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*SEQUENTIAL REPLICATION OF BACILLUS SUBTILIS CHROMOSOME, I. COMPARISON OF MARKER FREQUENCIES IN EXPONENTIAL AND STATIONARY GROWTH PHASES**

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The mechanism of DNA replication is fairly well understood,[1-3] while that of chromosomal replication is rather poorly known. The main difficulty is our ignorance of the molecular structure of chromosomes. The results of Taylor, Woods, and Hughes[4] show a bipartite and semiconservative nature of plant chromosomes. So far as the sequence of chromosomal replication is concerned, autoradiographic analyses of chromosomes of higher organisms have revealed no over-all polarity of replication,[5, 6] except for a suggestive case in *Crepis*.[7] Existence of polarity in small regions, however, is quite likely.[5, 8] For the *E. coli* chromosome, Maaløe[9] proposed a model in which replication proceeds along the chromosome in an oriented fashion. Although there was some suggestive evidence favoring such a model,[9] no direct proof had been obtained.

Based on isotopic transfer experiments in the *Bacillus subtilis* transformation system, we have concluded that the replication of different markers in *B. subtilis* does not occur in a random-in-time fashion but has a regular oriented pattern.[10, 11] In recent reports by Nagata[12, 13] the pattern of duplication of λ prophage in synchronized cultures of an Hfr strain of *E. coli* K12 has been interpreted as evidence for a polarity of chromosome replication. A similar conclusion has been reached by Cairns from autoradiographic studies on DNA replication of *E. coli*.[14]

This paper presents experimental results which give further support to the polarity of replication of the bacterial chromosome. The experiments consist of measurements of relative frequencies of genetic markers in an exponentially growing cell population of *Bacillus subtilis*, using the genetic transformation system developed by Spizizen.[15] If chromosomal replication has a polarity, there should be a difference in the frequency of genes depending on their position on the chromosome. This is exactly what we found in the present experiments.

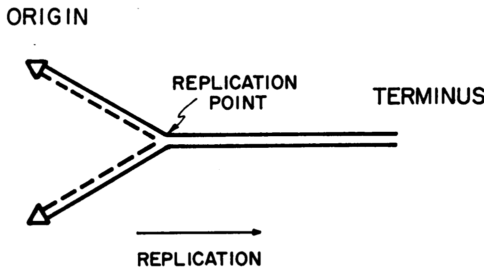

FIG. 1.—A replication model of the chromosome. Replication starts from one end (origin) of the chromosome, and proceeds toward the other end (terminus). Both strands are copied. The solid line represents the old strand and the broken one the newly synthesized strand. This model does not differ, in principle, from a circular chromosome model in which replication starts at a fixed point in one direction.

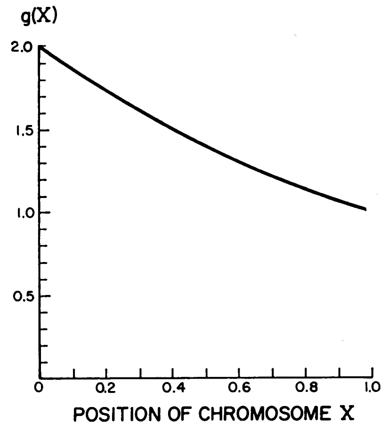

FIG. 2.—Relative frequencies of genetic markers at different positions on the chromosome in an exponentially growing population. The distribution (or mapping) function g(X) is not normalized; the integration of g(X) from X = 0 to X = 1 gives 1/ln2. This function was used for mapping markers (*see* Fig. 5).

The results gave clear evidence of an oriented replication of the chromosome and made it possible to construct a linear genetic map covering the whole range of the chromosome. Two small groups of linked genetic markers had been described in *B. subtilis* by joint transformation: the histidine-indole-tyrosine group[16–18] and the streptomycin-sporeless group.[19] The present experiments suggested the existence of another linked group, methionine and isoleucine, whose existence was then confirmed by joint transformation.

Materials and Methods.—Principles of the experiments: At the present stage of our knowledge of chromosomal structure[20,21] several alternative replication models are possible. The model which we chose to test experimentally is the one-end oriented, continuous replication model (Fig. 1). In this model, the chromosome replicates from one end (origin), proceeds continuously toward the other end (terminus) at an approximately uniform speed, and both strands replicate. For the sake of simplicity, the resting period in which no DNA synthesis occurs is assumed to be negligible.[9]

This particular model leads to several specific testable predictions. In an exponentially growing population without any synchrony of chromosomal replication, the frequency of chromosomes whose replication has just started is nearly twice as high as that of chromosomes which are about to complete their replication. Frequencies of chromosomes in the intermediate replication stages are intermediate between these two extremes (see appendix). This means that the frequency of a genetic marker in an exponentially growing population is a unique function of its location on the chromosome: i.e., the markers located toward the origin are more frequent than those toward the terminus. The frequency distribution of markers as a function of their position X on the chromosome is (see appendix): $g(X) = 2^{1-X}$.

The position X can vary from 0 (the origin) to 1 (the terminus); the corresponding values of $g(X)$ are 2 and 1, respectively. A graphic representation of the relation is given in Figure 2. If our model is correct, this relation can be used to map genetic markers in a linear order by experimentally measuring the relative frequency of different markers, $g(X)$, in an exponentially growing culture used as donor of DNA in a transformation experiment. Consequently, the function $g(X)$ is called the mapping function.

Experimental determination of marker frequency in a given transforming DNA preparation,

however, requires another condition. Since the efficiency of transformation varies from marker to marker due to differences in the integration efficiency,[22, 23] mere comparisons of numbers of transformants for different markers do not tell us the actual frequencies of the markers in the DNA preparation in question. This problem can be solved if we find a condition in which all markers are equally frequent. This condition will serve as the standard, and the relative frequency of markers in exponential cultures can be obtained by reference to the standard.

Conditions for equal marker frequency might be obtained by various methods. Removal of supplements from a growing culture of an amino acid-requiring mutant of *E. coli* might give the desired conditions.[9] In the present work, we chose the stationary phase as a possible example of the equal-gene-frequency situation. If all the cells in the stationary phase have complete, resting chromosomes, all markers should be equally frequent. Comparison of the relative transforming activity of DNA from exponential and stationary growth phases should disclose differences for different markers, if our replication model is right. If, on the other hand, the replication state or the age of the chromosome is distributed more or less similarly in the stationary phase as in the exponential phase, there should be no difference in marker frequency between DNA samples from the two growth phases.

A wild-type strain (W23) of *B. subtilis*[24] was used as the DNA donor strain throughout the work. Recipient mutants were isolated[11] from a Marburg strain (a derivative of strain 168 of Burkholder and Giles[25]) by an ultraviolet-penicillin method.[26, 27] Only mutants with low reversion frequencies, less than 10^{-7}, were used. DNA samples of the donor strain were prepared from cells in various growth stages. In order to stop further DNA replication, cells were heated at 60°C for 10 min immediately after sampling. They were chilled in ice, centrifuged, and suspended in a versene saline solution (0.15 *M* NaCl plus 0.1 *M* ethylenediamine tetracetate, pH 8.0) at a cell concentration of about 10^{10}/ml. The suspension was incubated at 37°C for 15 min with egg-white lysozyme (1 mg/ml). Lysis was completed by further incubation with duponol (final concentration 2 per cent) for 5 min at 37°C with constant shaking. DNA was prepared by a modification of Marmur's method[28] and was sterilized by keeping it in 70 per cent ethanol for several hours. The details of the methods will be described elsewhere.[11]

Transformation was performed mostly according to Anagnostopoulos and Spizizen.[24] In order to obtain competent recipient cells reproducibly, a growth curve was followed in each case by growing the recipient cells in a flask which allowed measurement of cell concentration without removal of samples. When the growth curve indicated the approach of the stationary phase, the cells were diluted tenfold with medium for a second growth phase[24] and shaken for 90 min at 37°C; then DNA was added. The cells were shaken further for 40 min and DNAase (5μg/ml) was added.

Experimental Results.—DNA was isolated from the donor strain at various times in the exponential and stationary growth phases (Fig. 3). Relative frequencies of transformants for various markers were compared for various samples of DNA from exponential and stationary phases. The results are presented in Tables 1 and 2. The results of Table 1 show that the ratios of transformants for one marker to those for another marker are independent of the DNA concentration used.

For each marker and for each DNA sample, the number of transformants obtained was normalized to the number obtained for the *met* (= methionine) marker. Then these normalized values were again normalized to the values for DNA sample #7, taken from a stationary phase culture (*see* Fig. 3). These final values show significant differences between different markers.

These differences indicate that at different stages in the growth of a culture there are significant differences between the frequencies with which different markers can be donated by transforming DNA. These differences should correspond, according to our hypothesis, to differences in the number of copies of the markers in the DNA and, hence, in the donor cells.

It is remarkable that for none of the markers tested (ten altogether, *see* Fig. 5) the normalized value for the exponential phase is more than twice the value in the

TABLE 1

TYPICAL DATA ON RELATIVE FREQUENCIES OF *leu*, *met*, AND *thr* MARKERS IN DNA FROM EXPONENTIAL AND STATIONARY GROWTH PHASES

DNA	Concentration	No. of Transformed Cells/10^7 Recipient Cells[a] *thr*	*leu*	*met*	Ratio *thr/met*	Average *thr/met* (relative[d])	*leu/met*	Average *leu/met* (relative[d])
No. 3[b]	0.1 μg/ml	6,670	8,050	4,970	1.34	1.41[c] → *1.62*[d]	1.62	1.59 → *1.22*
No. 3	0.01	1,070	1,140	730	1.47		1.56	
No. 3	0.001	61	82	32	1.91[e]		2.58[e]	
No. 5	0.1	5,110	7,180	5,540	0.92	1.02 → *1.17*	1.30	1.39 → *1.07*
No. 5	0.01	1,350	1,720	1,270	1.06		1.35	
No. 5	0.001	147	210	138	1.07		1.52	
No. 7	0.1	7,630	11,730	8,820	0.87	0.87 → *1.00*	1.33	1.30 → *1.00*
No. 7	0.01	1,670	2,580	2,070	0.81		1.25	
No. 7	0.001	253	352	268	0.94		1.31	
No DNA		7	0	0				

[a] Each value is the average of two plate counts. The recipient strain used is a triple mutant, *leu-met-thr* (Mu8u5u5).
[b] The numbers of the DNA samples are the same as those shown in Figure 3.
[c] The average ratio.
[d] The average ratio relative to DNA No. 7 which is taken as 1.
[e] These figures were discarded because of the small number of transformed colonies per plate.

stationary phase. The marker with the highest frequency in the exponential phase is *ade*, the lowest are *met* and *ileu*. The ratio between these two classes of markers is 2, which is the maximal ratio predicted by the proposed model.

A more detailed analysis of early and late stationary phases was made using the two extreme markers, *ade* and *met* (Fig. 4). The results indicate that the higher frequency of the *ade* marker in the exponential phase decreases gradually and finally reaches the stationary value. This is the expectation from our postulate that the stationary chromosome represents the completed form.

The quantitative results fit the predictions so well that the proposed model of chromosome replication gains strong support. Alternative models will be considered in the discussion, but none of them are as satisfactory as the proposed model.

The genetic map of *B. subtilis* shown in Figure 5 was constructed by calculating the position (X) of each marker from the observed normalized value of that marker in the exponential phase using the mapping function. It should be noticed that those markers that had previously been shown to be linked[16–18] (*his*, *ind*, *tyr*) be-

TABLE 2

RELATIVE FREQUENCIES OF VARIOUS MARKERS TO *met* IN EXPONENTIAL AND STATIONARY GROWTH PHASES

Relative frequency of marker to *met*[a]	DNA Samples: Exponential No. 1	No. 2	No. 3	No. 4	Stationary No. 5	No. 6	No. 7[b]	Overnight DNA[c]
ileu/met			*1.04*		*0.97*		*(1.00)*	
leu/met	*1.37*	*1.43**	*1.24*	*1.12**	*1.05*	*1.03**	*(1.00)*	*1.05*
thr/met	*1.74*		*1.67*		*1.15*		*(1.00)*	
ade/met	*1.96*	*2.10**	*1.92*	*1.90**	*1.22*	*1.40**	*(1.00)*	*1.05*
his/met	*1.55*		*1.28*				*(1.00)*	*1.05*

[a] The recipient strains are triple mutants derived from a double mutant *leu-met* (Mu8u5): *leu-met-ileu* (Mu8u5u1); *leu-met-his* (Mu8u5u2); *leu-met-ade* (Mu8u5u6); *leu-met-thr* (Mu8u5u5).
[b] The ratios (marker/*met*) for DNA No. 7 are taken as 1 in this set of data and the other values are normalized to them. These values are the averages of several repeated measurements, except those marked with an asterisk, which are based on one observation only.
[c] Relative marker frequencies in DNA from an overnight culture are shown for additional comparison.

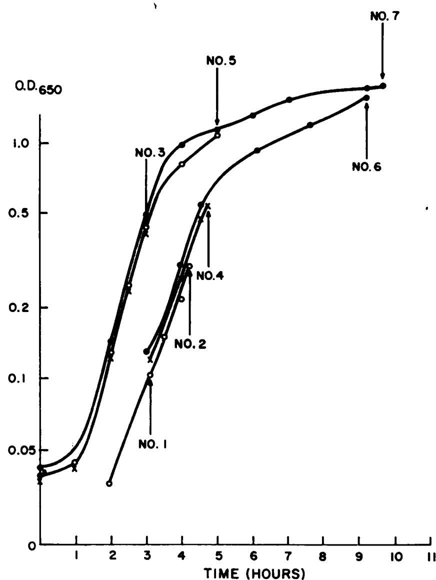

FIG. 3.—DNA samples from various growth stages of *B. subtilis* W23 in an enriched minimal medium. Cells were grown at 37°C with constant shaking in a minimal medium[24] supplemented with 0.05% casein hydrolyzate and 50 μg/ml of L-tryptophan. The volume was 1 liter for samples No. 1 to No. 4, 500 ml for No. 5 to No. 7. Cells of each sample were heated at 60°C for 10 min and DNA was prepared as described in the text.

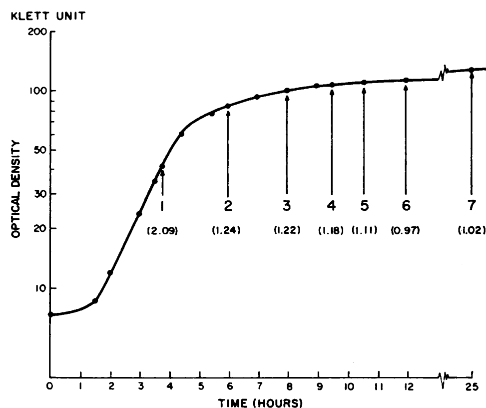

FIG. 4.—Detailed analysis of various stages of the stationary growth phase using *ade* and *met* markers. The figures in parentheses are the *ade/met* ratio. The culture and sampling conditions are similar to those of Figure 3. The recipient strain is a triple mutant strain, *leu-met-ade* (Mu8u5u6).

haved similarly in the present test, thus confirming the validity of the map (Table 3 and Fig. 5).

Extensive linkage tests were made among the markers used in the present experiment by searching for joint transformation of various combinations of the markers.[29] Besides the previously known *his-ind-tyr* linkage group, a new linkage between *met* and *ileu* was discovered (Table 4). This is in complete agreement with the proposed map (Fig. 5).

Discussion.—The method and results reported here and in a previous note[10] support an oriented replication of the *B. subtilis* chromosome and present a new opportunity to study the genetic structure and replication mechanism of chromo-

TABLE 3

MARKER FREQUENCIES OF *tyr*, *ind*, AND *his* IN VARIOUS STAGES OF GROWTH

DNA	No. of Transformed Cells[c]			Ratio			
	tyr	*ind*	*his*	*tyr/his*		*ind/his*	
No. 1[a]	243	281	291	0.84	*0.85*	0.97	*0.94*
No. 2	262	279	257	1.02	*1.03*	1.08	*1.05*
No. 3	437	484	427	1.02	*1.03*	1.13	*1.10*
No. 4	337	378	351	0.96	*0.97*	1.07	*1.04*
No. 5	752	807	760	0.99	*1.00*	1.06	*1.03*
No. 6	606	598	599	1.01	*1.02*	1.00	*0.97*
No. 7	941	999	955	0.99	*(1.00)*[b]	1.03	*(1.00)*[b]

[a] The numbers of the DNA samples are the same as those shown in Figure 3.
[b] The *tyr/his* and *ind/his* ratios of No. 7 DNA were taken as 1.
[c] The recipient strain used was a triple mutant, *tyr-ind-his* (Mylylu7).

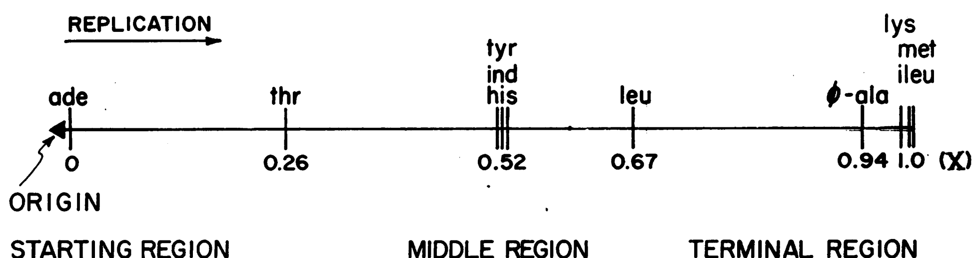

FIG. 5.—A genetic map of *B. subtilis* obtained by the method described in the text. The position of each marker on the chromosome is obtained from the average of several experimental values. The gene orders for *tyr, ind,* and *his,* and for *ileu* and *met,* are not yet known. The markers *phe* and *lys* were mapped by using the following double mutants as recipient strains: *leu-phe* (Mu8u7), and *lys-phe* (Mu12u17).

somes. Excluded are the models assuming random-in-time replication of different regions of the chromosome and those in which only one chain of DNA is copied, while the other chain remains single over a considerable length. The latter class of models is also made unlikely by isotopic transfer experiments[2] and autoradiographic studies[14] in *E. coli*.

Two modified forms of the oriented replication model, however, cannot be excluded completely. One is the "subunit-model" in which the chromosome consists of subunits which replicate more or less independently of one another. The replication of each subunit should, however, have polarity to satisfy the present results. In this scheme, the *ade* locus would be located close to the origin of one of the subunits, and *met* and *ileu* close to the terminus of some subunit. A subunit structure of chromosomes, in which the replication of each subunit proceeds in sequential order from one end of the chromosome to the other, is not distinguishable from the model proposed in this paper and does not require special consideration at the moment. In another model, replication would start from both ends of the chromosome or of its subunits (two-end model). In this case, the markers that we have mapped in the region close to the origin would physically occupy either end of the chromosome, and those mapped in the terminal region would occupy the middle region.

These two schemes would drastically change the genetic map. Although a subunit model cannot be excluded, the behavior of linked markers in the present experiments indicates that the subunits, if any, may be fairly large in size. The two-end model cannot readily be excluded by experiments of the present type unless more markers are accumulated and mapped. However, two lines of evidence on the

TABLE 4

A NEW LINKAGE GROUP (METHIONINE-ISOLEUCINE) IN *B. subtilis*

Conc. of DNA (μg/ml)	No. of Transformed Cells per 10^6 Recipient Cells[d]					Per Cent Cotransfer Index[a]	
	leu	*ileu*	*met*	*ileu-met*	*ileu-leu*	*ileu-met*	*ileu-leu*
0.04	334[b] ± 13[c]	158 ± 7	162 ± 2	27 ± 2	0	9.6	<0.2
0.008	229 ± 25	106 ± 3	100 ± 19	18 ± 5	0	9.6	<0.3
0.004	136 ± 6	48 ± 5	52 ± 11	9 ± 1	0	9.0	<0.5
No	0	0	0	0	0	...	...

[a] The cotransfer index was calculated according to Nester and Lederberg[16] and is expressed in percentage.
[b] Average number of colonies from 3 plates.
[c] Extreme values are shown to indicate the extent of errors.
[d] The recipient strain used was a triple mutant *leu-met-ileu* (Mu8u5u1).

E. coli chromosome tend to exclude these possibilities and support the one-end continuous replication model. One is an autoradiographic study by Cairns, who observed only one replication "fork" per chromosome.[14] The other is a study by Nagata,[12, 13] showing that in synchronously growing cultures of Hfr strains of *E. coli* K12 there is a correlation between the position of the prophage λ on the host chromosome and the time of prophage replication.

The equal frequency of markers at the stationary phase predicts that the chromosome will replicate synchronously when cells in the stationary phase are transferred to a fresh medium. In the previously reported cases of cell synchrony in bacteria,[30,31] it was an unsettled question whether or not chromosome synchrony accompanied cell synchrony. It is now possible to check the degree of chromosome synchrony in these cases by comparing the relative frequency of the two end markers or of any two markers located at different positions on the chromosome.

The age (replication-stage) distribution of chromosomes in the exponential phase described here assumes that no sizable fraction of the generation time is spent in the resting stage in which the chromosome does not engage in replication. In other words, the mapping function holds for growth conditions in which the resting period is negligible. This appears to be true for the present experiments, because the ratio of *ade* to *met* gives the value of 2. In general, however, the frequency ratio of any two markers in the exponential phase will be smaller the larger the fraction of the generation time occupied by the resting period, since a part of the population will have different gene frequencies, as described in this paper, while the resting part of the population will have equal frequencies for all markers. Using the two extreme markers, *ade* and *met*, it should be possible to detect the presence and to calculate the length of the resting phase in other culture conditions.[32]

As previously discussed by others,[9, 31, 33] there should be some regulating mechanism that controls chromosomal replication so that the normal DNA:mass ratio can be kept fairly constant in various growth conditions. It is attractive to assume that there is at the tip of the chromosome a region which signals the start of the chromosomal replication. The activation for such a signal gives an analogy of a repressor-inducer type mechanism.

Summary.—Relative frequencies of various genetic markers in the DNA of *B. subtilis*, strain W23, in exponential and stationary growth phases were compared using a transformation system. If the chromosome replication has a polarity, the frequency of each marker in the exponential phase should be a function of its location on the chromosome. The results indicate that such polarity exists in *B. subtilis*. On the basis of the results, a genetic map has been constructed in which the adenine marker is located near the point of origin, from which the chromosome starts replicating, and methionine and isoleucine near the terminus. The results also indicate that chromosomes in the stationary phase are in completed form.

Appendix.—The replication model described in this paper (Fig. 1) leads to the following quantitative description of the population.

In an exponentially growing population, the frequency of chromosomes which have replicated up to a position x is described by

$$f(x) = (\ln 2)2^{1-x} \quad (1)$$

The distribution function is equivalent to the age distribution function of cells solved by Powell[34] for organisms which replicate regularly by binary fission.

The frequency of a particular marker which occupies a position X of the chromosome is given by

$$g(X) = \int_0^X f(x)dx + 2\int_X^1 f(x)dx = 2^{1-x} \quad (2)$$

Here the function $g(X)$ is not normalized, so that the total number of markers is equivalent to $1/\ln 2$, while the total number of chromosomes is taken as 1 (equation 1). For graphic representation, see Figure 2. A detailed presentation of the above functions and their applications to other cases will be presented elsewhere.[32]

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* The abbreviations, *ade, his, ileu, ind, leu, lys, met, φ-ala* (or *phe*), *thr*, and *tyr* were used for mutants requiring adenine, histidine, isoleucine, indole, leucine, lysine, methionine, phenylalanine, threonine, and tyrosine, respectively.

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