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Segregation of Deoxyribonucleic Acid in Bacteria: Association of the Segregating Unit with the Cell Envelope

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Cells of the gram-positive organism *Lactobacillus acidophilus* R-26 were labeled with ^{3}H-thymine to measure the segregation of radioactive deoxyribonucleic acid (DNA) into daughter cells. Such cells were found to contain 8 conserved units of DNA which would correspond to two replicating chromosomes per cell. Fluorescent antibody (FA) against this organism was used to demonstrate that portions of the cell surface (2 to 4 units per cell) were conserved during growth and division. The permanent association of DNA with these conserved cell surface units was measured by combining autoradiography with FA techniques. DNA synthesized immediately before FA labeling was not associated with the fluorescent cell surface, whereas DNA synthesized a generation previously was. The results are consistent with a model in which DNA becomes permanently fixed to the cell surface when it is first used as a template.

Autoradiographic measurements of the distribution of radioactive deoxyribonucleic acid (DNA) among dividing cells led to a model for chromosome segregation in bacteria (12, 13).

According to this model (Fig. 1), chromosome replication cannot be initiated unless both polynucleotide strands of the DNA molecule are attached to a segregation apparatus of the cell [in bacteria, this has been assumed to be an element of the cell surface, possibly the cell membrane (8)]. When replication is completed, each newly formed chromosome is permanently attached to the cell by the polynucleotide strand which served as a template during replication. Thus, the unit of segregation is a single polynucleotide strand which becomes attached to the cell permanently when it is used for the first time as a template in replication.

This model is consistent with the segregation of markers located on different replicating elements, such as episomes and chromosomes (9; Rownd, *personal communication*), and has correctly predicted chromosomal segregation patterns in *Bacillus subtilis* (5) and in eukaryotic systems (12*a*, 14).

In the present paper, a direct test of the model in Fig. 1 is described. We have used *Lactobacillus acidophilus* R-26, a gram-positive organism which requires thymine when grown in the absence of folic acid (17). Segregation of its DNA, therefore, can be studied by autoradiography. We have succeeded in preparing fluorescent antibody (FA) which will combine with this organism. In the experiments which follow, we have studied the segregation of radioactive DNA vis-à-vis the cell surface.

MATERIALS AND METHODS

Cultures of *L. acidophilus* R-26 were maintained on solid medium and grown in the defined medium lacking folic acid as described by Soška (17). Exponential growth with a generation period of 60 min was routinely observed with a Coulter counter, model B.

FA was prepared by injecting rabbits (intravenously) twice a week with 2×10^8 cells of a saline vaccine (prepared from a stationary-phase culture which was treated for 24 hr with 0.2% Formalin, and then washed and suspended in saline). When test bleedings indicated a high agglutination titer, the animals were bled from the heart, and the antibody was purified by precipitation with 18% Na_2SO_4 (10, 11). Fluorescein iso-thiocyanate was coupled to the antibody (4), and the FA was purified by dialysis and passage through Sephadex G-25.

Methyl labeled ^{3}H-thymine was obtained from New England Nuclear Corp., Boston, Mass. Radioactivity of this compound has been shown to be incorporated exclusively into the DNA of *Lactobacillus* (18).

Cells were transferred into and out of media containing ^{3}H-thymine by collecting 1 to 5 ml of culture on membrane filters (18) on which they were washed

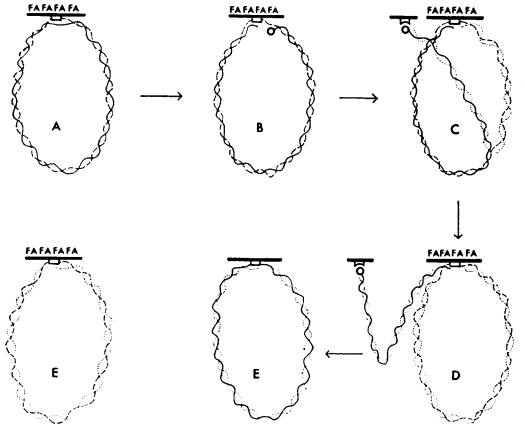

FIG. 1. *Model for chromosome replication and segregation. (A) The completed chromosome is attached to the cell envelope by a single polynucleotide template strand represented by the dashed line. The cell envelope is labeled with fluorescent antibody (FA). (B-D) Replication is initiated when the nonattached recently synthesized strand (solid line) is attached to a newly synthesized portion of the cell surface (not labeled with FA). (E) When replication is completed, the chromosome containing the original template strand (dashed line) is still attached to the FA-labeled cell surface, whereas the chromosome containing the template used for the first time (solid line) is now attached to a new cell surface which is not labeled with FA.*

with four 5-ml samples of prewarmed medium. They were suspended by shaking the membrane filter in prewarmed medium, the volume of which was adjusted to obtain a desired titer. The time required for such transfers never exceeded 2 min. The same method was used to transfer cells into 2.5% KCl buffer for treatment with FA. Excess FA was removed by centrifugation. The FA-labeled cells were washed twice with 5-ml samples of warm 2.5% KCl and were resuspended in sterile distilled water to disperse the cells. All centrifugations were carried out at room temperature. The removal of FA was completed within 20 min.

Glass petri dishes containing Lactobacilli Agar [3.8% Lactobacilli Broth plus 1.8% agar (Difco)] were prepared. The surface of the agar was washed with petroleum ether to remove lipids, and a 0.4% solution of collodion in amyl acetate was poured over the agar surface. This solution was immediately decanted, and the inverted plates were allowed to dry for 3 to 4 hr over wet paper towels. Microcolonies were grown by spreading a drop containing 10^5 FA-treated cells on these collodion-covered petri dishes and incubating at 39 C. When colonies had grown to an appropriate size (30 to 50 cells), they were fixed by formaldehyde vapor for 10 min. The collodion film was then floated off the agar onto a water surface and was picked up on a glass microscope slide.

For autoradiography, slides of microcolonies were dipped in Kodak NTB-2 liquid emulsion and stored in a cool dry container to expose.

After an appropriate exposure (60 days for the experiments in Table 1, 100 days for the experiments in Table 2), the slides were developed, and the silver grains were located in a Wild M-20 phase-contrast microscope. Fluorescence was observed in a Leitz ultraviolet fluorescence microscope.

RESULTS

Segregation of DNA in Lactobacillus. It was necessary to study the segregation of DNA in *L. acidophilus* before beginning experiments with FA. This was carried out by growing cells for three generations in 40 μc per 4 μg per ml of radioactive thymine (continuous prelabel) and then transferring them to nonradioactive medium for further growth (chase). Samples of individual cells were taken for autoradiography after one to eight generations of growth in nonradioactive medium. In another experiment, a culture was pulse labeled for 10 min with 200μc per 4μg per ml of ^{3}H-thymine and was then grown in the presence of nonradioactive thymine. Samples for autoradiography were taken after every generation of growth in nonradioactive medium.

Each replicating chromosome should contain four nucleotide strands, two of which are labeled by a pulse of radioactivity (Fig. 2). Almost all of this DNA is labeled during three generations of growth in ^{3}H-thymine. When, during growth, each nucleotide strand is separated eventually into a different cell, we can expect a cell with one replicating chromosome which has been pulse-

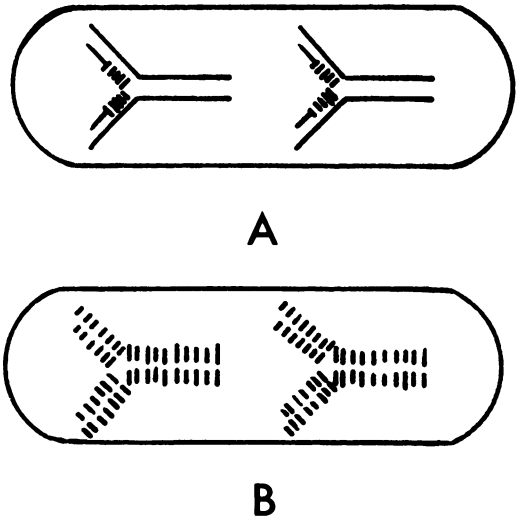

FIG. 2. *Cell containing two replicating chromosomes. (A) A pulse of radioactivity (vertical lines) labels four of the eight available conserved subunits. (B) Prolonged growth in ^{3}H-thymine labels all of the eight subunits of the two replicating chromosomes.*

labeled to yield eventually two labeled cells, whereas a cell containing one totally labeled replicating chromosome can be expected to yield between two and four labeled progeny depending upon the stage of DNA replication at which it is transferred to nonradioactive medium.

The results of the experiment on distribution of DNA are presented in Table 1 and Fig. 3. They are similar to results obtained with *Escherichia coli* (13, 20). Once nonradioactive cells appeared, the frequency of radioactive cells decreased exponentially with each generation of growth in nonradioactive medium. Moreover, the amount of radioactivity per *labeled* cell eventually became constant, indicating the presence within such cells of a conserved unit of radioactivity. An estimate of the number of such units originally present may be obtained from these data (13).

At the time at which unlabeled cells first appeared and the average amount of radioactivity per labeled cell became constant, the number of conserved units available for distribution over the population became limiting and was exceeded by the cell number. This occurred between the second and third generation for pulse-labeled cells and between the third and fourth for continuously labeled cells. Thus, the pulse-labeled cells must have contained about four conserved radioactive DNA units, and the prelabeled cells, about eight.

When the linear portion of the curves in Fig. 3 are extrapolated to where they intersect the ordinate (0 generation), the value obtained gives the percentage of conserved radioactive units originally present in each cell. [The rationale for this extrapolation has been discussed elsewhere (13).]

This estimate also indicates the presence of about four radioactive units in pulse-labeled cells and eight in continuously prelabeled cells.

These results indicate the presence of two replicating chromosomes per cell. Moreover, since a pulse of radioactivity labels all of the cells, we may conclude that DNA replication occurs during the entire division cycle.

When the number of labeled cells began to decrease, they did not decrease to 50% within a span of one generation (Fig. 3). This indicates that some chromosome fragmentation (which may be analogous to sister-chromatid exchange in higher cells) is occurring during replication. A similar situation has been observed with *E. coli* (6, 7, 13). The data do not justify an exact estimation of the extent of chromosome fragmentation in *L. acidophilus*. However, the data in Fig. 3 can be best fitted by assuming that each labled nucleotide strand produces a fragment about once every 7 to 10 generations [a value similar to that reported in detailed studies of *E. coli* (6)]. Such fragmentation is reflected in the slow decrease in the amount of radioactivity per labeled cell in the values presented in Table 1 for pulse-labeled cells after three or more generations of growth in nonradioactive medium or for prelabeled cells after four or more generations of growth. The relative constancy of the last values for pulse-labeled cells in Table 1 reflect the insensitivity of low numbers of grains per labeled cell in estimating fragmentation. Thus, when the average value of grains per cell is less than 1, the

TABLE 1. *Distribution of radioactive DNA in daughter cells during successive generations of growth in nonradioactive medium*[a]

Generations after labeling	Pulse-labeled cells			Three generations of prelabeled cells		
	No. of cells examined	Per cent without grains	Avg no. of grains per labeled cell	No. of cells examined	Per cent without grains	Avg no. of grains per labeled cell
0	>1,000	<1	—	>1,000	≪1	—
1	>1,000	<1	—	>1,000	<1	—
2	536	4.8	5.33	>1,000	<1	—
3	574	23	2.8	562	3.02	6.5
4	539	53	2.44	516	17.6	3.2
5	540	70	1.84	515	52	2.81
6	533	81.2	1.86	505	74	2.62
7	582	89.4	1.9	504	87.3	2.5
8	535	94.4	1.9	—	—	—

[a] Cells were pulse-labeled or prelabeled with ^{3}H-thymine as described in the text. They were then transferred to nonradioactive medium and cell division followed in the Coulter counter. Samples were taken after each doubling of the cell number. The culture density was maintained below 10^8 cells/ml by appropriate dilution with fresh medium. Autoradiographs were prepared from each sample and were developed after 60 days of exposure.

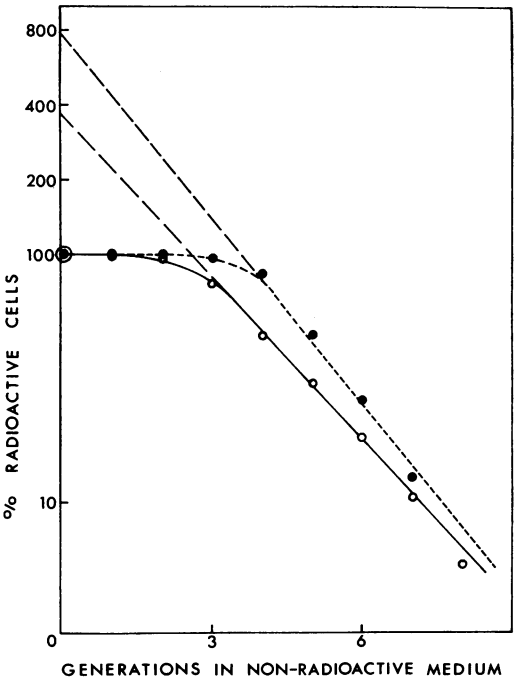

FIG. 3. *Distribution of radioactive DNA among progeny cells during growth in nonradioactive medium. The data from the experiment described in Table 1 are shown. The solid symbols on the dotted curve present data from cells prelabeled with ^{3}H-thymine for three generations. The open symbols on the solid curve present data from cells pulse-labeled with ^{3}H-thymine. The logarithm of the percentage of labeled cells is graphed as a fraction of the number of generations of growth in nonradioactive medium. An extrapolation to zero generations yields an estimate of the percentage of conserved units present. Since 100% of the population was labeled, the values of 400 or 800% indicate an average of 4 or 8 radioactive units per cell (13).*

number of grains per labeled cell will asymptotically approach 1 in accordance with a Poisson distribution.

Conserved surface units of Lactobacillus. Cultures of *L. acidophilus* were labeled with FA by incubating them for 1 hr in 2.5% KCl [this diluent does not injure *Lactobacillus* (18)] in the presence of the FA (8μg of serum protein per ml). They were then collected by centrifugation, washed, and resuspended in nutrient medium. After several hours of growth, weakly fluorescent and strongly fluorescent cells were observed. Although this indicated that some cell-wall material was being conserved during growth and division, several problems were encountered: (i) Clumps of cells caused by antibody action occurred, making it difficult to observe the distribution over daughter cells. (ii) Weakly binding antibody might dissociate from the cells to which it was attached originally and recombine. (iii) It was difficult to ascertain that all cells were viable. Thus, antibody might be conserved on dead cells and dispersed over dividing cells.

These problems were overcome by observing cells growing on solid medium. When microcolonies were allowed to develop from FA-labeled cells, it was found that during division most of the fluorescence was conserved over a few viable cells, and a small part was evenly dispersed over all of the cells of the microcolony (Fig. 4a). An advantage of the microcolony technique lay in the ability to select colonies in which all of the

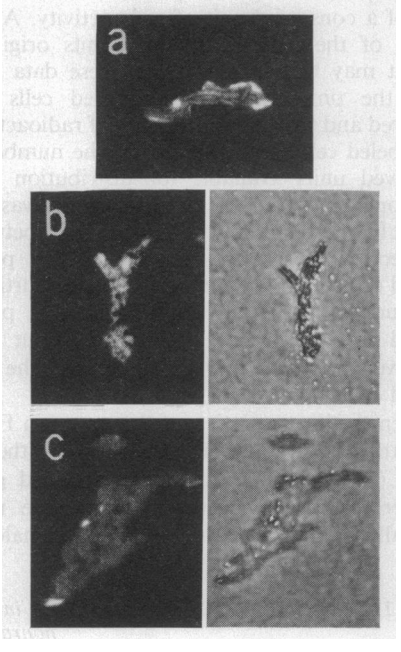

FIG. 4. *Photographs of microcolonies derived from fluorescent antibody-labeled cells. (a) A photograph in the fluorescent microscope of a microcolony derived from an FA-labeled cell (or cells) which was not overlaid with photographic film. (b) A microcolony derived from a cell labeled with ^{3}H-thymine and then immediately labeled with FA. This preparation is an autoradiograph. The fluorescence is shown in the photograph on the left, and the silver grains appear as white dots in the phase-contrast picture to the right. Note that neither of the two fluorescent spots (located in the two arms of the Y) are associated with silver grains which otherwise are distributed over the rest of the microcolony. (c) A microcolony derived from a cell labeled with ^{3}H-thymine, grown for 1.5 generations in nonradioactive medium and then labeled with FA. Of the three fluorescent spots in the left-hand picture, two are associated with silver grains (the one at the bottom and the one at the top), and the third (middle spot) is not.*

TABLE 2. *Association of conserved fluorescence with radioactive DNA*[a]

Assay	Time of ^{3}H-thymine pulse	
	Immediately before FA	1.5 generations before FA
No. of colonies examined	51	62
Avg no. of cells per colony	45	42
Total no. of fluorescent spots	139	201
Total no. of grain clusters[b]	337	237
No. of fluorescent spots with silver grains[c]	18	129
Grain clusters per colony	6.6	3.8
Fluorescent spots per colony	2.7	3.2
Fluorescent spots with grains observed (%)[c]	13	64
Fluorescent spots with grains calculated (%)[d]	16	46

[a] An R-26 culture was labeled for 20 min with 200 μc per 4 μg per ml of ^{3}H-thymine. One portion was incubated immediately for 1 hr at 39 C in 2.5% KCl containing FA (8 μg of serum protein per ml). The other was grown for 90 min in nonradioactive medium and then incubated with FA. After removal of the FA by centrifugation, the cells were dispersed and spread on agar plates overlaid with a film of collodion. After 6 hr of incubation at 39 C, the microcolonies were fixed with Formalin and transferred on the collodion to microscope slides. Autoradiographs were prepared, and after 100 days of exposure were developed. These were examined in the fluorescent microscope and in the phase-contrast microscope (see Fig. 4), and pictures were taken. These pictures supplemented by direct study in the phase-contrast microscope were used to map the location of silver grains in relation to the fluorescent spots. A summary of the data is presented.

[b] Grain clusters are areas in which three to five silver grains appear as a group over one bacterium.

[c] Association of one or more silver grains with a fluorescent area was scored as positive, i.e., a fluorescent spot with silver grains.

[d] Calculated from the age distribution of an exponential culture by considering those cells in which the 20-min pulse was incorporated at the end of a round of replication (therefore into a template strand) as well as at the beginning of the next cycle. It was assumed that chromosomes too close to the end of the cycle (one-fourth of the pulse) might not contain sufficient radioactivity to register.

$$16\% = 100\left[\frac{ln2\int_{x=1/12}^{x=1/3} 2^x\,dx}{1 + ln2\int_{x=1/12}^{x=1/3} 2^x\,dx}\right]$$

$$46\% = 100\left[\frac{1 + ln2\int_{x=1/12}^{x=1/3} 2^x\,dx}{(2 + ln2\int_{x=1/12}^{x=1/3} 2^x\,dx)(1 + ln2\int_{0}^{.5} 2^x\,dx)}\right]$$

cells were displayed as a sheet and which are of a uniform size. This tends to eliminate colonies derived from large clumps of cells. (We have not been able to avoid this clumping which occurs as a result of centrifugation of the cells when the FA is removed. Efforts to break up these clumps have reduced their frequency, but separation of the *entire* population into individual cells was not successful.) Examples of microcolonies are shown in Fig. 4a, b, and c.

Association of radioactive DNA with conserved units of the cell surface. According to the model in Fig. 1, polynucleotide strands of DNA being used as templates should be attached to the cell surface, whereas those in the process of being synthesized should not.

A culture of *L. acidophilus* was labeled for 20 min (30% of a generation period) with 200 μc per 4 μg per ml of ^{3}H-thymine. It was then grown for 90 min (1.5 generation periods) in nonradioactive medium and was suspended in 2.5% KCl containing FA. After 1 hr, the cells were centrifuged out of the antibody solution and were spread on collodion-covered solid media to make microcolonies. Another sample was labeled with ^{3}H-thymine for 20 min and was immediately placed in FA. Microcolonies were also made from these cells. The microcolonies were dipped in film and allowed to expose. After development, fluorescent centers were located and pictures were taken. The same colonies were also photographed under phase contrast to locate silver grains. Examples of such photographs are shown in Fig. 4. The photographic film quenched the fluorescence slightly, but not sufficiently to pose a serious problem. When the pictures were compared, each fluorescent spot was carefully checked for the presence of radioactivity. The data are presented in Table 2. Only those colonies were scored in which the bacteria were spread in a sheet and which had well-defined areas of fluorescence.

It is clear that fluorescence is associated with radioactivity most frequently in cells in which the DNA was labeled more than a generation before the addition of FA.

As was to be expected from the data in Table 1, the amount of radioactivity per microcolony was much less in cells labeled 1.5 generations prior to antibody labeling than in those labeled just before. Nevertheless, this radioactivity was concentrated in fluorescent cells in the former but not in the latter.

The term $ln2\int_0^{.5} 2^x dx$ represents additional nonradioactive nucleotide strands which may be expected to act as templates and which were synthesized between the end of the radioactive pulse and the time of FA treatment.

DISCUSSION

The experiment in Table 2 demonstrates that DNA forms a permanent association with a part of the cell envelope which is synthesized during the generation after synthesis of the DNA itself. This is consistent with the model in Fig. 1 which assumes that such an association does not occur until the polynucleotide strand is used as a template in replication (which would occur in the generation after its synthesis).

From the data in Table 2, it is possible to estimate the number of conserved units of cell surfaces as between two and four per FA-labeled cell. As was to be expected, the number of such units was the same in pulse-labeled and pulse-prelabeled cells, since in both experiments FA labeling took place immediately before cells were allowed to form microcolonies. These results are similar to those obtained by others for gram-positive organisms (1–3).

On the other hand, there were about half as many grain clusters in pulse-prelabeled cells as in pulse-labeled cells. This is a result of the cell division which occurred between incorporation of the radioactive label and the formation of microcolonies. The absolute number of grain clusters observed is higher than would be predicted by assuming two replicating chromosomes per cell (Fig. 2). However, this could result from fragmentation of the radioactive DNA during replication, a phenomenon observed previously in *E. coli* (6, 7, 13) and in higher organisms (15, 16, 19). Thus, the data in Table 1 and Fig. 3 indicate that a labeled nucleotide may fragment once every 7 to 10 generations. The average number of cells per colony (ca. 45) indicates between five and six generations of growth. Thus, we would expect that fragmentation would have produced an increase of about 80 to 90% in the expected number of grain clusters.

Our data do not establish a direct attachment of DNA to the cell wall (nor do we wish to suggest this), since attachment to the membrane or other internal structure and, in turn, attachment of this to the cell wall would yield the results which we have observed. Studies of the type undertaken by Jacob, Ryter, and Cuzin (9) in which tellurium is used to label the cell membrane indicate that it is also conserved during cell growth.

Our studies have established a union between template DNA and some structure of the cell surface. The nature of this structure must await its isolation with the DNA template still attached.

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LITERATURE CITED

1. CHUNG, K. L., R. Z. HAWIRKO, AND P. K. ISAAC. 1964. Cell wall replication. I. Cell wall growth of *Bacillus cereus* and *Bacillus megaterium*. Can. J. Microbiol. **10**:43–48.
2. COLE, R. M. 1965. Symposium on the fine structure and replication of bacteria and their parts. III. Bacterial cell-wall replication followed by immunofluorescence. Bacteriol. Rev. **29**:326–344.
3. COLE, R. M., AND J. J. HAHN. 1962. Cell wall replication in *Streptococcus* pyogenes. Science **135**:722–723.
4. COONS, A. H. 1958. Fluorescent antibody methods, p. 400–422. *In* J. F. Danielli [ed.], General cytochemical methods, vol. 1. Academic Press, Inc., New York.
5. EBERLE, H., AND K. G. LARK. 1966. Chromosome segregation in *Bacillus subtilis*. J. Mol. Biol. **22**:183–186.
6. FORRO, F., JR. 1965. Autoradiographic studies of bacterial chromosome replication in amino acid deficient *Escherichia coli* 15T⁻ Biophys. J. **5**:629–649.
7. FORRO, F., JR., AND S. A. WERTHEIMER. 1960. The organization and replication of deoxyribonucleic acid in thymine deficient strains of *E. coli*. Biochim. Biophys. Acta **40**:9–27.
8. JACOB, F., S. BRENNER, AND F. CUZIN. 1963. On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. **28**:329–348.
9. JACOB, F., A. RYTER, AND F. CUZIN. 1966. On the association between DNA and membrane in bacteria. Proc. Royal Soc. (London) Ser. B **164**:267–278.
10. KEKWICK, R. A. 1939. The electrophoretic analysis of normal human serum. Biochem. J. **33**: 1123–1129.
11. KEKWICK, R. A. 1940. The serum proteins in multiple myelomatosis. Biochem. J. **34**:1248–1256.
12. LARK, K. G. 1966. Regulation of chromosome replication and segregation in bacteria. Bacteriol. Rev. **30**:3–32.

12*a*. LARK, K. G. 1967. Non-random segregation of sister chromatids in *Vicia faba* and *Triticum boeoticum*. Proc. Natl. Acad. Sci. U.S. *in press*.

13. LARK, K. G., AND R. BIRD. 1965. Segregation of

the conserved units of DNA in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S. **54**:1444–1450.

14. LARK, K. G., R. A. CONSIGLI, AND H. C. MINOCHA. 1966. Segregation of sister chromatids in mammalian cells. Science **154**:1202–1205.
15. MARIN, G., AND D. M. PRESCOTT. 1964. The frequency of sister-chromatid exchanges following exposure to varying doses of ^{3}H thymine or X-rays. J. Cell Biol. **21**:159–167.
16. PEACOCK, W. J. 1963. Chromosome duplication and structure as determined by autoradiography. Genetics **49**:793–801.
17. SOŠKA, J. 1966. Growth of *Lactobacillus acidophilus* in the absence of folic acid. J. Bacteriol. **91**:1840–1847.
18. SOŠKA, J., AND K. G. LARK. 1966. Regulation of nucleic acid synthesis in *Lactobacillus acidophilus* R-26. Biochim. Biophys. Acta **119**:526–539.
19. TAYLOR, J. H. 1960. Asynchronous duplication of chromosomes in cultured cells of Chinese hamster. J. Biophys. Biochem. Cytol. **7**:445–464.
20. VAN TUBERGEN, R. P., AND R. B. SETLOW. 1961. Quantitative radioautographic studies on exponential growing cultures of *Escherichia coli*. Biophys. J. **6**:589–625.