

Chromosomes in *Bacillus subtilis* Spores and Their Segregation During Germination

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Spores of a thymine-requiring mutant of *Bacillus subtilis* 168 (leucine⁻, indole⁻, thymine⁻) were uniformly labeled with ³H-thymidine. These were seeded on thin-layer agar plates where they germinated into long-chained microcolonies. Autoradiograms were used to measure the distribution of labeled deoxyribonucleic acid in the chains of cells, which ranged in length from 2 to 32 cells. Four major grain clusters appeared in most chains. These clusters were homogeneous in size; their grain numbers were distributed symmetrically from 9 to 15 with an average of 12.0. When three or fewer major clusters appeared in short chains, some of them were composed of two subclusters. However, there were always four clusters per chain when these subclusters were counted as individuals. Groupings containing two to eight grains appeared, as well as the four major clusters in longer chains. These minor groups were fragments of the major clusters. In contrast to the symmetrical distribution of major clusters, fragmented clusters were distributed at random, indicating random fragmentation. The total number of major and minor clusters increased at a constant exponential rate when measured against total cell number per chain, i.e., number of generations. It was calculated from the rate that a detectable fragmentation, at least 16% of a conserved unit (defined as a single strand of the complete chromosome), occurred every 6.0 generations. These results led us to conclude that each *B. subtilis* spore contained four conserved units or two completed chromosomes. Segregation of the four units into progeny cells was almost random. The one notable exception was a conserved unit which frequently appeared in a terminal cell to which an empty spore coat was attached. The presence of two chromosomes in the spore is consistent with our proposed structure of the completed chromosome, in which two sister chromosomes are covalently linked at the initiation region. This double chromosome may be incorporated into the spore without further structural change.

Various gram-positive bacteria produce spores as their dormant form. It has been assumed that a spore contains one set of genetic information, i.e., one complete chromosome. This proposal was substantiated in *Bacillus subtilis* by measuring frequencies of various genetic markers through transformation (9, 10, 17). Furthermore, elaborate electron microscopic examination of the sporulation process in *B. subtilis* clearly indicated that one of the nuclei in the sporulating cell was incorporated into each matured spore (11).

Recently, Yoshikawa reported a new model for the structure of replicating chromosomes of *B. subtilis*. According to this model (Fig. 9), at the end of each replication cycle the two daughter chromosomes remain covalently bonded (16). It is of interest to determine whether or not such

chromosomes would be incorporated into spores without further structural change. This was attempted by measurement of the numbers of chromosomes or conserved units per spore. (The manner of chromosomal replication in *B. subtilis* is semiconservative. Each strand of the parental chromosome becomes a conserved unit in the two daughter chromosomes.)

Chemical measurement showed that the deoxyribonucleic acid (DNA) content of each *B. subtilis* spore was 5.1×10^{-9} μg (2, 5). This agrees fairly well with the amount of DNA obtained for one nucleus in a growing cell after correcting for replicating regions (4). From these data, the molecular weight of *B. subtilis* DNA was calculated as 3.0×10^9 to 4.0×10^9 daltons, assuming that the spore and nucleus each contained only one chromosome. On the other hand,

measurement of chromosome length by electron microscopy (A. T. Ganesan, Ph.D. Thesis, Stanford Univ., Stanford, Calif., 1963) and autoradiography (2) gave 1.3×10^9 to 1.8×10^9 daltons as the molecular weight of one chromosome. In this report, these discrepancies are clarified by employing methods entirely different from those described in previous reports.

The numbers of conserved units in *B. subtilis* spores were measured by uniformly labeling with ^3H -thymidine (TdR) and then following segregation of the label in progeny cells during germination. Autoradiographs of microcolonies clearly showed four conserved units in one spore. However, Ryter and Jacob recently observed two conserved units per spore by use of autoradiographs of *B. subtilis* spores germinated in a liquid medium (13). The four conserved units in our experiments were fairly stable during cell divisions. Fragmentation frequency was low but constant. It was calculated that one detectable fragmentation (at least 16% of a conserved unit) of each conserved unit occurred every 6.0 generations.

MATERIALS AND METHODS

Strains. A thymine-requiring mutant of *B. subtilis* 168, leucine⁻, indole⁻, thymine⁻ (MY2Y1U2), was used throughout this investigation (15).

Preparation of ^3H -TdR-labeled spores. The thymine-requiring mutant was grown overnight in medium C (15), enriched with L-leucine (50 $\mu\text{g}/\text{ml}$), L-tryptophan (50 $\mu\text{g}/\text{ml}$), TdR (2 $\mu\text{g}/\text{ml}$), and 50 $\mu\text{C}/\text{ml}$ of methyl- ^3H -TdR (6.7 c/mmole, New England Nuclear Corp., Boston, Mass.), and in 0.05% potato extract (Difco). The cells were harvested, washed with medium C, and concentrated 20 times. The concentrated cells were plated on 2% agar plates of the same composition. Spores were harvested and purified after 4 days at 37 C, as reported previously (14). These purified spores had a specific activity of 6.7×10^{-9} $\mu\text{C}/\text{spore}$.

Preparation of microcolony from the labeled spores. A thin layer was applied to a glass slide by dipping it into melted agar medium [Antibiotic Medium No. 3 (Difco) enriched with TdR (20 $\mu\text{g}/\text{ml}$), L-leucine (50 $\mu\text{g}/\text{ml}$), L-tryptophan (50 $\mu\text{g}/\text{ml}$), and Difco agar (1.5%)]. When the agar was set, the plates were stored in a high-humidity chamber at 30 C. Microdrops of a suspension of the ^3H -TdR-labeled spores, containing 2×10^6 spores/ml, were seeded on these plates, and the plates were then incubated in the chamber at 30 C. The spores germinated into chains of 2 to 30 cells within 4 to 5 hr. Empty spore coats were usually found attached to one of the terminal cells of the chains. The growth rate during germination was dependent on the amount of water in the agar. Growth was stopped by heating plates at 80 C until the agar dried completely. Precipitated salts and other extraneous matter were dissolved by soaking the slides in 5% chromic acid for 2 min. They

were then gently rinsed with water, air-dried, and stored in a desiccator at 4 C.

Autoradiography. The slides were covered with Kodak autoradiographic stripping film AR-10 at 15 to 18 C, dried, and kept in a dark box with silica gel at 4 C. A period of 1 to 2 weeks was allowed for exposure. The films were developed with Kodak D-19 developer for 20 min at 15 C, and the numbers of cells and grains were counted with a Nikon microscope model S.

RESULTS

Microscopic observations. Typical examples of microcolonies observed under the phase-contrast microscope are shown in Fig. 1-6. Cells germinated on agar plates perpendicularly to the spore's long axis, and each spore grew into a long multicelled chain. The empty spore coats were often attached to one end of these chains. Segregation of spore DNA into progeny cells during germination was easily followed because of this chain formation. For this purpose, spores were uniformly labeled with ^3H -TdR, and autoradiographs were made of microcolonies which grew from the labeled spores (Fig. 1-6). Generally, four grain clusters, similar in size, were observed in every chain. These four clusters were clearly seen in the two-cell chains (Fig. 1B) and consistently appeared in chains of 20 or more cells (Fig. 4-6). Short chains of two to four cells sometimes contained two or three clusters per chain (Fig. 1A, 1C, 1D, 2B, 2D, and 3A). When there were clusters of two, both clusters contained approximately twice as many grains each as were present in the individual clusters in the four-clustered chains. In clusters of three, one of the clusters contained twice as many grains as were present in each of the other two. These large clusters were grouped into two subclusters which were well separated and easily recognizable (Fig. 1A, 2D, and 3A). It is believed that these two subclusters represent two labeled chromosomes in one cell since it is known that cells grown in this medium have two nuclei per cell (4). Small two- to four-grain clusters were found in larger chains along with the four major clusters (Fig. 3B, 3C, 3D, and 4B). It was assumed that these were fragments of labeled DNA, probably resulting from crossing-over between two sister chromosomes.

Numbers of grain clusters in microcolonies of various lengths. Microscopic observations led to the general impression that each spore had four conserved units which segregated during germination without extensive fragmentation. The number of grain clusters per chain was counted in many chains of various sizes to reach quantitative conclusions. To measure fragmentation fre-

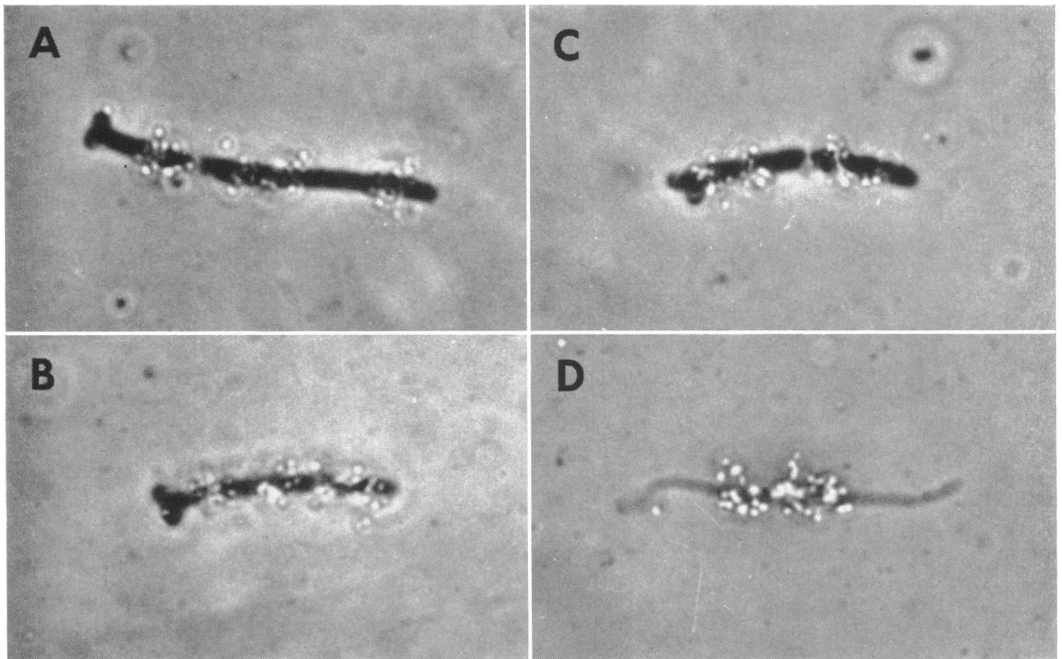


FIG. 1. Autoradiogram of chain of two to three cells arising from the germination of ^3H -labeled spores (see *Materials and Methods* for preparation of spores and autoradiogram). Photographed with phase-contrast microscope. $\times 1,000$. Focus is on cell plane. (A) Three clusters appeared; the cluster in the center consisted of two subclusters. (B) Four separate clusters are seen in two cells. (C) Three clusters appeared. (D) Two large clusters are seen; two subclusters are beginning to appear in one of them.

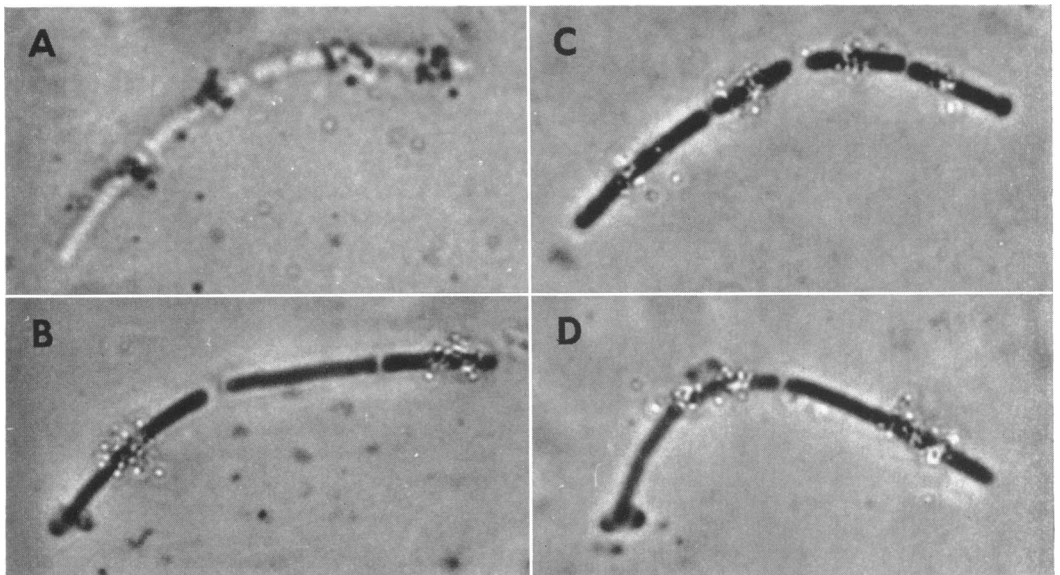


FIG. 2. Autoradiograms of four-cell chains arising from ^3H -TdR-labeled spores. Photographed as Fig. 1. (A) Four clusters, each in an individual cell. Focused on grain plane. (B) Two large clusters. Two subunits are clearly seen in one of them. (C) Similar to A, except focused on cell plane. (D) Two clusters with two subclusters.

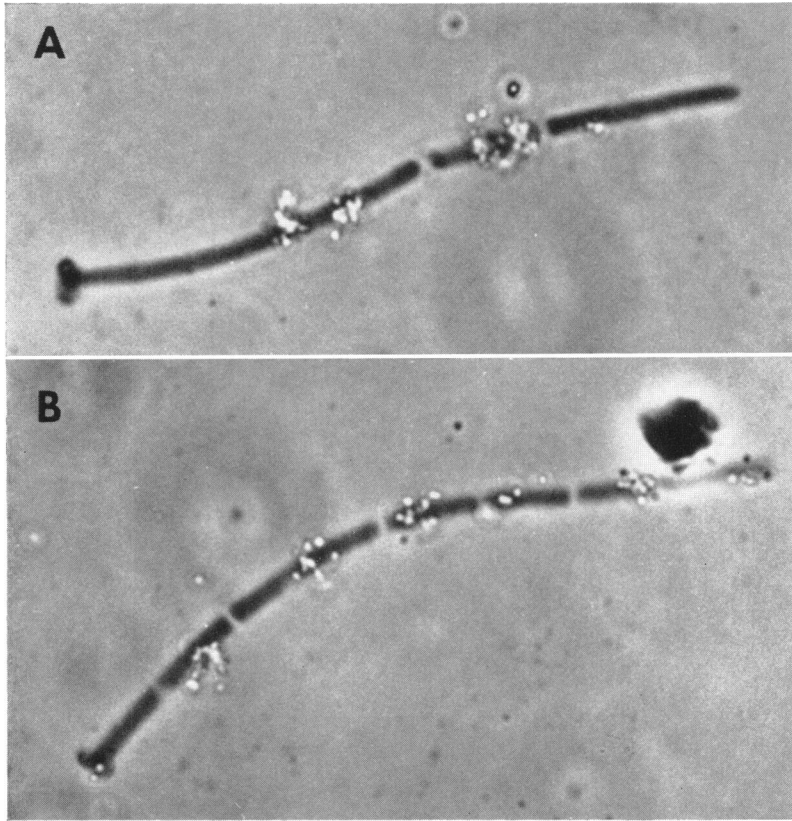


FIG. 3A and B. Autoradiogram of four- to six-cell chains arising from $^3\text{H-TdR}$ -labeled spores. Photographed as Fig. 1. (A) Four subclusters are clearly shown, two in the same cell, indicating that the cell has two nuclei. (B) Four clusters distributed one per cell. One of the cells has a three-grain fragment.

quency, any groupings of more than two grains were scored as individual clusters. Two grain clusters correspond to approximately 16% of a conserved unit, as discussed below. A single grain is difficult to distinguish from the background, so fragments of less than 16% of a conserved unit were ignored (*see* Table 1). Chains of various sizes were divided into four groups, each of which represented an intermediate state between generations. Such groupings are statistically more meaningful, because total cell number per chain does not necessarily indicate the precise germination stage of the chain; for instance, a four-cell chain could be either at the beginning or almost at the end of its second generation. Furthermore, as shown in Fig. 1–6, each individual cell in a chain is at a different generation stage.

A graphic representation of Table 1 is shown in Fig. 7, which is based on data in Table 1 analyzed in two different ways, depending on which of the two possible numbers of spore chromosomes was

assumed. If each spore contained but one chromosome, at zero generation there would be two molecular hybrid chromosomes in one cell, each with one labeled strand and one nonlabeled strand (Fig. 7, top). In the top portion of Fig. 7, chains containing three to four cells represent 1.5 generations, and those containing five to eight cells represent 2.5 generations. In these cases, the clusters of double-grain count would be conserved units and the four major clusters would be derived fragments. If, on the other hand, each spore had two chromosomes, four molecular hybrid chromosomes (two per cell) would be present at zero generation (Fig. 7, bottom). In the bottom portion of Fig. 7, chains containing three to four cells represent only 0.5 generation and those containing five to eight cells represent 1.5 generations. When two or three major clusters are observed in shorter chains, closer examination nearly always reveals a total of four subclusters (Fig. 1A, 2D, and 3A). The average number of

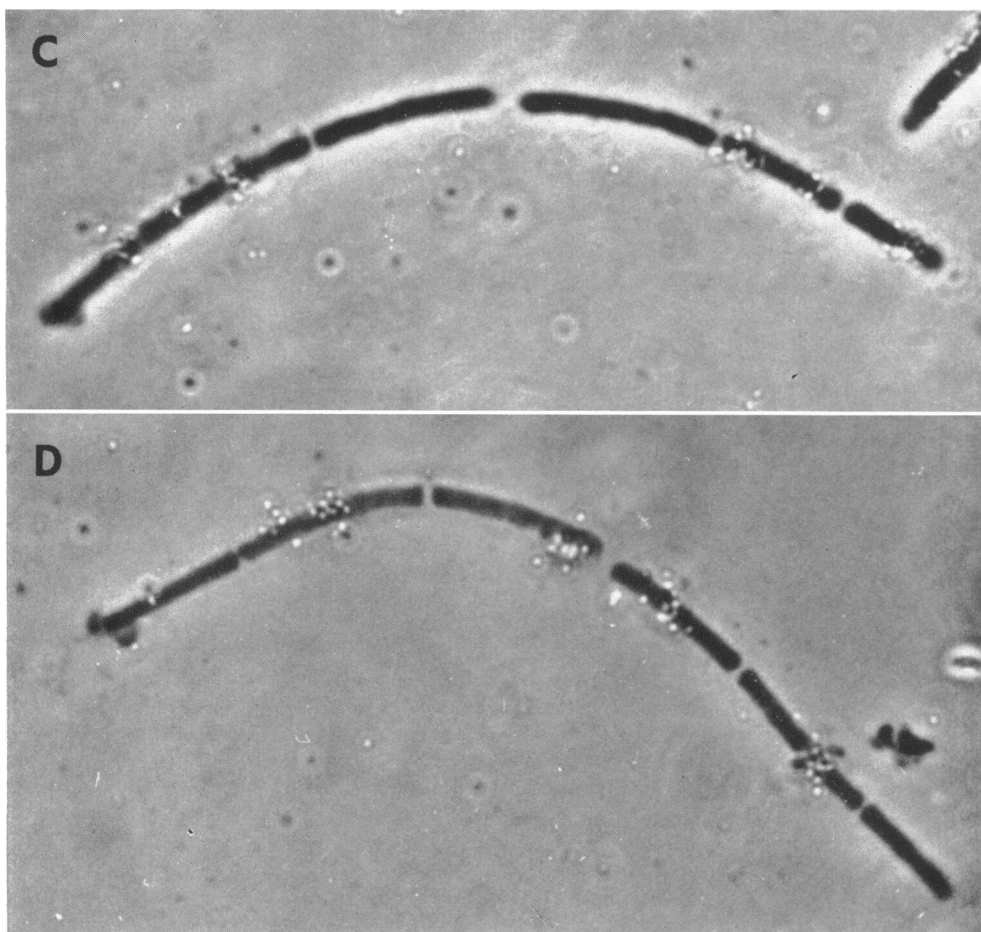


FIG. 3C and D. Autoradiogram of four- to six-cell chains arising from ^3H -TdR-labeled spores. (C) Two fragments together with four major clusters. (D) One large fragment is seen together with four major clusters.

clusters per chain was corrected, taking this into account (Table 1, last column) and was used in Fig. 7. (bottom).

If the analysis of the data is based on the assumption that each spore contained but a single chromosome, it would have to be concluded that fragmentation of the radioactive chromosomes occurred twice as fast in the early divisions after germination as in the later ones (Fig. 7, top). Moreover, the improbable assumption is required that the fragments formed during these first generations were regularly of very similar size, while later fragmentation gave rise to pieces of unequal size (Fig. 8). On the other hand, grain clusters per chain increased at a constant exponential rate for more than four generations, if it is assumed each spore contains two chromosomes (Fig. 7, bottom). This led us to conclude that one

spore contained four conserved units, or two complete chromosomes. It is possible to calculate the frequency of detectable fragmentation (at least 16% of a conserved unit) as one fragmentation per conserved unit per 6.0 generations (Fig. 7, bottom). The frequency would be two to three times greater than the present value if all fragments smaller than 16% were included in the calculation.

Segregation of the conserved units. Ninety-eight chains containing various numbers of cells were randomly chosen, and the numbers of grains in each cluster were counted (*see* Fig. 8). Four major clusters were found in most chains, as stated previously. The numbers of grains per cluster varied from 9 to 15 with an average of 12.0, and the distribution of these clusters was quite symmetrical (Fig. 8), indicating homogeneity of size. On

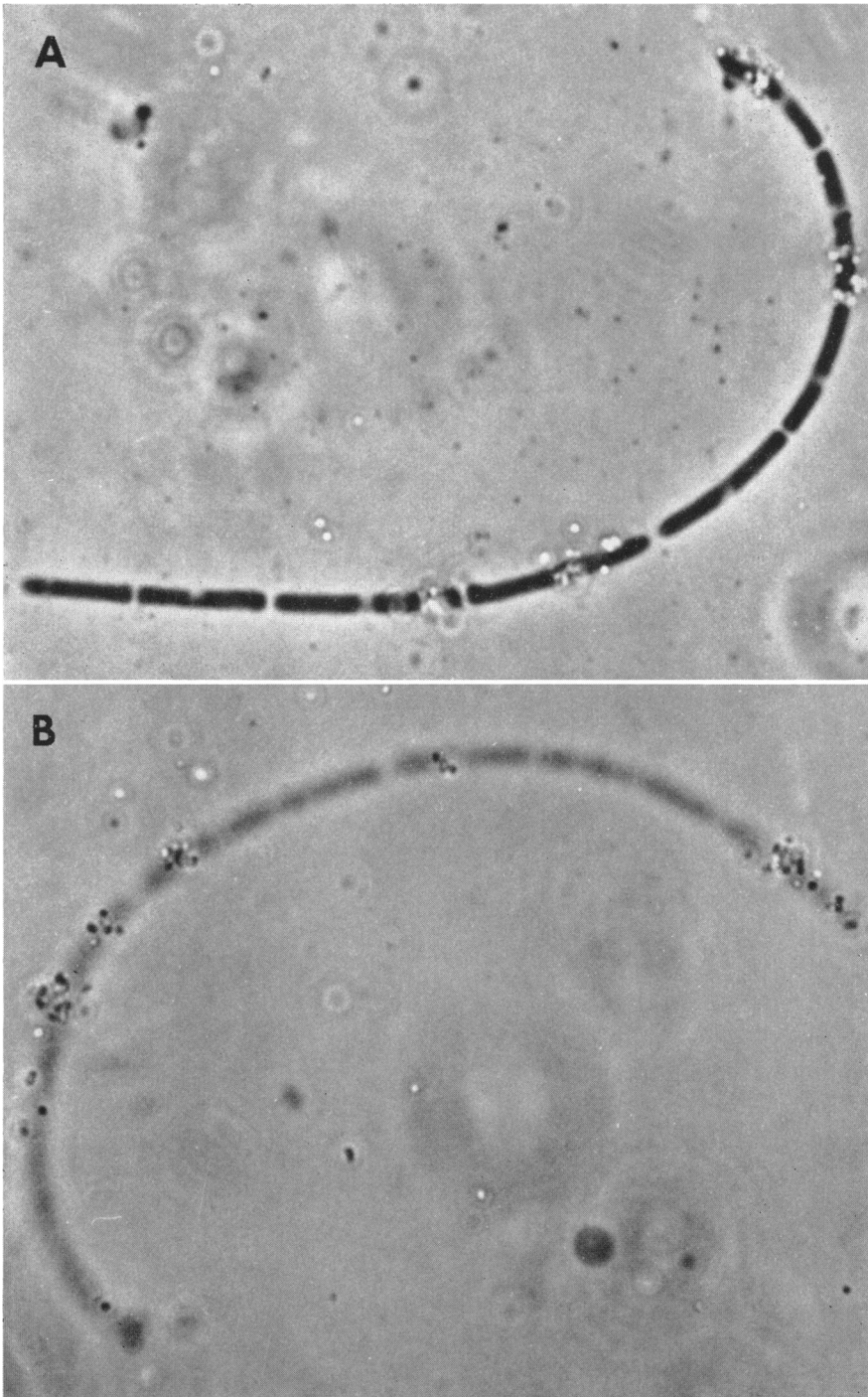


FIG. 4. Autoradiograms of 11- to 12-cell chain arising from $^3\text{H-TdR}$ -labeled spores. Photographed as Fig. 1. (A) Focused on cell. Only four major clusters are shown. (B) This is a rare example of extensive fragmentation. Two major clusters are still visible. Focused on grains.

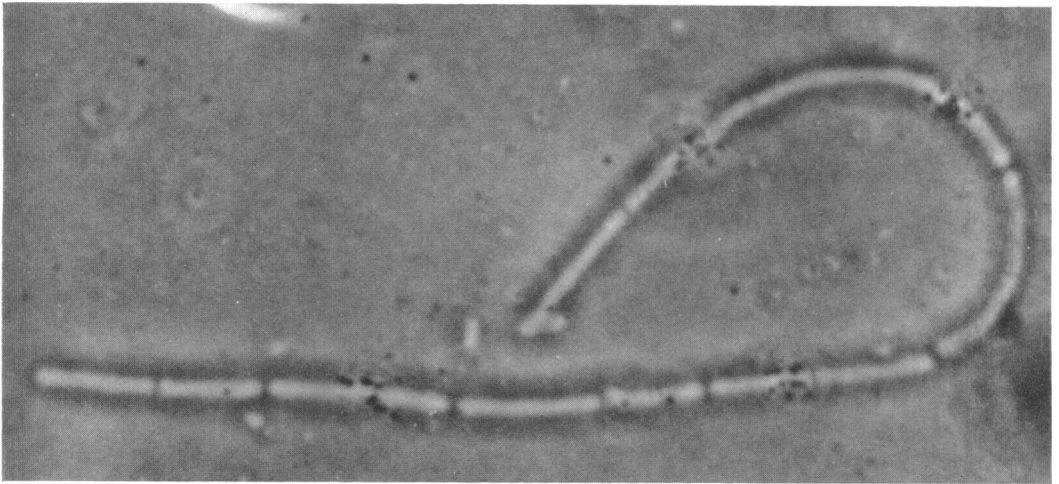


FIG. 5. Autoradiogram of 14-cell chain arising from $^3\text{H-TdR}$ -labeled spore. Photographed as Fig. 1 with focus on the grains. Only four clusters appear clearly.

the other hand, clusters containing eight or fewer grains were distributed at random, indicating random fragmentation. These data strongly support the conclusion that four major clusters were derived from the four original conserved units in the spore.

The four conserved units segregated almost randomly into progeny cells except when they segregated into terminal cells to which the empty spore coats adhered. These cells were labeled more frequently than would be expected from random distribution (see Table 2). This indicated that, in the spore, one conserved unit, or one strand, was more tightly bound to the cell structure than were the other three.

DISCUSSION

From the data presented in this paper, it was concluded that each *Bacillus subtilis* spore contains four conserved units of DNA, each representing a complete single strand of a chromosome. The principal alternative interpretation of the data would hold that the four segregating radioactive units resulted from fragmentation of two units due to crossing over between sister chromosomes. Such fragmentations have been reported (6-8). Fragmentation did indeed occur during germination, and its frequency was calculated. However, if there were two chromosomes per spore, they would have had to fragment twice as frequently during the first three generations as later. Furthermore, according to the symmetrical distribution of the four major clusters, it would have to be assumed that the two conserved units

would break into two equal-sized pieces (Fig. 8). Such regular fragmentation is highly unlikely if it is caused by random crossing over between sister chromosomes. In fact, fragmentations which occurred later were random, and the calculated frequency was one fragmentation per conserved unit per 6.0 generations. Forro has reported a random fragmentation frequency of 0.5 to 0.7 events per chromosome per generation in *E. coli*. If those fragments smaller than 16% of a conserved unit had been included in the calculations, our results would be in agreement with his. Our findings of two chromosomes per spore disagree with Ryter's and Jacob's recent conclusion of one chromosome per spore (12, 13). The reasons for this difference are not known at present.

As we previously reported, during the initial germination period, the spore grew out into a chain of two cells, after the first round of chromosomal replication ended (14). Therefore, because each cell has two nuclei in the medium used (4), four complete chromosomes were synthesized during the first round of replication. The existence of four conserved units in each spore is consistent with these previous findings.

Electron microscopic examination showed one nucleus in each *B. subtilis* spore (11). Therefore, the four conserved units within each spore must be in close contact. Our model of a completed chromosome is one in which two daughter chromosomes are covalently linked at the initiation region (see Fig. 9). If such a double chromosome were present in a sporulating cell, it could be incorporated in the spores as such.

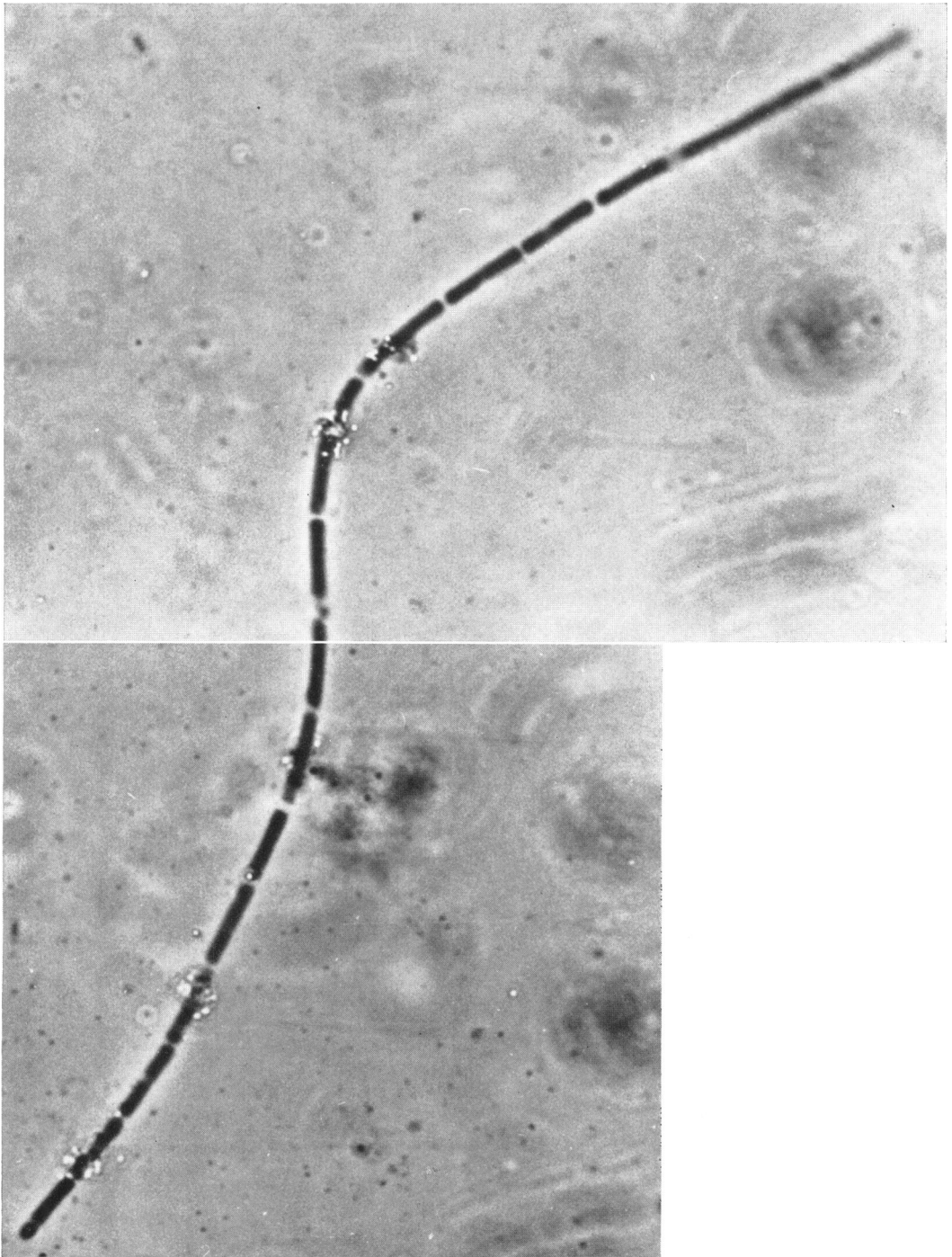


FIG. 6. Autoradiogram of 18-cell chain arising from ^3H -TdR-labeled spore. Photographed as Fig. 1 with focus on cells. Four major clusters and one small fragment are seen.

TABLE 1. Distribution of number of grain clusters per chain arising from $^3\text{H-TdR}$ -labeled spores

Groups	No. of cells per chain	No. of grain clusters per chain	No. of chains found	Avg no. of grain clusters per chain ^a	Corrected avg of grain clusters per chain
I	3-4	2	8	3.6	4.0
		3	14		
		4	44		
		5	2		
II	5-8	3	10	4.5	4.6
		4	34		
		5	30		
		6	12		
III	9-16	4	24	5.0	5.0
		5	56		
		6	24		
		7	2		
IV	17-32	4	6	5.7	5.7
		5	44		
		6	22		
		7	2		

^a An average number of clusters per chain was calculated for the chains in each group.

The chemically determined molecular weight of the *B. subtilis* chromosome should be re-examined. The value of 3×10^9 to 4×10^9 daltons calculated from growing cultures (4) is too large if each spore has two chromosomes. The molecular weight calculated from chromosome length (2) is consistent with the assumption that there are two chromosomes per spore. A discrepancy between these values based on growing cells and those based on spore data might arise from uncertainty as to the exact state of the replicating chromosome in growing cultures. For instance, a new round of DNA replication has been assumed to begin just after each cell division. However, midgeneration doubling of the rate of DNA synthesis in synchronized *E. coli* cultures was reported by Clark and Maaløe (1). If a new round of replication starts at r_0 generation within one generation ($0 \leq r_0 \leq 1$), the amount of DNA of exponentially growing singly nucleated cells would be $2^{1-r_0}/\ln 2$. To arrive at the true molecular weight, the amount of DNA per cell obtained from exponentially growing cells should be divided by $2^{1-r_0}/\ln 2$ rather than by $1/\ln 2$. If $r_0 = 0.5$, the true molecular weight would be 0.7 times the reported value (4).

Escherichia coli and *B. subtilis* have both been

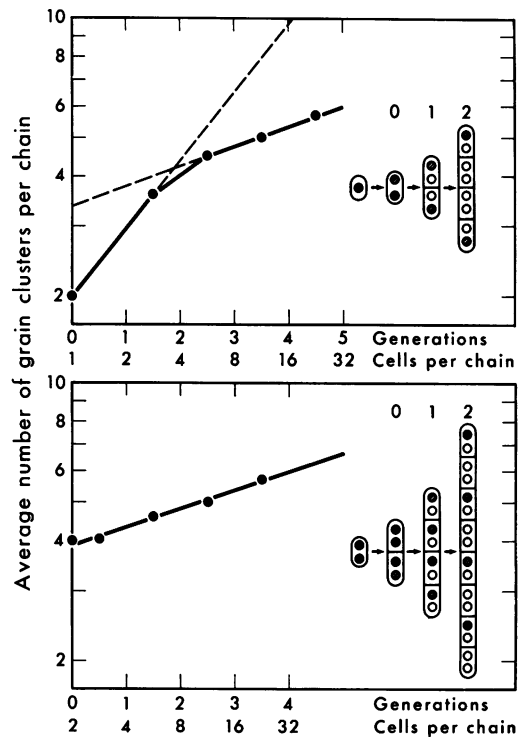


FIG. 7. Change in number of clusters per chain during growth of chains from spores labeled with $^3\text{H-TdR}$. Data from Table 1 are plotted: (top) assuming one chromosome per spore, (bottom) assuming two chromosomes per spore. Cells with hybrid chromosomes were designated as individual cells at zero generation because fragmentation was detectable only in the following generation. Two models of segregation are shown, assuming that the spore contains one (top) or two chromosome (bottom). The fully labeled chromosome is shown as black circle. A chromosome with hybrid DNA is shown as a shaded circle. An unlabeled chromosome is shown as an open circle.

reported as having two nuclei in a single cell which segregate into progeny cells in an orderly fashion (3, 8). According to Lark, the older strand in each chromosome is permanently attached to the cellular structure, possibly the cell membrane. We tested this hypothesis by analyzing the distribution of the four conserved units in four-cell chains during germination. If Lark's hypothesis were true, each of the four cells would contain one of the original conserved units. However, 32% of the chains which we studied by radioautography had one or more cells without grain clusters (see Table 1). This indicates that two nonlabeled chromosomes synthesized from two hybrid chromosomes segregated together into the same cell. This is inconsistent with Lark's proposed segregation model

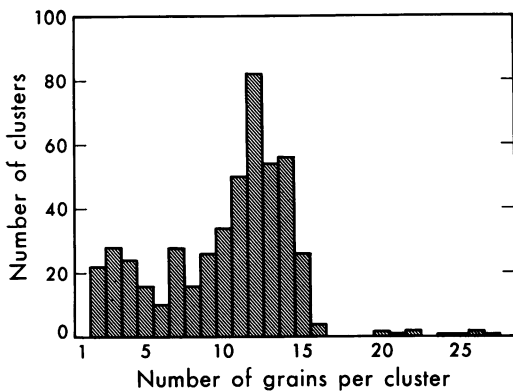


FIG. 8. Distribution of grain numbers in major clusters and fragments. Ninety-eight chains containing varying numbers of cells were randomly chosen for counting the numbers of grains in each individual cluster. Number of cells per chain varied from 4 to 31 with an average of 12.3. The average number of grain clusters per chain was 5.0.

TABLE 2. Segregation of grain clusters in chains arising from ³H-TdR-labeled spores

Source of data	Fraction of chains with clusters in			
	Two ends	One end		Zero ends
		Spore end	Distal end	
Observed ^a	0.07	0.33	0.13	0.47
Calculated (random) ^b	0.07	0.19	0.19	0.55
From Eberle and Lark (3) ^c	0.26	0.46		0.28

^a The total number of chains counted was 255. The average number of clusters per chain was 5.4, and the average number of cells in the chains was 20.5.

^b Probability of any individual cell containing a grain cluster is $5.4/21 = 0.26$. Random frequencies were calculated as the terms obtained in the expansion of $(0.26 + 0.74)^2$.

^c These figures represent the observed distributions of radioactive DNA in chromosomes of *B. subtilis* arising from cells containing two fully labeled replicating chromosomes. The value of 0.46 represents the fraction of chains with clusters in one end. In this case, two ends were indistinguishable.

(8). The following alternatives should be considered to explain our finding. First, the attachment of the chromosome to the cell structure may be temporary, or the attachment of one of the two chromosomes by one of its strands may be random with respect to whether the attachment

takes place by the old or the new strand. Second, the relative location of the two chromosomes could be such that they can segregate into the same cell. Ryter and Jacob have reported such random segregations (14).

As shown in Table 2, the four conserved units segregated almost randomly except for the one that frequently appeared in a terminal cell to which an empty spore coat was attached. This showed that one of the four conserved units was permanently attached to the original cell structure in the spore. Therefore, we must assume a random but permanent attachment of one of the four strands at the completion of each replication cycle. It is possible to construct a segregation model which fits these findings. However, at present, too many assumptions are necessary to make a reliable segregation model.

Autoradiography was used to study DNA replication in germinating spores of *B. subtilis*. Two complete chromosomes, corresponding to four conserved DNA units, were present in each spore. Upon germination, these units segregated. Fragmentation occurred, and the rate of fragmentation was constant. Three of the units segregated at random into progeny cells, but the fourth unit tended to remain attached to the cell structure of the spore.

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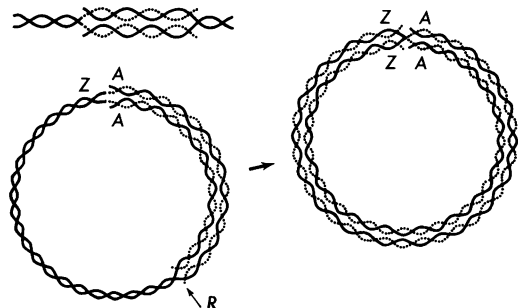


FIG. 9. A model of the replicating and completed chromosome. Both replicating (left) and completed chromosomes (right) are circular. Newly synthesized DNA strands (dotted lines) are joined covalently to the terminus of the parental strands (solid lines). The linear diagram shows part of the replicating chromosome at the initiation region. A and Z designate genes at the start and finish. The model was described in detail previously (16).

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