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THE MOLECULAR SYNCHRONY AND SEQUENTIAL REPLICATION OF DNA IN ESCHERICHIA COLI*

BY TOSHIO NAGATA[†]

DEPARTMENT OF ZOOLOGY, COLUMBIA UNIVERSITY

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Cytological and genetic observations on a number of organisms have contributed to formulating the concept of a sequential replication of the chromosome starting from one or more fixed points.[1,2]   Especially in bacteria, as Maaløe[3] pointed out, this possibility found support in a mass of data strongly indicating a definite regularity in the synthesis of DNA.[4,5]   Advantage of the bacterial system was taken in the present work which employed two strains of *Escherichia coli* K-12 Hfr, as well as one of F⁻, and analyses were made of the problem of molecular synchrony and the sequential replication of the chromosomes.   A system has been established for determining the course of change in the number of prophage λ per bacterium during a replication cycle of DNA in populations of lysogenic bacteria under conditions of synchronous growth.   A substantial amount of evidence indicates that the prophage λ is indeed a DNA structure, occupies a definite site on the K-12 linkage map, replicates in harmony with the host chromosome, and can be induced by various agents.[6-8]   The existence of λ as an episome allows us to regard it as a gene on the bacterial chromosome and at the same time provides us with a means of enumerating its intracellular number at any given time.   Thus, the kinetics of this measure should directly reflect the pattern of the replication of the host chromosome itself.

Bacterial Strains.—All the bacteria used are derivatives of *Escherichia coli* K-12 (Table 1).

Superinfecting phage: $\lambda v_1 h$, a weak virulent and host range mutant of λ,[9] was kindly provided by Dr. R. K. Appleyard.   In contrast to the wild type (v_1^+) which forms turbid plaques, the mutant v_1 gives rise to clear plaques.   High titer stocks of this phage were prepared by the method of confluent lysis.[10]

Media: (a) *M-9* is a minimal salt solution composed of 7.0 g anhydrous Na_2HPO_4, 3.0 g KH_2-

TABLE 1

BACTERIAL STRAINS

Strain	Remarks	Origin	Donor*
HfrH(λ)	m^-		M. Demerec
HfrCS101(λ)	m^-	58–161	M. Demerec
F$^-$Z260(λ)	m^-	(F$^+m^-$)	N. D. Zinder
HfrH(λ, *424*)	m^-	HfrH(λ)	. . .
HfrCS101(λ, *424*)	m^-	HfrCS101(λ)	. .
F$^-$Z260(λ, *424*)	m^-	F$^-$Z260(λ)	. . .
C600	$t^-l^-B_1^-$, sensitive to λ & *424*	679–680	R. K. Appleyard
C600(λ)	$t^-l^-B_1^-$, sensitive to *424*	C600	. . .
C600(*424*)	$t^-l^-B_1^-$, sensitive to λ	C600	F. Jacob

The symbols designate as follows; m, methionine; t, threonine; l, leucine; B_1, Vitamin B_1; (λ), lysogenic for λ; (*424*), lysogenic for *424*. The double lysogens for λ and *424* were isolated after infecting each λ lysogen with the temperate phage *424;* C600(λ), likewise after infecting C600 with λ.
* The author is greatly indebted to these donors for their generosity.

PO_4, 1.0 g NH_4Cl and 1 liter of distilled water. After autoclaving, a separately autoclaved stock solution of $MgSO_4 \cdot 7H_2O$ is added to a final concentration of 5×10^{-4} M, the pH being 6.8–7.0.

(b) *M-9(0.05):* To the *M-9*, glucose and DL-methionine are added to final concentrations of 0.05% and 50 μg/ml, respectively. It is used to grow tube cultures and gives a final yield of bacteria of 5×10^8 cells/ml.

(c) *M-9(0.1)* is same as *M-9(0.05)*, except that the final concentration of glucose is 0.1%; it is used to grow flask cultures and gives a final yield of bacteria of 10^9 cells/ml.

(d) *YTB* is a growth medium for indicator bacteria as well as for postinduction incubation, and is composed of 5 g Yeast Extract (Difco), 10 g Bacto-Tryptone (Difco), 5 g NaCl and 10 ml of a 25% $MgSO_4 \cdot 7H_2O$ solution, dissolved in 1 liter of distilled water.

(e) *YTA:* To the *YTB*, 1.7% Bacto-Agar (Difco) is added.

(f) *Soft agar:* Same as *YTA* except that the agar content is 0.7%. It is used to plate phage particles for plaque counting by the agar layer technique.[11]

Methods: (a) *Synchronization of bacterial growth* was achieved by employing a fractional filtration technique originally described by Maruyama and Yanagita;[12] several important modifications were made, however. In order to start each experiment with a bacterial population as homogeneous as possible, a single colony was isolated and grown on a fresh *YTA* slant for about 6–8 hr at 37°. From this young clone, a small number of cells was inoculated into 5 ml *M-9(0.05)* for overnight incubation with aeration at 37°. After washing the overnight culture twice, cells were inoculated into 300 ml of fresh *M-9(0.1)*. The culture was incubated at 37° on a shaker for more than 10 hr whereupon it reached a stationary phase; thereafter, incubation was continued for 120 more min. This timing is important in that a relatively homogeneous and healthy population of cells largely determines success in synchronization. After centrifugation of the culture, cells were washed once and recovered as *ca.* 1.4-1.7 ml of thick suspension which was ready for filtration. A filter paper pile was prepared with 18 sheets of No. 126 Toyo filter paper sandwiched between 2 sheets of No. 1 Toyo filter paper at the top and 1 sheet at the bottom. The pile was tied with a piece of string, placed in a funnel, and an inner cylinder was screwed above it. The pile must be moistened with 3 ml of prewarmed *M-9* for 10 to 15 min before use. The thick suspension of cells, prepared as above, was first adsorbed on the top sheets of the pile. This was achieved within 2 min, whereupon the inner cylinder was screwed tightly and 10 ml of prewarmed *M-9* was added; simultaneously the suction decompression was applied. Two or three successive elutions with 10 ml *M-9* were found necessary in order to obtain a good recovery of cells in the filtrate (usually 10–20%). The entire process of elution should take only about 1 min. The filtrate was centrifuged, an appropriate amount of the pellet was inoculated into 300 ml of fresh prewarmed *M-9(0.1)*, and incubation in a 37° shaker-waterbath followed immediately.

(b) *Measurement of DNA net synthesis* was carried out by taking samples at every 10 min during the postfiltration incubation; the samples were subjected to the method described by Burton.[13]

(c) *The absolute number of prophage λ per bacterium* was determined by the technique[14] which involves induction of λ and superinfection with $\lambda v_1 h$. The Poisson distribution was introduced

as a correction factor in order to make possible the analysis of the lysate of a population instead of just single bursts.[15] Every 5 min in the postfiltration incubation 6 ml samples were withdrawn and irradiated with UV for 20 sec with a Westinghouse sterilamp (46 erg/sec/mm²). Four ml of the irradiated suspension were inoculated into 1 ml of 5 × *YTB* where a suitable number (depending upon the multiplicity desired) of superinfecting phage $\lambda v_1 h$ was present. Incubation followed immediately in a 37° waterbath with aeration by bubbling. After 15 min of post-ultraviolet incubation, a 0.1 ml sample was taken to determine the number of unadsorbed $\lambda v_1 h$. After 130 min, the lysate was chloroformed; the supernatant was then plated on very thick *YTA* (not less than 1 cm) with a growing culture of *E. coli* K-12 C600.

(d) *Determination of the relative number of two prophates, λ and 424:* In order to avoid the ambiguity caused by the superinfection technique which requires some correction procedures, the system was improved by eliminating superinfection and introducing a second prophage, *424*, as a reference for λ. All the strains, already lysogenic for λ and used before, were further lysogenized with *424*. The resulting double lysogens were subjected to exactly the same procedures for synchronization, viable counts, and DNA determination. For prophage enumeration, samples were simply induced by UV; the titers of phage λ and *424* in the resulting lysate were determined by plating the supernatant on *YTA* with the appropriate indicator bacteria. These were *E. coli* strains K-12 C600 (*424*) for λ and C600(λ) for *424* (because of their immunity differences); their plating efficiencies were not significantly different.

Results.—Kinetics of prophage duplication during one cycle of DNA replication: After precautions in every step of the procedure for synchronizing cell division, all strains responded by a satisfactory stepwise growth. DNA increased exponentially through several generations with a probable break at each onset of cell division; thus, one DNA replication cycle corresponded almost exactly to one generation of cell division. This roughly agrees with the finding with nonsynchronized populations that DNA synthesis occupies more than 80 per cent of a cell generation.[4] The kinetics of increase in turbidity also roughly corresponded with that of DNA.

(a) *Change in the absolute number of prophage λ per bacterium:* In all the samples for prophage determination, taken at different times during the cell growth and infected with various multiplicities of $\lambda v_1 h$ (ca. 2–10), practically all the cells in a population were lysed. The average burst size increased linearly from about 80 to 120 as a function of time within one replication cycle. No significant difference was observed in the burst size obtained after induction alone and after induction plus superinfection. It was proved in a system equivalent to the present one that both genotypes of λ, $v_1{}^+$ and v_1, are not subject to differential selective forces during their vegetative multiplication together.[16] Therefore, the ratio of the number of turbid plaques to the number of clear ones, obtained by plating the lysate, can be taken as a direct function of both the multiplicity of superinfecting phage and the number of intracellular prophage at the time of induction and superinfection; the last parameter can be calculated by knowing the first two.[15]

Figure 1a shows the pattern of prophage kinetics in HfrH(λ). The number of prophage per bacterium remained at a constant value until the net increase of DNA reached approximately the 50 per cent level. Then an abrupt doubling in prophage number was achieved at a time when the increase in total DNA was 80 per cent; toward the beginning of the next replication cycle the number returned sharply to the initial value. In another strain of Hfr, CS101 (λ), however, the number of prophage doubled quickly during a very short period after the beginning of the replication cycle, remained constant throughout the rest of the cycle until the onset of the next (Fig. 1b). At the time of the doubling in prophage number, the DNA increase was only at a level of *ca.* 10 per cent. The F⁻(λ) displayed yet
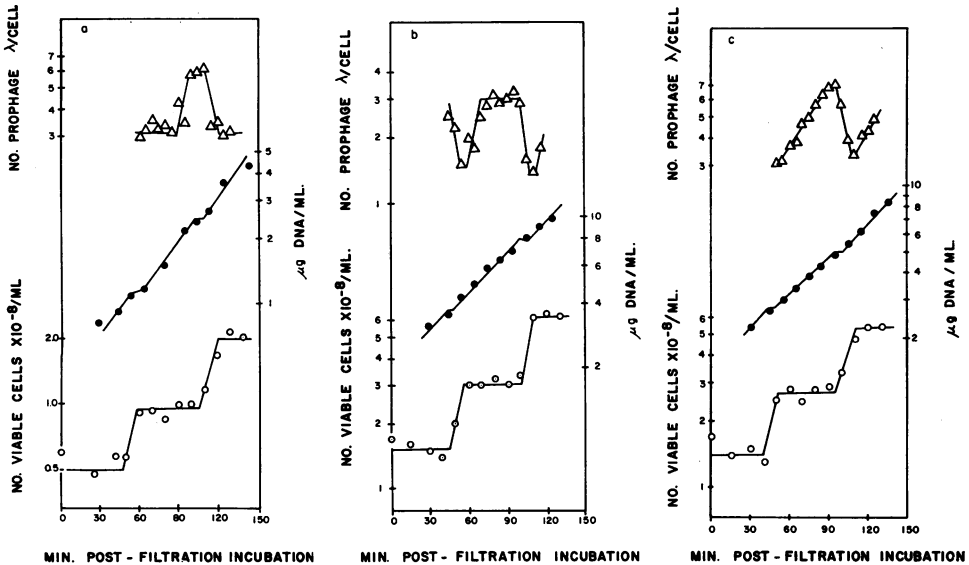
FIG. 1 *a–c.*—Change in the absolute number of prophage λ per bacterium over one replication cycle of DNA in synchronized populations of *E. coli* K-12. The intracellular number of λ(△) was determined by the induction-superinfection technique described in *Methods*. Synchronization was achieved by fractional filtration; DNA (●) was determined by the Burton-Dische method; the number of viable cells (○), by plating samples on agar plates. (*a*) HfrH(λ) with average multiplicities of superinfection, *n*, of 4.8–8.9. (*b*) HfrCS101(λ) with *n* = 6.4–11.3. (*c*) F⁻Z260(λ) with *n* = 2.8–4.8.

another pattern of prophage kinetics (Fig. 1c) where no abrupt doubling of the number was observed. When cells were grown nonsynchronously, these specific prophage patterns were completely distorted to the extent that the number of prophage per cell did not significantly change. It can be concluded, therefore, that the observed difference in the prophage kinetics is significant; each of the unique patterns is characteristic of each strain of bacteria, attributable only to the synchronous growth of the cells, and hence reflects the molecular synchrony of DNA itself.

(b) *Change in the relative number of prophages, λ and 424, per bacterium:* Upon induction of bacteria doubly lysogenic for λ and *424*, both prophages gave rise to progeny phages in the lysate. The average burst size was not significantly different among the different strains; it was also the same for single lysogens carrying either of the prophages as for double lysogens, growing synchronously or not. The same type of consistency was also observed for the per cent induction of λ (more than 96%) and *424* (25.7 ± 1.4%). DNA synthesis stops immediately after irradiation with the inducing dosage of UV and is arrested for about 30 min during post-UV incubation. All these facts justify taking the ratio of λ/*424* in a lysate as a measure of the intracellular ratio of the two prophages at the moment of sampling and induction.

In HfrH(λ, *424*) (Fig. 2a), the ratio stayed at a constant value nearly halfway through the course of increase of DNA, whereupon it was sharply reduced to almost one half. Then it resumed the initial value at a time when the DNA had increased about 80 per cent. On the contrary, the ratio in HfrCS101 (λ, *424*) (Fig.
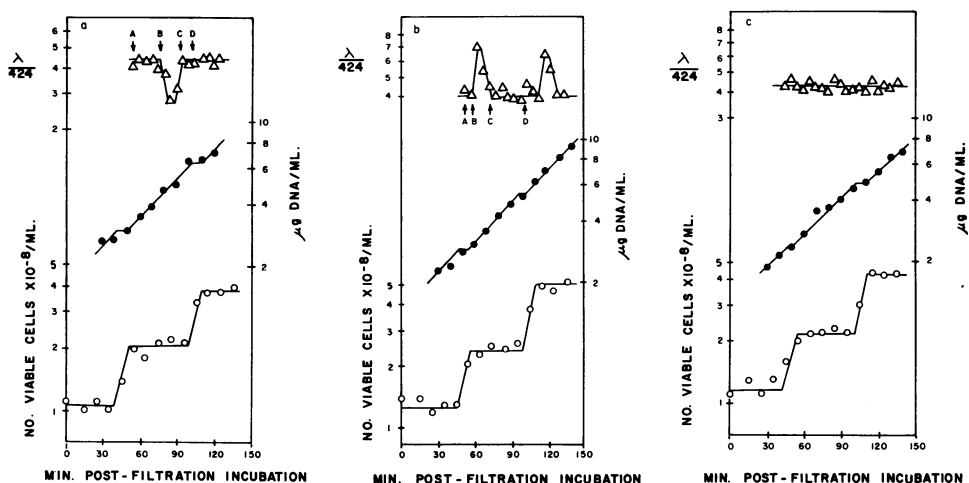
FIG. 2 a–c.—Change in the relative number of prophages λ and 424 per bacterium during one replication cycle of DNA in synchronized populations of E. coli K-12. The relative number was expressed in terms of the ratio (Δ) of phage λ to phage 424 in the lysate obtained after induction of double lysogens with UV. All the conditions for synchronization, DNA (●) determinations and viable counts (O), are similar to those described for Figure 1. (a) HfrH(λ, 424); the sharp decrease in the ratio is significant (P ≪ 0.01) by t test. (b) HfrCS101(λ, 424); the sharp increase in the ratio is significant (P ≪ 0.01) by t test. (c) F⁻Z260(λ, 424); the ratios scattered around a mean of 4.35 ± 0.11.

2b) doubled almost immediately at the beginning of synchronous growth, returned to the original level when the DNA had increased about 40 per cent, and thereafter remained constant until the beginning of the next generation. In F⁻(λ, 424) (Fig. 2c), the ratio remained constant throughout the cycle. These observations are all consistent with the previous findings and distinguish even more clearly the relative time of duplication of the prophages in each Hfr strain.

Polarity in the replication of bacterial chromosomes: It is known that the point of rupture on the continuous circular chromosome of E. coli K-12, the location of the F factor on the resulting linear structure, and hence the direction of genetic transfer by each chromosome at mating are characteristic of each Hfr strain.[7, 17] The relative positions of λ and 424 on such a linkage map of each Hfr were compared with their relative time of duplication expressed in terms of per cent increase of DNA with a marked agreement between the two sets of values (Table 2). Moreover, the fact that the timing of λ duplication in Hfr strains determined by two different methods was proved to be consistent suggests the validity of the observations. All these results lead to the conclusion that the replication of the Hfr chromosome is synchronized and has a definite polarity starting from its posterior end where the F factor is attached and proceeds to the forward end. There is an assumption here, however, that the map distance of genetic loci including prophages, expressed in terms of the time of entry upon conjugation, designates their geographical positions on the actual DNA molecule; this may be the case, especially in view of P³² decay experiments where it was shown that the kinetics of genetic transfer at conjugation could be correlated with the kinetics of nucleotide transfer.[7, 19]

The prophage kinetics of F⁻ (Fig. 1c) shows perfect harmony with the mutation kinetics of E. coli 15 (F⁻) thymineless bacteria induced with 5-bromouracil during

TABLE 2

COMPARISON OF THE RELATIVE POSITIONS OF TWO PROPHAGES AS DETERMINED BY TIME OF
DUPLICATION WITH THEIR POSITIONS ON THE LINKAGE MAP

Strain	HfrH (λ, 424)				HfrCS101(λ, 424)			
Points on Fig. 2a, b	A	B	C	D	A	B	C	D
μgDNA/ml	2.9	4.4	5.4	6.0	2.9	3.1	4.0	5.9
Δ DNA to point A	0.0	1.5	2.5	3.1	0.0	0.2	1.1	3.0
% increase of DNA	0.0	48.3	80.5	100.0	0.0	6.7	36.7	100.0
Markers on the linkage map	F	424	λ	t	F	λ	424	lac
Distance from F	0'	57'	92'	108'	0'	6'	41'	108'
% distance	0.0	52.8	85.0	100.0	0.0	5.5	38.0	100.0

Point A represents the start of a generation cycle; B, the time of the first prophage duplication; C, that of the second prophage duplication; and D, the end of the cycle. μgDNA/ml for each point were read from the DNA curves in Fig. 2a and b. Markers on the linkage map: F designates the F factor attached to the tail end of the chroromosome; 424 and λ, the prophages; t, threonine; and lac, lactose. The last two genes occupy positions at the head ends of the chromosomes.[7, 17] The distance of each marker from F is expressed in terms of time of entry upon conjugation in minutes.[18]

a synchronous growth caused by thymine starvation.[20] Recent autoradiographic studies[2] have revealed that even the replication patterns of the chromosomes are quite similar in F⁻ and Hfr.   These facts seem to indicate that the F⁻ chromosome replicates also with polarity but starts at random from any site on the continuous circular structure.   Accordingly, if one used thymineless Hfr for the mutation study, a pattern of induction kinetics similar to that of prophage should be expected; some preliminary results are at hand suggesting this is indeed the case in such a system where a mutagenic base analogue was pulsed into the synchronously replicating DNA.[21]

Discussion.—Although the possibility of a "conservative" replication of DNA *in vivo* has not yet been completely ruled out,[22] evidences have been accumulated which indicate that the replication is "semiconservative"; this was most clearly shown in bacteria,[23] where "side-to-side" hybrids were formed rather than "end-to-end" association of subunits.[24] A question arises, however, as to whether or not this mechanism also characterizes the replication of DNA molecules at higher levels of organization, e.g., in the whole intact bacterial chromosome.   The autoradiographic studies of Cairns[2] are illuminating in this regard, and his conclusion was that the long molecule of DNA (more than 700 μ) is double-stranded, and replicates from one fixed point in a semiconservative fashion.   These results are readily compatible with the present prophage data.

Based on the very likely assumption that the long molecule of DNA also undergoes semiconservative replication, an attempt was made to construct molecular models (Fig. 3) of chromosome replication in order to account for the prophage data. In Model I, one expects λ to duplicate when DNA replication is 85 per cent complete in HfrH and 5.5 per cent in HfrCS101, whereas the observed values are 80 per cent and 6.7 per cent, respectively (cf. Table 2), showing a marked agreement. In view of studies on the *in vitro* synthesis of DNA,[25] however, one might argue that replication starts from both ends of the molecule with unwinding of the double helix, leaving each of the partner strands temporarily single.[26] Although it was argued that this need not necessarily apply,[27] the possibility still remains also at the chromosomal level and was examined in Model II. If we assume that the temporarily single-stranded molecule of λ DNA, attached to the host molecule in a yet unknown way, can be induced, as well as the newly formed double-stranded λ, to
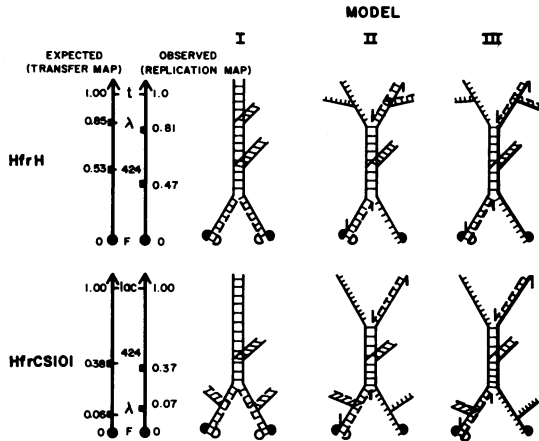
Fig. 3.—Molecular models for the sequential replication of Hfr chromosomes. The solid lines in these models indicate the parental DNA strands and the broken lines, new growing strands. The thick lines in Model III indicate the particular strands assumed to carry information for responding to UV induction to produce normal phage progeny (see text). Arrows in the Models II and III show the antiparallel polarity in the backbones of the double-stranded DNA determined by the phosphodiester bonds of the deoxyribonucleosides. Although the molecular nature of the attachment of the prophages to the host chromosome is not known, the prophages are indicated as branches to make the illustrations clear.

give rise to healthy progeny phages, the expectation would be that λ prophage duplicates upon a 5.5 per cent increase in DNA in CS101, in agreement with the observation (6.7%). But λ should double when DNA has increased by 15 per cent in HfrH, in disagreement with the observation (80%); the two observations are evidently contradictory. If we assume otherwise, that single-stranded λ cannot be induced, then the expected value for HfrH (85%) agrees with observed (80%); but this is not the case for CS101, expected (94.5%) versus observed (6.7%). Again there is an obvious contradiction, indicating that the model is invalid. Yet another possibility could be entertained; namely, if we assume that only one of the two strands carries information for responding to the inducing agent, then Model III can be formulated. Suppose only the upward strand in each strain of Hfr carries the information, and replication takes place in a manner similar to Model II. Suppose further that the new chain growing upward from the end where F factor is located also carries the information, then this particular chain is the one which decides the timing of λ duplication as detected by induction. This model is as compatible with the prophage data as Model I and cannot be ruled out by the prophage technique alone. Against this hypothesis, however, is evidence that both members of a base pair must be changed upon mutation for a mutant phenotype to be expressed.[28] Furthermore, the Kornberg enzyme could still operate in the way postulated in Model I, if the bacterial chromosome consisted of a large number of subunits, each of which replicated in an antiparallel fashion. The results of Cairns also support Model I. Here the most challenging question concerns the nature of the controlling mechanism that determines the replication polarity; what factor plays the role of primary importance—the DNA polymerase, hydrogen bond formation, unwinding itself, or some unknown factor?

Summary.—The question of the mechanism of chromosome replication in bacteria has been studied with lysogenic strains of *Escherichia coli* K-12 Hfr and F⁻. Bacterial growth was synchronized by the fractional filtration technique, the smaller

cells being recovered. One replication cycle of DNA corresponded to a synchronized cell generation. Kinetic studies of prophage duplication over one replication cycle of DNA revealed: first, that the pattern of kinetics is distinctively strain specific; second, that this is brought about only by synchronization; and finally, that the specific time of prophage duplication, expressed in terms of per cent increase of DNA, can be directly correlated with the known genetic linkage map.

These findings led to the conclusion that the replication of the chromosome in *E. coli* K-12 is synchronized and sequential, especially in Hfr, starting from the posterior end where the F factor is attached and proceeding to the forward end.

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[15] When a population of bacteria lysogenic for $\lambda v_1{}^+$ is induced and at the same time superinfected by λv_1 with an average multiplicity of n, the distribution of fractions of bacteria superinfected with actual multiplicity i follows the Poisson equation, $P_{(i)} = n^i e^{-n}/i!$ When x is the initial number of prophage per bacterium, the ratio, R, of $\lambda v_1{}^+/\lambda v_1$ in the progeny population can be related as $R = \Sigma[xP_{(i)}/\langle x+i)]/1 - \Sigma[xP_{(i)}/(x + i)]$. A solution of this equation is not easily obtained, but one can make approximate computations within a biologically reasonable range of x, e.g., 1 to 10, against selected values of n, e.g., 2 to 10. The log-log plot of the resulting values of R against x for each n gives fairly linear relations which allow a relatively accurate interpolation for estimating x from the experimental values of R and n.

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

BY HIROSHI YOSHIKAWA AND NOBORU SUEOKA

DEPARTMENT OF BIOLOGY, PRINCETON UNIVERSITY

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.