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Clonal Analysis of Cell Division in the *Bacillus subtilis div IV-B1* Minicell-Producing Mutant

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Spores of the *Bacillus subtilis* minicell-producing mutant *div IV-B1* were germinated and grown to microcolonies in chambers which facilitate continuous observation of the developing clones with a phase-contrast microscope. Time lapse photographs were taken of 46 clones, covering the period from the beginning of outgrowth until at least two rounds of cell division had been completed. Cell lineages were constructed from contour length measurements of the photographs. These data include cell lengths, division site locations, and cell numbers in clones of various ages. From these data we have determined that the probability of a minicell being produced at any division by the *div IV-B1* mutant is 0.31. The location of the abnormal division site which generates the first minicell produced in the outgrowing clone appears to be random with respect to the existing cell poles. In contrast, the location of the second abnormal division site, and hence the second minicell, is not random but rather occurs preferentially in proximity to the first minicell. This clustering of abnormal events suggests that division site location is related to pole age (generations), although other influences on minicell clustering cannot be ruled out at present.

Two very useful properties of bacterial minicells are: (i) their size and (ii) the fact that they contain no detectable parental cell deoxyribonucleic acid (DNA). These properties have been exploited by a number of investigators who have purified minicells produced by mutants of either *Escherichia coli* (1, 5-7) *Salmonella typhimurium* (15, 16) *Haemophilus influenzae* (14), or *Bacillus subtilis* (9, 12, 13), and studied them in various experimental anucleate systems. Limited attention has been given, however, to the mechanism of minicell production, and it remains to be explained satisfactorily why minicells are free of the parental genome.

Several lines of evidence suggest that the cell divisions which gives rise to minicells are normal in all respects except for their location along the long axis of the cell (2, 12). Instead of forming approximately midway between the two existing cell poles and thereby generating two daughter cells of approximately uniform length, in minicell-producing mutants cell divisions arise in close proximity to an existing cell pole. This very unequal division results in a large, genome-containing cell and a tiny anucleate minicell. It appears, therefore, that the location of division site along cell length in bacteria is under genetic control. Such mutations have been given the designation *div IV* (8, 17).

A cell division which results in a minicell fails to increase the number of viable cells in the culture. A *div IV* mutation which causes every division to be abnormal would generate many minicells but would eventually be lethal since there would be no opportunity for the minicell-producing cells to multiply. To be viable, a *div IV* mutant must be able to undergo some divisions which result in two genome-containing daughter cells, as well as abnormal minicell-producing divisions. The regulation of cell division site location in minicell-producing mutants is complex, therefore, because two categories of division are possible—those which result in anucleate minicells and those which result in two genome-containing daughter cells.

This communication (presented in part at the 13th Int. Congr. Genet., Berkeley, Calif., 1973) deals with a study of division site location in a *Bacillus subtilis div IV* mutant. We have taken advantage of the spore outgrowth system of *Bacillus* to examine the initial cell divisions during clone development. The system appears ideal for the determination of patterns in cell division mutants since it provides a unique and

uniform starting population—spores of the mutant. Also, it is possible to orient all cells produced by the outgrowing clone with respect to proximal and distal poles relative to the spore from which the clone emerges.

MATERIALS AND METHODS

Bacteria. *CU 403 div IV-B1* is a derivative of the *B. subtilis* 168 strain that carries *thy A*, *thy B*, and *met B* markers and the minicell-producing mutation *div IV-B1*. The isolation of the *div IV-B1* mutation and its transfer to *CU 403* have been described previously (13). Phase-bright spores of *CU 403 div IV-B1* were produced by growth on potato extract medium and purified as reported previously (4). Purified spores were pasteurized by heating at 70 C for 20 min in distilled water, and stored at 4 C.

Clone growth. Growth chambers were constructed of round cover slips (22 mm, no. 2) by our previously published procedures (13). The medium consisted of Trypticase soy agar (BBL, Cockeysville, Md.) containing 20 μg of thymine per ml. One drop of an appropriate dilution of spores in Trypticase soy broth was deposited in the center of the agar and spread evenly over the entire agar surface. The inoculum was allowed to dry at room temperature (25 C) until the agar surface was dull in appearance. The agar surface was then pierced with a sterile toothpick to create air reservoirs throughout, and sealed with a second cover slip. The chamber was placed on a standard microscope slide and transferred to a microscope stage incubator which maintained a temperature of 30 C at the slide surface. The cultures were observed with a Wild phase-contrast microscope.

Clone measurements. Time lapse photographs were taken with a 35-mm camera attached to the microscope. Overlapping photographic sequences were taken of clones extending beyond a single microscope field. All photographs were printed to a standard magnification. The prints were assembled to give continuous contour lengths of clones at each stage of development. Clone lengths were measured from the photographic prints by using a precision, wheel-type, map-measuring instrument (Minerva map measurer, Switzerland). The locations of cell divisions along clone length (and consequently individual cell lengths within the clone) were determined by the same procedure.

RESULTS

In the microscope growth chambers that we devised, spore germination at 30 C is followed several hours later by the appearance of the outgrowing cell. The initial cell to emerge consists of a short rod with two poles of normal morphology. Frequently, the spore coat remains attached to one of the cell poles. This relationship may be used to orient all of the subsequent cells produced by the clone. A photographic record is then made of the developing clone, as shown in Fig. 1A. Cell growth is accompanied by elongation. As the clone develops, divisions arise at various sites along the clone length. The locations of divisions and the production of minicells are easily observed. Figure 1B illustrates the manner in which these data are assembled for study. The pattern of cell division, the locations and frequency of minicell production within the clone, the growth of individual cells, and the fate of cell poles may be determined from these diagrams. We examined 46 clones of *CU 403 div IV-V1*, constituting a total of 518 cells, in this manner.

Minicells arise by the location of a cell division in close proximity to an existing cell pole. The probability of a minicell being produced may be calculated from the total number of divisions observed in all of the clones and the total number of minicells produced. We found 148 minicells produced by clones that underwent 472 cell divisions. The probability of a minicell being produced by any one division in a *CU403 div IV-B1* clone is therefore 148/472 or 0.31. The probability of a normal division is 1 − 0.31 or 0.69. If every division has the same probability of producing a minicell ($P = 0.31$), it follows then that on the average one would expect 3.22 divisions to occur for each minicell produced, resulting in slightly more than one minicell for every three genome-containing cells. We have observed 27 incidents in which young clones contain a single minicell. The average number of cells (including minicells) in these clones is 4.11 (Table 1).

The initial cell formed during spore outgrowth contains two poles which become the most distal and proximal poles in the clone relative to the spore coat. These are the oldest poles in the clone. The first division in a developing clone results in two new poles which become the second oldest poles in the clone. Each subsequent division similarly results in new poles that may be assigned a relative age in the clone's history. Figure 2 illustrates relative pole ages in a four-cell clone, assuming the second round of division is synchronous in both sister cells. The two poles produced by each division are termed sister poles and assigned the same age since they arise at the same time. These assumptions are useful in constructing the following simple model concerning the location of the first minicell produced within a clone.

If P = the probability of a minicell being produced per cell division and we assume the minicell can be formed at either the proximal or distal pole relative to the spore coat with equal likelihood, then $\frac{1}{2} P$ = the probability of a minicell being produced at either pole per cell division. At any particular location in the clone,

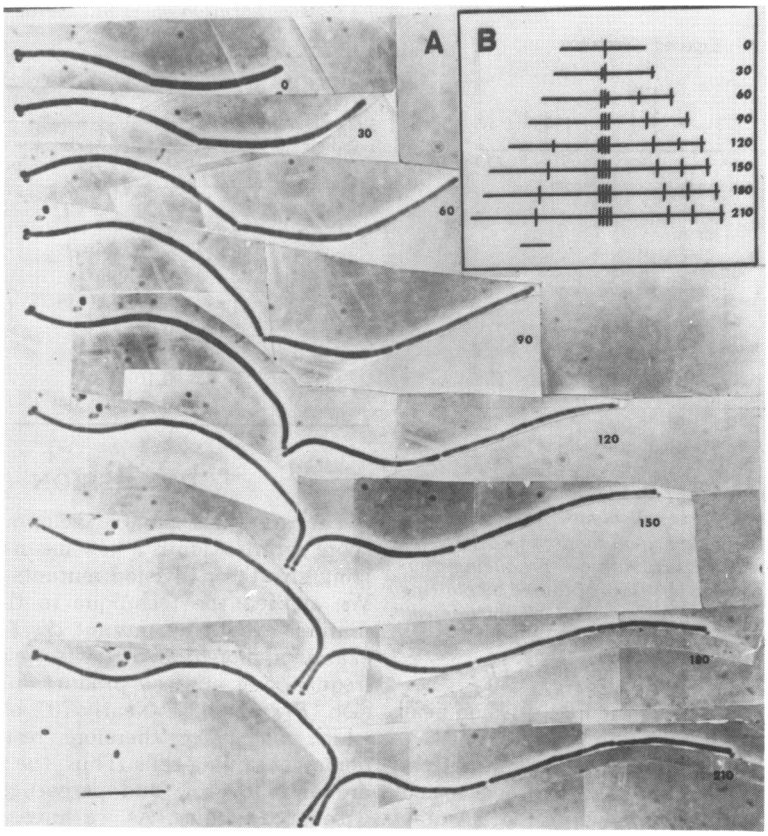

FIG. 1. *Growth and division pattern of a typical CU403 div IV-B1 clone following spore germination. (A) Phase-contrast micrographs of living cells growing in chamber described in text. Spore coat is attached to the left of the clone. Numbers indicate time (minutes) of incubation from time of first photograph. Bar = 10 μm. (B) Line diagram of the same clone obtained by plotting the contour lengths and division sites found in (A) with a wheel-type map-measuring instrument.*

TABLE 1. *First minicell appearance in div IV-B1 clones*

Total no. of cells per clone	No. of clones with			Total no. of cells	Total no. of cell divisions
	1 Mini-cell	2 Mini-cells	3 Mini-cells		
2	4			8	4
3	9			27	18
4	4	2		24	18
5	4	4		40	32
6	5	4	1	60	50
7			1	7	6
8		1	1	16	14
9		1		9	8
10	1	1		20	18
11			2	22	20

the age of the pole becomes important then in determining the probability that a minicell will be located at that position; for the older a pole is, the more cells it has been part of and the greater the chance that an abnormal division might arise next to it producing a minicell. If we assign a relative age to each pole as g_P = the age of a pole in generations, then $\frac{1}{2} P \times g_P$ = the probability of a minicell being located at a pole of age g.

Figure 3A illustrates this theoretical distribution of the relative frequency of minicells at each pole in four-cell clones. Figure 3B shows the locations of first minicells in the 27 clones mentioned above. Virtually all of the minicells are found at either the proximal or distal poles,

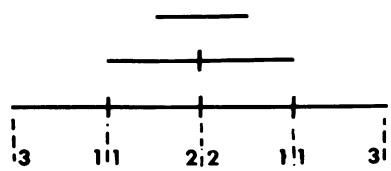

FIG. 2. *Relative pole ages in four-cell clones. First two rounds of division during out-growth are diagramed. See text for assumptions and details.*

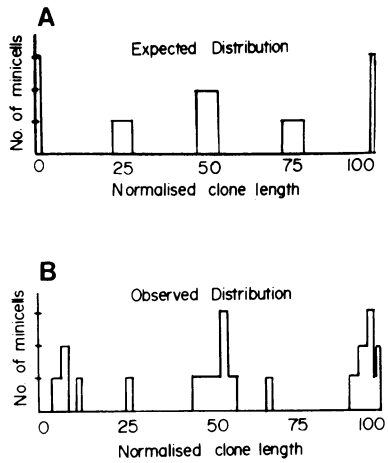

FIG. 3. *Theoretical (A) and observed (B) distribution of minicells in four-cell clones following spore germination. Model based upon random location with respect to cell poles of first minicell-producing division during out-growth. (B) Data obtained by plotting the normalized location of the first single minicell produced during clone development as summarized in Table 1.*

or at the poles created by the first division near the center of the clone length.

If the location of all minicells produced in developing clones is random with respect to cell poles, the kind of model shown in Fig. 3A may be extended to larger clones. In so doing, it becomes apparent that minicells should be found most frequently at certain positions within the clone, namely, at the proximal and distal poles, at the sister poles produced by the first division, at the sister poles produced by the second round of division, and so on in order of decreasing pole age in generations. Furthermore, the model predicts that clusters of two minicells produced by sister poles should arise frequently provided both sister cells undergo an equal number of divisions. Our data indicate that 59 of 148 minicells or 40% of all observed minicells were located in clusters rather than at separate locations within the clones. In 12 clones, minicells were observed to arise simultaneously from sister poles; in 5 of the 27 clones in which the first minicell production was observed, another minicell was soon produced from the adjoining sister pole, resulting in two-minicell clusters. Table 2 summarizes observations of the location of the second minicell produced in clones with respect to the location of the first minicell already present in each clone. These data indicate preferential clustering of minicells.

TABLE 2. *Location of second minicell in div IV-B1 clones*

Total no. of cells in clone	Next to first --00--		Elsewhere -0-//-0-		Total observed
	Observed	Expected	Observed	Expected	
4	0	1.00	3	2.00	3
5	4	1.25	1	3.75	5
6	3	1.80	6	7.20	9
7	2	0.83	3	4.16	5
8	1	0.14	0	0.86	1
9	1	0.25	1	1.75	2
10	0	0.22	2	1.78	2
11	0	0.10	1	0.90	1
12	1	0.09	0	0.91	1

DISCUSSION

The study of clone development following spore germination is a new means of characterizing growth or division mutants of *B. subtilis*. We applied the technique to the analysis of minicell-producing mutant *div IV-B1*. Having examined more than 500 cells, we calculated the frequency of minicell production per cell division (P) to be 0.31. Nearly 70% of the divisions which take place, therefore, result in two genome-containing cells. Thus, the mutant clones are able to grow and perpetuate themselves without difficulty. As we have previously reported, minicells are unable to grow (13). Thus, the cellular material compartmentalized in a minicell represents material that can no longer contribute to the growth and reproduction of the clone. The frequency of minicell production is important, therefore, in determining the viability of a minicell-producing mutant. Given a constant rate of clone length increase, the greater the value of P, the longer the generation time, in terms of viable cells, becomes. When $P = 1$, the mutation is lethal.

We have observed that minicells may arise at any cell pole produced by the outgrowing clone. The most proximal and distal poles are frequent sites at which minicells arise, although it is sometimes difficult to detect the minicell at the proximal pole because it is hidden by the spore coat. Most often the minicells which arise at these poles are produced not by the first division in the clone, but rather during the second or third rounds of division. Since these poles are descended from the initial cell to emerge from the spore, it appears that potential division sites may exist near the original poles which are carried through several generations before expression. Adler and Hardigree (2) have recently

suggested that *Escherichia coli* may contain division sites close to cell poles that are destined to become centrally located as the cells grow, and which function in the generation following their origin. Paulton (10, 11) has shown that, in rapidly growing *B. subtilis* cultures, newborn cells contain several already initiated cell division sites. Our data are in accord with both of these reports.

In 27 of the 46 clones which we examined, the first abnormal division produced a single minicell. The locations of these minicells within each clone are shown in Fig. 3B. Virtually all of the minicells are found at either the oldest or second oldest poles in the clones. Despite the small sample size, these data fit, reasonably well, the expected distribution of minicells based on the random location model which takes into account pole age in generations. It appears, therefore, that the first minicell to arise in developing clones may appear at any of the cell poles in the clone but that the older a pole is in terms of generations, the greater the chance a minicell will arise next to it. As might be expected from the $P = 0.31$ value, the first minicells to appear are found early in clone development. The value of P will obviously be related to the size of young clones, in cell numbers, in which the first minicell appears.

We frequently observed the production of minicells in clusters such as the one shown near the center of the clone in Fig. 1. Such clusters almost always arise as a result of minicell production by adjacent sister poles. In view of the fact that adjacent sister poles arise simultaneously, they should, at least for the first few generations of their existence, remain identical in generation age (until the sister cells become asynchronous). It follows then that sister poles should have identical probabilities over this period for minicell production. An amount of clustering would be expected, therefore, in relation to the sister pole age correlation as predicted by the model shown in Fig. 2 and 3. We have observed, however, a number of cases in which clusters of three or more minicells arise or in which adjacent minicells are produced from the same parental cell. Of 17 cases in which there are two-minicell clusters produced from sister poles, four clones have been observed to progress to three-minicell clusters as shown in Fig. 1. These incidents, although few in number, suggest that pole age may not be the only factor influencing the production of minicells in clusters. It is possible that some condition responsible for multiple minicell production exists in localized regions of the original cell from which the clusters will arise several generations later. Our previously published autoradiographs of DNA location in *div IV-B1* clones (13), which indicate the absence of DNA in certain regions, may provide a clue to this hypothetical condition. Possibly the absence of DNA is related to the production of minicells.

The dual capacity of *div IV-B1*-carrying cells to divide normally or abnormally raises some interesting questions concerning the regulation of division site selection. In the *E. coli* minicell-producing mutant, Adler has claimed that either a minicell-producing or a normal division can occur in a given cell, but not both (2). Although we did not take photographs at the short time intervals used by Adler in producing films of *E. coli* minicell production, we observed what appear to be a number of incidents in which a long cell divides simultaneously to produce several progeny, one of which is a minicell. Whether or not these events actually initiate simultaneously or sequentially, it still remains to be explained how the *div IV-B1* mutation operates to cause 30% of divisions to be abnormal in location.

Any model which attempts to define the mechanism for selection of division site location in *B. subtilis* minicell-producing mutants should take into account the fact that *B. subtilis*, unlike *E. coli*, *Salmonella*, and *Haemophilus*, which also can produce minicells, contains genetic information regulating events similar to the initial stages of cell division in close proximity to a cell pole, that is, genes which regulate sporulation (3, 17). The genetic potential for locating a division near a cell pole must, therefore, exist in all *B. subtilis* cells, although these gene(s) are normally turned off during vegetative growth. When sporulation is initiated, an array of genes become active which modify what begins as an apparent cell division near a pole into the complex events resulting in a spore. It is possible, therefore, that minicell production results from the expression of some, but not all, of the spore information during vegetative growth. The *div IV-B1* mutation does map near a group of mutations regulating spore production (13, 17), but this may simply be coincidental.

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