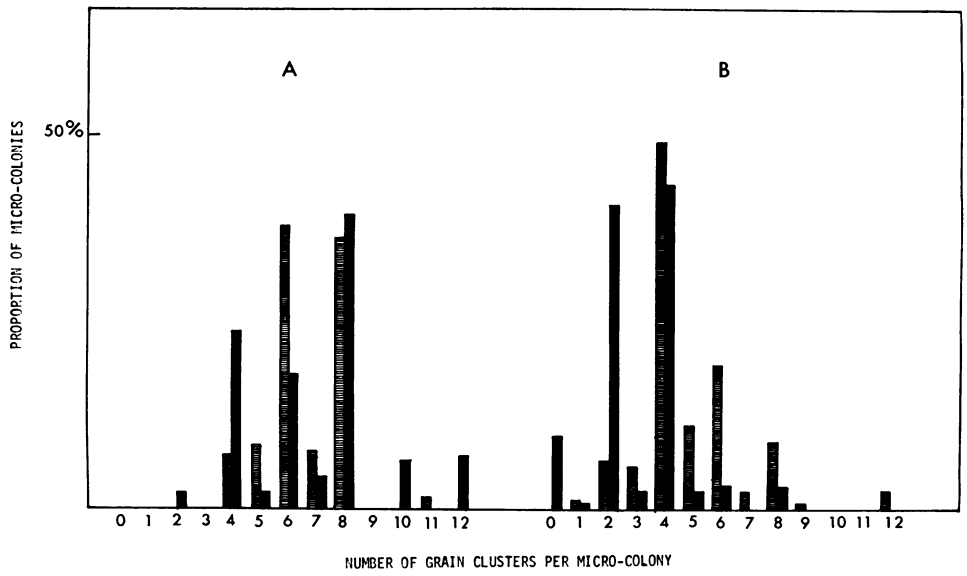

FIG. 1.—Estimation of the number of conserved DNA units present in *B. subtilis* cells growing in glucose and in acetate media. The frequency of grain clusters per microcolony is presented. (*A*) Completely labeled cells, (*B*) pulse-labeled cells. Glucose (□) or acetate (■) cultures were labeled for 3 generations (complete labeling) or for 0.1 generation (pulse labeling). They were then transferred to agar slides and microcolonies allowed to develop. Autoradiographs were prepared and after appropriate exposure the number of grain clusters per microcolony was counted. The experimental details are presented in *Materials and Methods*.

More than 95% of the cells from glucose cultures occurred as isolated single individuals, a single cell thus yielding a microcolony. On the other hand, 70% of the cells in an acetate culture were present as unseparated pairs. Thus 55% of the microcolonies grown from cells of an acetate culture arose from pairs of cells, whereas 45% arose from single cells.

The per cent of the microcolonies which show no label in pulse-label experiments gives an indication of the fraction of the division cycle during which cells are not synthesizing DNA. The results, in Table 1, indicate that only 20 per cent of the acetate-grown cells or 25 per cent of the succinate-grown cells are unlabeled. Thus it would appear that even in cells with very long generation times the cells are replicating DNA during a major fraction of the division cycle. The pulse of radioactive thymine was administered for 0.1 of a generation period during which cells may be entering or leaving a synthetic gap. Since such cells will be labeled, the gap in which synthesis does not occur may be as large as 0.3–0.35 of the division cycle ((0.2 or 0.25) + 0.1).

The average number of grain clusters in colonies arising from acetate-grown cells indicates that each parent cell initially contained one replicating site where label entered into two conserved units. Glucose-grown cells would appear to contain two such replicating sites. Since the glucose cells also contain twice as much DNA as acetate cells, it is reasonable to conclude that these sites are on individually replicating and duplicate chromosomes.

This was confirmed in an experiment in which completely labeled, or pulse-labeled glucose-grown cells were transferred to nonradioactive medium and sampled after one, two, three, four, or five generations of growth. Individual cells were examined by radioautography after prolonged exposure. The frequency of unlabeled cells

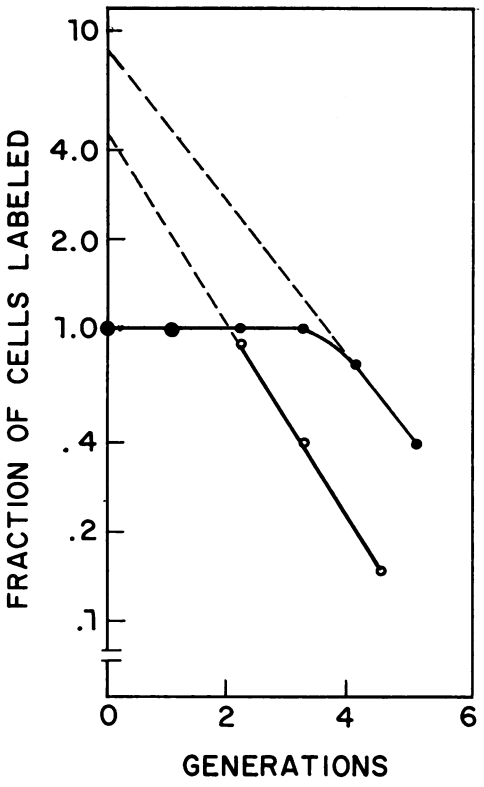

FIG. 2.—Estimation of the number of radioactive conserved units of DNA present in glucose-grown *B. subtilis* cells prelabeled with H^3-thymine for three generations or for 8 min (pulse).

A glucose culture of *B. sublilis* was incubated for three generations with H^3-thymine (200 μc/40 μg/ml). Another culture was incubated for 8 min with H^3-thymine (400 μc/20 μg/ml). Both cultures were transferred to nonradioactive medium and sampled immediately or after 1, 2, 3, 4, 5, or 6 generations of growth (followed as increase in cell number in the Coulter counter). Autoradiographs of individual cells were prepared from each sample. After an exposure sufficient to yield an average of between 10 and 15 grains per radioactive cell, the fraction of radioactive cells (with at least one grain) was measured. The logarithm of this fraction is graphed as a function of the generations of growth in nonradioactive medium. (●) Completely labeled culture, (○) pulse-labeled culture.

was measured at each generation. In Figure 2 the logarithm of the fraction of unlabeled cells is graphed as a function of the number of generations of growth in nonradioactive medium. Cells without label appear after three generations for the completely labeled cells and after two generations for the pulse-labeled cells, indicating that at the time of transfer to nonradioactive medium, eight radioactive units of DNA were present in the completely labeled cells, four in the pulse-labeled cells.

Indeed, if the linear portions of the curves are extrapolated to the ordinate, they intersect it at values of approximately 4 and 8 for pulse-labeled and for continuously labeled cells, respectively (the rationale for his extrapolation was discussed by Lark and Bird[18]). A single replication fork should incorporate radioactivity into two conserved units of DNA during the period of a pulse label, whereas a completely labeled replicating chromosome contains four radioactive conserved units of DNA—two completed polynucleotide strands which serve as templates and two unfinished strands in the process of completion.

Because of the long generation period, such experiments could not be carried out using acetate-grown cells.

Discussion.—From the data presented, we conclude that cells of *B. subtilis* growing in glucose minimal medium contain two semiconserved units of DNA (genomes), while at slower growth rates, as in acetate and succinate minimal media, only one such genome appears to be present.

The number of conserved units of pulse-labeled DNA per cell segregated into subsequent progeny indicates that there is one growing point per genome in both the glucose- and acetate-grown cells

From the chemical determinations of the amount of DNA per cell, it is possible to calculate the molecular weight of the genome. An average of the values given in Table 1 is 3.9×10^9 daltons for a nonreplicating, completed unit. In one other experiment (not described), the DNA content was determined in exponentially growing cells by H^3-thymine incorporation, as described by Lark and Lark.[13] This gave a value of 3.52×10^9 daltons for a nonreplicating completed chromosome. (These numbers were obtained by dividing the value for the replicating chromosome by $\sqrt{2}$ or 1.41. However, with slow-growing cells there may be a gap of 30 per cent, and depending on whether the gap is before or after replication, the correction factor could be more or less with values which could be as low as 1.3 or as high as 1.6). Our values for the molecular weight of the *subtilis* chromosome are somewhat higher than those of Dennis and Wake[4] who found that spores contained 3.0×10^9 daltons of DNA. Massie and Zimm[19] found that stationary Penassay cultures of *B. subtilis* W23, with one to two nuclei per cell, contained from 3.8 to 4.2×10^9 daltons per cell. Since W23 is reported[7, 8] to have chromosomes in the completed state in the stationary stage, the values of Massie and Zimm presumably would be for a single completed chromosome and in general agreement with our determinations.

The data from the microcolony autoradiographs of pulse-labeled *B. subtilis* cells show that, even at very slow growth rates (doubling times of 270 to 600 minutes), most of the cells are synthesizing DNA. Thus in *B. subtilis*, as in *E. coli*,[9] the rate at which DNA replication progresses along the chromosome appears to vary with the growth rate, decreasing at slower growth rates.

If we take a length of about 2000 μ (3.9×10^9 daltons) for the genome of *B. subtilis*, our data indicate that it may be replicated at rates which range from about 40 μ/minute during dichotomous replication in Penassay medium to 6 μ/minute in cells growing in acetate or succinate media.

The value we have obtained for the molecular weight of the genome, *B. subtilis*, is more than twice the value reported for the length of the *subtilis* chromosome estimated by autoradiography.[4] Two explanations are possible: (1) The autoradiographic data are subject to the criticism that the *subtilis* chromosome may be sheared during preparation for autoradiography. (2) Our data yield a value for the DNA content of the entire *subtilis* genome, and it is possible that this is composed of more than one replicon, the largest of which is 750 μ. Recent data on *E. coli*[18, 20–22] as well as mammalian cells[23] indicate that all of the replicons (independent replicating units[24] present in a cell will segregate to maintain, as a group, those DNA molecules containing previously synthesized polynucleotide strands of a common age (those synthesized in the previous generation, those synthesized two generations previously, those synthesized three generations previously, etc.). Thus, if the genome of *B. subtilis* were composed of more than one replicon, these would segregate as one unit and could not be distinguished from a single replicon on the basis of their segregation pattern.

The genetic markers studied by others[3, 7, 8, 16] have not as yet been established as associated with a single replicon, although many of them have been shown to occur as linked clusters and most have been demonstrated to be replicated in a

sequential order[7, 8, 16, 3] during spore germination or growth from the stationary phase. Future experiments combining physical with genetic techniques may indicate whether one or more replicons compose the *subtilis* genome.

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