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Polarized Relationship of Bacterial Spore Loci to the "Old" and "New" Ends of Sporangia

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The frequency of association of spore loci with the "old" and "new" ends of rod-shaped sporangia in batch cultures of *Bacillus megaterium* ATCC 19213 was estimated by phase contrast microscopy. The analysis was facilitated by (i) the association of most of the sporangia into chains of two to five sporangia and, (ii) the occurrence of two types of cross wall distinguishable by their degree of splitting. It was concluded that a newly formed spore is located at the "old" end of a sporangium. By inference, the sporulation division septum locus is distal to the ultimate normal cell division septum, i.e., proximal to the "old" pole of the *B. megaterium* sporangium. This result is discussed in relation to deoxyribonucleic acid segregation during sporulation.

End or polar walls of rod-shaped bacterial vegetative or sporangial cells are derived from the cross wall portions of cell division septa. One of the two poles is derived from the last cell division cross wall, whereas the opposite pole is from a previously formed cell division cross wall. In terms of time of origin, these ends may be referred to as the "new" and "old" ends of the rod, respectively, since they appear during different cell generations. However, in terms of biosynthesis, ends may be of similar ages when end walls are subject to turnover as reported for *Bacillus subtilis* (2). Similarly, this temporal definition of "old" and "new" ends need not imply any relationship with the mode of wall extension.

Electron microscopy studies of spore morphogenesis have shown that the stage II septation and subsequent developmental stages are asymmetrically localized at one pole of the sporangial cell (13, 14). This striking observation raises the question of whether a spore develops at either the old or the new end of the sporangium, or whether it can be randomly located at either end. In 1955, Streshinskii (12; paraphrased in reference 10) reported that in several *Bacillus* species the sporangial loci of spores have a polarized relationship to the cell division septum partitioning sister sporangia. The polarization is such that each spore is localized at the old cell pole.

This orderly topological relationship implies that the loci of sporulation septa and cell division septa may be determined by the same mechanism. If so, the relationship supports the hypothesis that the earliest stages of sporulation represent a modified cell division (7). In view of this theoretical importance, the pattern of spore loci in the strain of *Bacillus megaterium* used in previous studies of the hypothesis (5, 6) was qualitatively scanned. However, some apparently anomalous spore loci patterns were observed that necessitated a quantitative study before they could be reconciled with a polarized spore locus pattern. My data are the first numerical data in support of a polarized spore locus pattern in *B. megaterium* since previous reports about *B. megaterium* (3, 9, 12) were pictorial. This investigation emphasizes the importance of the role of an enumerative and statistical approach to spore location in species showing apparent anomalies, for example *B. subtilis* (E. Freese, personal communication, and Fig. 1D of reference 1). In *B. subtilis*, spore loci are partially polarized relative to the cell division septum joining pairs of sporangia (J. Mandelstam, personal communication). Of course, it is conceivable that, of those spore-formers having terminal spores, some may have spore loci that are not polarized either distally or proximally to the old pole, but that are randomly located at either pole. In that event, it would still be possible to use spores as topological markers in studies of cell division in species with nonrandom spore loci such as *B. megaterium*.

MATERIALS AND METHODS

Strain and culture conditions. A strain derived from *B. megaterium* ATCC 19213 originally obtained from the laboratory of R. A. Slepecky was used in this study. General culture techniques have been de-

scribed previously (4, 5, 6). Modifications are mentioned in the footnotes to the Tables.

Microscopy. Phase-dark forespores (prespores), phase-bright spores, and cross wall types were detected by phase contrast microscopy (4).

Pictographs of chains and cross wall types. Cross walls were categorized into two types designated by appropriate representative symbols: I, apparently younger, unseparated cross walls which are rarely indented; X, apparently older cross walls definitely separating into two halves. The outer cell termini of chains, which are the halves of completely split cross walls, are represented by curved lines. Using these symbols, the cross wall patterns of the two most commonly observed chain types, two-celled chains (duplets) and four-celled chains (quadruplets), are conveniently represented as (I) and (I X I), respectively. The parentheses represent the terminal walls of chains. These pictographs are essentially equivalent to outline drawings of medial longitudinal thin sections of cell chains. Lines representing side walls are omitted. The polar loci containing or lacking spores can be indicated by adding to the pictographs the symbols: s, spores; o, absence of a spore, e.g., (s oIo s) represents a duplet with each cell having a spore at the pole distal to the cross wall separating each cell and no spore proximal to the cross wall.

RESULTS AND DISCUSSION

Chain length. Under the culture conditions used (Table 1), 86% of the stationary phase cell chains formed by *B. megaterium* contained two to four cells. This type of chain length distribution simplifies the analysis of the relationship of spore loci to the old and new ends of sporangia, if the cross walls are not more than one- or two-cell-generations old. Thus, it is assumed that during cell separation old cross walls split before young cross walls. The next two sections present data indicating the validity of this assumption.

Cross wall types. Two distinct types of intercellular cross wall (see above) occur in all chain types. Examples of these cross walls can be seen in a previously published phase contrast photomicrograph (Fig. 2 and frame C1 of reference 4) of *B. megaterium* cells sporulating after growth in 0.1% sucrose-salts medium. Classification of cross walls was simplified by the enhanced contrast which is due to progressive lytic clearing of the cytoplasm as sporulation progresses.

Cross wall distribution pattern. Table 1 indicates the possible distribution patterns of the two intercellular cross wall types symbolized as X and I. Strikingly, the observed patterns are nonrandom so that the various possibilities are restricted to only one pattern per chain type. This is especially apparent with the four-celled chains where only one out of eight possible patterns occurs. With the two- and four-celled chains, the nonrandom distribution patterns of the two cross wall types statistically support the idea that old cross walls split before young cross walls and that cross walls of similar ages tend to split at the same time. The same reasoning can be applied to the chains with an odd number of cells (see below). Chains with six or more cells represent only about 8% of the total chains formed. These longer chains were not analyzed for cross wall pattern since they were infrequent. This infrequency combined with an average 14% frequency of nonsporulation in long chains made analysis difficult.

TABLE 1. *Cross wall distribution pattern in cell chains*[a]

No. of cells per chain[b]	Cross wall pattern[c]	No. of chains:	
		Observed	Expected[d]
2	(o oIo o)	53	26
	(o oXo o)	0	26
3	(o oIo oXo o)	22	11
	(o oXo oXo o)	0	6
	(o oIo oIo o)	0	6
4	(o oIo oXo oIo o)	40	5
	Seven others	0	35
5	Not classified	8	
6 to 12	Not classified	10	

[a] Chains sampled about 8 h after end of growth in 0.3% (wt/vol) sucrose-salts medium at 30 C. Inoculum cells prepared by dilution of logarithmic growth phase cells stored at −80 C in 25% (vol/vol) glycerol. Final glycerol concentration in the sucrose salts medium was 0.0005% (vol/vol).

[b] Single cell frequency was less than 1%.

[c] Symbols defined in text. Only potential spore loci (o) are shown. Table 2 shows the actual spore locus pattern of these chains.

[d] Expected values calculated from the total number of chains in each chain type assuming a random association of the two cross wall types I and X. Numbers are corrected to the nearest whole value.

Sporulation. About 90% of the cells examined contained phase-bright or phase-dark spores (Table 2). With rare exceptions, the spores occupied polar loci characteristic of some but not all species of the bacterial sporeformers. The sample analyzed in Table 1 and 2 was deliberately taken before phase brightening was complete (42%) to avoid the possibility of spores shifting their location due to sporangial cytoplasm autolysis prior to release of mature spores.

Chains with even cell numbers. Table 2 shows that, of 53 two-celled chains (duplets) examined in a randomly selected sample of 133 chains, only two chains did not fit the pattern expected for an old end spore location. One of these duplets lacked one spore. The other duplet had two phase-bright spores and the individual cells looked very clear. This suggested that the sporangia were very mature and that one of the spores had shifted away from its old end location (see section on lysis). However, even accepting these two chains as valid exceptions, the fact remains that in duplets spores occupy almost exclusively loci at the old ends of sporangia. The same conclusion can be reached with the data for quadruplets (four-celled chains), though here the proportion of chains missing a spore is greater than in the duplets. However, apart from the missing spores, the spore loci patterns of the quadruplets missing a spore, e.g., (s oIo sXo oIo s), resembled the pattern observed for quadruplets with a full spore complement that is the (s oIo sXs oIo s) pattern. It seems unlikely that the quadruplets missing single spores represent chains with spore loci patterns that are inconsistent with a nonrandom spore locus pattern. That would imply a selective inefficiency of sporulation at the new end position. Considered together, or separately, the duplet and quadruplet spore loci patterns show that spore loci do not occur in a random manner in chains of cells and suggest that spores are located at the old ends of sporangia.

Chains with odd cell numbers. The class of three-celled chains (triplets) forms the most serious putative exception to the idea that spore loci occur nonrandomly at the old ends of sporangia. Since the triplet class in Table 1 contained only 22 chains, a further sample of 48 chains was taken. This sample showed approximately the same values for total spore frequency

TABLE 2. *Spore loci distribution pattern in cell chains*[a]

No. of cells per chain	Possible spore loci patterns[b]	No. of chains:		Phase-bright spores (%)[d]	Sporulation (%)[e]
		Observed	Expected[c]		
2	(s oIo s)	51	13	63	99
	(s oIs o)	1	26		
	(o sIs o)	0	13		
	Spores missing[f]	1			
3	(s oIo sXo s)	13	2	24	85
	Other patterns (7)	0	11		
	Spores missing	9			
4	(s oIo sXs oIo s)	24	2	36	89
	Other patterns (15)	0	22		
	Spores missing	16			
5	(s oXo sXo sXs oXo s)	2		40	83
	Other patterns (30)	0			
	Spores missing	6			
6 to 12	Not classified	10		37	86
2 to 12	All chains	133		42	90

[a] Chains produced as described in Table 1.

[b] The possible number of spore loci patterns for a chain of n cells is 2^n. These patterns are indicated or enumerated for the particular cross wall pattern characteristic of each chain type (see Table 1). Cross wall pattern not determined for chains with five or more cells. Symbols are defined in text.

[c] Expected numbers for each chain type are rounded to the nearest whole number and are based only on the number of chains having one spore in every cell. Expected values, not calculated for chains with five or more cells, are for random loci patterns.

[d] Number of cells with phase-bright spores divided by the total number of cells and multiplied by 100. Calculated for each chain type and for all chains.

[e] Number of phase-bright plus phase-dark spores divided by total number of cells and multiplied by 100. Calculated for each chain type and for all chains.

[f] Out of a total chain sample of 133 only 33 had one spore missing and only five had two spores missing. Apart from the missing spores, most chains had spore loci and cross wall patterns identical with their full spored counterparts.

(85%), phase-bright spore frequency (25%), and proportion of chains lacking spores as the triplets in Table 1. Thus, the two samples were pooled for analysis. Forty of the 70 pooled triplets showed 100% sporulation and had a (s oIo sXo s) spore loci cross wall pattern. Twenty-nine of the remaining triplets lacked one spore and one lacked two spores. However, none of the 29 one-spore deficient triplets had spore loci incompatible with a (s oIo sXo s) pattern. The distinctive pattern (s oIo sXo s) can be harmonized with the other results by assuming that the triplet arose from a duplet. One member of the duplet sporulated but the sister cell divided once more and then its two progeny sporulated. The first cross wall partially split to an X-type and the second cross wall, being younger, was an I-type. The duration and time of this event presumably resulted in a delay in sporulation relative to the other chain types because only 25% of the spores were phase bright compared with a value of 42% for the general population (or the doublet value of 63%). Furthermore, 58% of the cells in the putative singlet subfraction had phase-bright spores as opposed to only 9% in the putative doublet subfraction; the respective sporulation frequencies of the two subfractions were 93 and 83%. These values are consistent with the proposed relationship of the triplet cells to one another.

Chains with five or more cells. Due to the overall sporulation rate being 90%, increasing numbers of cells in a chain enhanced the possibility that the chain would contain a cell without a spore. Also, the frequencies of chains with a given number of cells greater than five were low. These two facts made analysis of such long chains difficult. However, preliminary inspection of their spore and cross wall patterns suggested that reasoning such as that applied to the triplets would be necessary in the case of chains with odd cell numbers to fit their patterns with the old end spore location pattern hypothesis. For instance, of the eight quintuplets observed, two had a complete spore complement, five were missing one spore, and one was missing two spores. Allowing for the missing spores, the general pattern was (s oXo sXo sXs oXo s). The exact cross wall pattern was not determined, hence the arbitrary use of X-type cross walls in the previous pictograph. Nevertheless, the pattern suggests that, in general, quintuplets might be analyzed in terms of doublets and triplets. Long chains with even cell numbers could be analyzed like even-celled short chains or like the published pictures of even-celled long chains (3, 9).

Statistical analysis. Results were analyzed statistically using the binomial probability distribution. To do this, the class with the highest frequency of occurrence was compared with a class consisting of all other possibilities. The number of other possibilities varies with the number of cells per chain, so each chain type was analyzed separately.

The intuitively obvious fact, that the cross wall patterns frequencies shown in Table 1 are highly significant departures from random, can be confirmed using this test for the duplet, triplet, and quadruplet chains. The same comment applies to the spore loci patterns (Table 2) for chains with a full complement of spores. Classifying chains missing spores as members of the class consisting of all other possibilities is unjustifiable, especially when their spore loci patterns approximate those of the high frequency class.

It can be safely concluded that spore loci are nonrandomly distributed in sporangia when compared with the locations of their neighbors in the cells of the same chain. This conclusion, coupled with the nonrandom cross wall patterns and the interpretations of these patterns presented previously, suggests that spores are located at the old ends of sporangia.

The cross wall patterns, while facilitating interpretation, e.g., of the triplets, are not absolutely essential for making a choice between an "old" or "new" end location. Table 3 shows the frequency of spores located at the outer cell termini of chains of different lengths. Out of 266 possible loci adjacent to the end walls of chains, 257 had spores. The nine loci missing spores were in terminal cells with no spores except in the case of one doublet. Since, unless one assumes that there is some highly preferential splitting of young cross walls relative to older cross walls, the outer cell termini of chains must be old termini, it must be concluded that spores are nonrandomly located at the old ends of sporangia.

TABLE 3. *Frequency of missing spores at the terminal loci of the different classes of chains*[a]

No. of cells per chain	No. of terminal loci missing spores[b]		
	0	1	2
2	105	1	0
3	41	3	0
4	76	4	0
5	16	0	0
6–12	19	1	0

[a] 133 chains as in Table 1 and 2.

[b] Terminal locus means the position proximal to the end wall of a chain.

Lysis and spore loci. An approximate indication of the magnitude of the effect of sporangial cytoplasm lysis on spore location was obtained. A batch sporulation culture was checked at a time when many spores had been released by total sporangial lysis. Doublet and quadruplet chains, that were still essentially intact and that had a full complement of spores, were analyzed for spore position and cross wall type (Table 4). About 10% of the sampled chains had spores that had apparently shifted location. Most of these spores were located in the center of the sporangium. This result illustrates the advisability of examining the loci of developing spores as early as possible. Ideally, given Nomarski interference optics, the locus of developing spores can be examined as early as sporulation stages II and III with this strain of *B. megaterium* (4).

Significance. Statistically, the data suggest that spores are formed at the old ends of sporangia in this strain of *B. megaterium*. This conclusion agrees with that reached by other investigators using different strains of *B. megaterium* with longer chains but lacking the anomalies noted here (3, 9, 12). Therefore, the sporulation septum must be nonrandomly located at the end that is away from the site of the previous cell division septum (new end). It seems unlikely that such an orderly spatial relationship is accidental. Therefore, there may be a common underlying mechanism controlling the two kinds of septation, especially since the two types of septa form partitions between segregating deoxyribonucleic acid (DNA) molecules. However, an important difference between the two kinds of septation is that the sporulation septum is polarly located in the sporangium, whereas the division septum is centrally located in a vegetative cell. The cell division models that have been proposed so far cannot be readily adapted to explain the asymmetric location of the sporulation septum (3).

The septation location problem may be secondary to the problem of how the spore DNA is located near the old pole. It is known that the shape and position of the DNA molecules are important in sporulation (1). Nevertheless, there seems to be no correlation with the segregation pattern of "old" and "new" chromosomes into spores. Thus, the chromosome segregation pattern is apparently random, at least in *Bacillus cereus* strain 2 (8). The spore locus pattern for *B. cereus* strain 2 is not known. It is apparently nonrandom in another *B. cereus* strain (12). Whether sporulation DNA segregation is random or determined is an important question in comparing early sporulation events with those of the normal cell division cycle. However, the situation concerning DNA segregation in normal division is not entirely established, although the most recent evidence strongly supports a partially deterministic model (11), which contrasts with the apparently random sporulation DNA segregation (8).

It is interesting to speculate on whether mutants with altered spore loci could exist and how they could be isolated. At this time, their detection would appear to be possible only on a serendipitious basis. Disporic mutants (3) may be candidates for the topological mutant category.

TABLE 4. *Spore loci in residual sporangia during autolysis*[a]

No. of cells per chain[b]	Spore and cross wall patterns[c]	No. of chains
2	(s oIo s)	29
	(s oIs o)	1
	(o sIs o)	0
	(s oIo s o)[d]	4
	Other patterns	0
4	(s oIo sXs oIo s)	13
	(s oIo sXo s oIo s)	2
	Other patterns	0

[a] Culture grown as described in Table 1. Glycerol concentration was 0.02% (vol/vol) due to carry-over with inoculum. The sample was taken 17 h after growth ended.

[b] Only duplet and quadruplet chains with a full spore complement were sampled.

[c] Phase brightness or darkness of spores was not recorded.

[d] One spore of one cell had an approximately central locus.

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