Cell Division Suppression in the *Bacillus subtilis* *div IV-A1* Minicell-Producing Mutant

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Growth and division patterns of *Bacillus subtilis* wild-type (*div IV-A1*⁺) and minicell-producing mutant (*div IV-A1*) clones were studied after spore germination during microcolony development in chambers that facilitate continuous observation with a phase contrast microscope. Data obtained from 13 *div IV-A1*⁺ clones were used to derive the equation $DE = [(\mu m - 17.6)/8.8]$, which expresses the relationship of cell divisions present in clones of various lengths. This equation was used to determine the number of divisions expected in *div IV-A1* clones if the mutant clones were able to divide as often as wild-type clones. The observed number of divisions present in mutant clones was found to be only 25.27% of the number expected on the basis of this equation. Although individual *div IV-A1* clones varied in the percentage of division equivalents expressed, there appeared to be no correlation between the overall clone growth rate and the number of divisions expressed. Culturing *div IV-A1*⁺ and *div IV-A1* clones together in the same growth chamber revealed that there were no diffusible interactions influencing the division phenotypes of either mutant or wild-type cells. At later stages of growth, mixed microcolonies containing cells of both genotypes were formed. A length analysis of individual cells in these populations indicated that the relative division suppression of mutant compared with wild-type cells characteristic of the initial stages of clone development was maintained. It is likely, therefore, that the excessive length of minicell-producing cells (*div IV-A1*) is a reflection primarily of division suppression in the mutant and not simply of mislocation of division along cell length.

The length distribution of rod-shaped cells in growing populations is regulated by both genetic and physiological influences. In *Bacillus subtilis* we have shown that the timing relationship of cell division initiation to cell growth, the initiation process itself, and the location of division site along cell length are under genetic control (5, 8, 9, 14). In addition, Paulton (12) has shown that the time of cell division maturation in wild-type cells is constant over a range of growth rates. Thus, as cultures progress through the normal growth stages, the division initiation clock changes when referenced to cell length increase, resulting in the commonly observed short cells found in stationary-phase cultures.

We have studied the growth and division patterns of wild-type and minicell-producing mutant strains of *B. subtilis* after spore germination during outgrowth in chambers that permit microscopic examination of the developing clones. A comparison of the *div IV-A1* mutant with its isogenic *div IV-A1*⁺ parent strain has revealed that, in addition to aberrant division

site location, the *div IV-A1* mutant is highly suppressed in division ability. This suppression is primarily responsible for the extreme length of minicell-producing cells in *div IV-A1* cultures.

MATERIALS AND METHODS

Bacteria. CU 403 *div IV-A1* is a derivative of the *B. subtilis* 168 strain that carries *thyA thyB* and *ilvD* markers in addition to the *div IV-A1* mutation, which governs minicell production. Strain CU 403 *div IV-A1*⁺ is the parent strain into which the *div IV-A1* mutation was transferred as previously reported (14). Strain CU 403 *div IV-A1*⁺ carries *thyA thyB ilvD* and *metB*. Purified spores of both strains were pasteurized by heating at 70 C for 20 min in distilled water and were stored at 4 C.

Clone growth. Growth chambers were constructed of round cover slips (22 mm, no. 2) as described previously (5, 10, 14). The medium consisted of Trypticase soy agar (BBL, Cockeysville, Md.) containing 20 μ g of thymine per ml. The chambers were inoculated with spores by the procedures detailed in reference (5). Individual chambers received spores of either mutant or wild-type strains or a mixture of

both. The chambers were incubated at 30 C on a Wild phase contrast microscope stage in the standard manner.

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. The developed negatives were projected with a stripfilm projector onto the surface of a large sheet of newsprint. The images were traced with a marker pen and the drawings were assembled to give continuous contour lengths of the clones. Clone lengths were measured from the drawings with a precision, wheel-type, map-measuring instrument (Minerva Map Measurer, Switzerland). The locations of cell divisions along clone length were determined by the same procedure.

Data analysis. A Pdp8e minicomputer (Digital Equipment Corp., Maynard, Mass.) was used to determine the regression line derived from a plot of division number versus clone length in strain CU 403 *div IV-A1*⁺ clones. A simple program was constructed from this line that computes the number of divisions expected in clones of various lengths. This program was used to calculate the number of divisions expected by using clone lengths obtained from mutant (*div IV-A1*) analysis. The actual number of divisions present in the mutant clones was then compared with the number of divisions expected if the mutant could divide as often relative to cell length increase as the wild type. The percentage of division equivalents expressed is defined as the ratio of observed to expected divisions determined in this manner.

RESULTS

The CU 403 *thyA*⁻ *thyB*⁻ *met*⁻ *ilv*⁻ strain was selected as the genetic background into which we routinely transfer mutations that regulate aspects of cell growth and division before analysis. We chose this strain for several reasons. (i) It contains a thymine requirement that facilitates isotopic labeling of deoxyribonucleic acid for synthesis and location studies. (ii) The methionine and isoleucine auxotrophic requirements are convenient for preparing strains that carry the mutation we wish to study. Our method in general consists of transforming the CU 403 strain with deoxyribonucleic acid from a donor containing the growth or division mutation to prototrophy for either the methionine or isoleucine requirement. The transformants are then screened for the mutation of interest. (iii) In previous studies we have found that the *tag-1* mutation is well expressed in the CU 403 strain (13). In view of these facts, we have attempted therefore to transfer new mutations whenever feasible into the CU 403 strain. Our previous studies of the *div-IV* mutations were conducted with the mutations in this genetic background (5, 10, 11, 14).

Growth and division patterns are conveniently studied in clones developing after spore germination in simple growth chambers that we devised. We have found this procedure a valuable one in the characterization of mutants with defects in either growth or division (5, 10). Some of our findings with the *div IV-B1* minicell-producing mutant have been published (5). We have now obtained similar data for the *div IV-A1* mutation in the CU 403 genetic background. In Fig. 1, the relationship of clone length to the number of divisions present in the clone for the CU 403 strain is shown. These data were obtained from 13 clones grown on various days in different growth chambers. The line drawn through the points in Fig. 1 represents a regression analysis of these data and indicates a slope of 8.8 and a y-intercept of 17.6. The equation in Fig. 1 (referred to as equation 1) is obtained from the regression analysis and simply expresses the number of divisions in clones of various lengths. Equation 1 may be used to predict either the number of divisions expected in clones of known length or the average length of cells at the time of division. We use the term division to indicate the physical compartmentalization of a cell into progeny as visualized by the presence of a septum. The actual separation of sister cells used as a reference in other studies of fluid-grown populations cannot be applied to clones grown on semisolid agar surfaces in our system.

Equation 1 was used to calculate the number of division equivalents expected, using clone

FIG. 1. Relationship of clone length to cell division in the CU 403 *div IV-A1*⁺, wild-type strain. Data were derived from 13 clones grown from spores as described in the text. The line was drawn by using regression analysis. The equation represents the regression line and computes the number of divisions expected given the clone length in micrometers. This equation is the basis for calculation of the expected number of division equivalents in mutant clones.

length data obtained from the analysis of *div IV-A1* mutant clones. Table 1 indicates the manner in which the percentage of division equivalents expressed was determined. In the case of clone 4-21-A, only five divisions arose during the period of observation. For the purposes of these calculations, the actual number of divisions includes and does not distinguish "normal" divisions from those resulting in minicell production. In clone 4-21-A, for example, one of the five divisions observed was a minicell-producing division. In terms of number of divisions, minicell-producing and normal divisions are given equal weight. The total of 10 actual divisions shown in Table 1 represents the sum of divisions observed at each stage of clone development, not the total number of divisions produced by the clone. The third column in Table 1 represents the calculated number of divisions expected by using equation 1. At the final stage of observation, for example, clone 4-21-A had grown to a total length of 416 μm , corresponding to 45.2 expected divisions. Thus, in the total analysis of clone 4-21-A development, nearly 90 divisions should have been observed if the wild-type ratio of growth to division was maintained. In actuality only 10 divisions were recorded. The percentage of division equivalents expressed for clone 4-21-A is therefore approximately 11. Similar analysis of 43 *div IV-A1* clones revealed that in total the *div IV-A1* mutant expressed about 25% of the divisions predicted by equation 1.

The growth chamber in which the *div IV-A1* clone 4-21-A was cultured was seeded with spores of the wild-type strain (CU 403 *div IV-A1*⁺) as well as those of the minicell-producing mutant. Two wild-type clones, designated 4-21-B and 4-21-C, happened to lie close to clone 4-21-A and were suitable for analysis. The

combined data obtained from clones B and C were used to obtain a regression analysis equation similar to that shown in Fig. 1. This equation was used to predict the number of divisions expected in neighboring clone 4-21-A, assuming the mutant clone was able to divide as often as these two wild-type clones. The results are shown in column 4 of Table 1. The values are very similar to those derived from equation 1. Clones B and C are useful, therefore, as internal standards indicating how well clones are able to divide in this particular chamber in comparison with the average division ability of similar clones in other chambers.

The growth rate of individual clones in our culture chambers depends in part upon the particular location of the clone within the chamber (5, 14). In general we attempt to select clones located close to the periphery of the chambers, where the cells are better oxygenated and growth rates are most rapid. Under such conditions, mutant and wild-type clones grew at very similar rates (Fig. 2). The three clones shown in Fig. 2 (which correspond to the clones in Table 1) had length doubling times of A = 68 min, B = 57 min, C = 60 min. The majority of *div IV-A1* clones studied in these experiments had length doubling times close to 60 min.

In total, *div IV-A1* clones were able to express only about one-quarter of the divisions found in wild-type clones of comparable length (Table 2). We have observed, however, that individual *div IV-A1* clones vary in the percentage of division equivalents expressed. This variation is shown in Fig. 3, where the division equivalents expressed are plotted as a function of the growth rate. There appears to be no correlation between the two parameters. At present we cannot adequately explain why individual *div IV-A1* clones vary in their division ability. The variations in growth rates are in part a ramification of the particular site within the chamber at which the clone is located and to some fluctuations in the temperature at which the clones were incubated.

Variations in the percentage of division equivalents expressed in *div IV-A1* clones are also indicated in Fig. 4, in which the relationship of clone length to divisions present is shown. A regression analysis of these data indicates a slope of 26.4 and a y-intercept of 44.6, as indicated by the line drawn through the data points on Fig. 4. The equation derived from this line is also included in the figure. In comparison with Fig. 1, Fig. 4 indicates the additional length to which *div IV-A1* clones must grow in order to produce an equivalent number of divisions as wild-type clones. The ratio of slopes

TABLE 1. Calculation of division equivalents in *div IV-A1*^a

Length of clone 4-21-A (μm)	Actual no. of divisions	DE based on sum of <i>div IV-A1</i> ⁺ clones	DE based on clones 4-21-B and -C in same chamber
66	1	5.5	5.4
143	1	14.2	13.8
232	3	24.3	23.6
416	5	45.2	43.8
Total	10	89.2	86.6

^a Percentage of division equivalents (DE) expressed for clone 4-21-A = 11.2 to 11.5; percentage of division equivalents expressed for 43 clones of *div IV-A1* = $725/2868.75 = 25.27$.

FIG. 2. Growth of mutant div IV-A1 (A) and wild-type (B, C) clones in the same growth chamber. Spores of both genotypes were seeded in the chamber as described in the text. The three clones were located close to one another in the growth chamber.

found in Fig. 4 and Fig. 1 is approximately 3.

Division suppression in the div IV-A1 mutant during the earliest stages of clone development may be demonstrated by comparison of the clone lengths at the time of first division. On the average, minicell-producing clones were 3.7 times the length of wild-type clones at the time the first division became apparent (Table 2, part A). Since a number of mutant clones produced a minicell at the first division, the average length of nucleated daughter cells produced by the first division was not simply half the total clone length as in the case of wild-type clones. The ratio of nucleated cell lengths was 4.39 instead of 3.70, as would be expected if no minicells were produced (Table 2). The figure 4.39 is an index, therefore, of the length difference between wild-type and minicell-producing cells grown under the same conditions.

The ratio of mutant to wild-type nucleated cell lengths may be examined at later stages of growth by simply allowing growth chambers seeded with spores of both strains to continue incubation until microcolony formation is produced. In a number of instances, cells of neighboring clones co-mingle to form mixed microcolonies. If the two neighboring clones happen to be mutant and wild type, the mixed microcolonies consist of minicell-producing and normal cells in close proximity to one another (Fig. 5). It is apparent from these figures that there were no diffusible interactions between the cells of both genotypes which influence their growth and division phenotypes. In such mixed

microcolonies, the mutant cells are easily distinguished from the wild-type cells on the basis of length and minicell production. Cell length measurements obtained from photographs of

FIG. 3. Relationship of the percentage of division equivalents expressed to growth rate in div IV-A1 clones. Percentage of division equivalent expressed is the ratio of observed to expected (on the basis of equation 1) divisions for the entire period of analysis of each clone (see Table 1). Growth rate is obtained from a plot (on semilog paper) of the clone length in micrometers versus time in minutes. Rate is expressed as the clone length doubling time in hours.

FIG. 4. Relationship of clone length to cell division in the CU 403 div IV-A1 minicell-producing mutant. Data were obtained from 43 clones grown from spores as described in the text. The regression line and its equation are included.

TABLE 2. Comparison of cell lengths in *div IV-A1* and wild-type clones

Stage of clone development	<i>div-IV-A1</i>	Wild-type	Ratio
A. Initial			
Measurement			
No. of clones	43	13	—
Avg clone length (μm) at first division	76.23	20.6	3.70
Avg cell length (μm) at first division	38.11	10.3	3.70
Avg length (μm) per nucleated cell at first division	45.25	10.3	4.39
B. Later, during mixed microcolony formation			
Measurement			
No. of nucleated cells	401	88	4.55
Total length (μm) of nucleated cells	1,184.5	1,112.8	1.06
Avg length (μm) per nucleated cell	2.95	12.6	4.28

similar mixed microcolonies are shown in Table 2, part B. The ratio of average nucleated cell lengths was 4.28, a figure very similar to that obtained at the earliest stage of clone development.

Finally, we determined the probability of minicell-producing division in 60 CU 403 *div IV-A1* clones. There were 432 observed cell divisions and 169 minicell-producing divisions; the probability of a minicell division was 0.39. Approximately 40% of all divisions observed were located in close proximity to a cell pole, thereby generating minicells.

DISCUSSION

The production of minicells by bacterial mutants is a ramification of the mislocation of division sites along cell length. We and others have therefore attempted to exploit the minicell-producing systems in the hope of learning more about the manner in which division site location is determined and is under genetic regulation (1-3, 5-7, 14-16, 18). As an approach to such problems, we devised a simple method of culturing clones grown from spores in chambers that enables us to observe and photograph the developing clones. A number of parameters concerning growth and division may be determined from the analysis of data obtained in this manner. In the present study the *B. subtilis div IV-A1* minicell-producing mutant, as well as the parent wild-type strain into which the *div IV-A1* mutation was transferred, was examined. During the course of these studies it became apparent that the lengths of minicell-producing cells of the *div IV-A1* mutant were much greater than the lengths of wild-type cells grown in like manner. Similar observations have been reported for other minicell-producing mutants of different bacterial species (1-3, 6, 7, 16, 18). In one instance a model was devised that ascribes the excess length solely to the mislocation of

division sites along cell length (16). The analysis presented in this paper indicates that in the *div IV-A1* system extreme length of minicell-producing cells is primarily a result of division suppression rather than division mislocation.

A growth-division relationship comparison of wild-type and mutant cells was examined as follows. The relationship of clone length to number of divisions present in the clone was determined for wild-type clones during early stages of outgrowth after spore germination. From these data a regression line was calculated and the equation of that line was written in a form that yields the number of divisions expected given the total clone length in micrometers. This equation (Fig. 1) was used to predict the number of divisions expected by using clone lengths obtained from the analysis of *div IV-A1* mutant clones. A minicomputer was used to carry out the computations. A comparison was then made between the predicted number of divisions and the actual number of divisions present in the mutant clones. The results indicate that only approximately 25% of the expected divisions were present in the mutant clones. It is unlikely that abnormal division site location is the cause of this suppression for the following reasons. (i) Division suppression is observed during the initial stage of clone development before the first division and doesn't depend on the first division being minicell producing. (ii) Clonal histories indicate that *div IV-A1* cells produced by divisions at normal locations, as well as those resulting from a minicell-producing division, are both suppressed in division ability and consequently extremely long in comparison with wild-type cells. (iii) The frequency of minicell production ($P = 0.39$) is too low to account for the extreme length of *div IV-A1* cells during the early stages of clone development. It appears, therefore, that division suppression is independent of division site mislocation in the *div IV-A1* system.

FIG. 5. Mixed microcolonies comprised of *div IV-A1* and wild-type cells. Neighboring clones of different genotypes were allowed to grow in a growth chamber until cells of both types were in contact with one another. *div IV-A1* cells are easily distinguished from wild type on the basis of minicell production and cell length. Similar photographs were used to determine cell lengths in such mixed populations. Bar = 10 μ m.

A number of bacterial mutants suppressed in division have been described previously (4, 8, 9, 15). It has, moreover, been reported that slow growth tends to favor division in some of these mutants in comparison with their suppressed division ability at rapid growth rates (2, 15). In view of these reports, we searched for a correla-

tion between growth rate and division ability in *div IV-A1* clones. Our experience has been that slow growth does not per se rescue division ability in the *div IV-A1* system. We have observed a variation in the percentage of division equivalents expressed in individual mutant clones, but we cannot at present explain the

mechanism of this variation. We are currently searching for modifying genes and environmental conditions that influence the overall division ability of this mutant or the frequency of minicell-producing divisions.

The method of coculturing mutant and wild-type clones in the same chamber provides a unique opportunity to: (i) search for diffusible interactions between strains that influence growth or division patterns; (ii) make immediate comparisons of growth and division patterns under uniform environmental conditions; and (iii) obtain an internal standard in each growth chamber that enables better comparisons to be made between results obtained from different chambers. In the present experiments we applied this technique to show that *div IV-A1* cells are not influenced by, and do not influence in turn, neighboring wild-type cells. In addition, we have shown that the same relative division suppression operates in *div IV-A1* mutant cells growing in mixed microcolonies as we have found in cells newly emerged from spores. From these observations we conclude that division suppression is part of the mutant phenotype regulated by the *div IV-A1* mutation.

In view of the close proximity on the *B. subtilis* linkage map of the *div IV-A1* locus to another gene (*div I*) known to regulate cell division initiation (17, 19), the question arises as to whether the *div IV-A1* mutant either carries a second mutation in the *div I* gene or exerts a polar effect on the *div I* gene. Neither of these possibilities can be ruled out at the present time, although we feel they are unlikely for the following reasons. (i) Minicell production in *div IV-A1* mutants has never been separated from division suppression although the *div IV-A1* mutation has been transferred genetically into several different strains. (ii) The reported phenotypic properties of *div I* mutants (temperature-sensitive division initiation [17]) are not those found in *div IV-A1* division suppression. (iii) Finally, the fact that other minicell-producing systems are frequently associated with division suppression (6, 7, 17) makes it unlikely that multiple-gene defects are required. We are searching, therefore, for a common mechanism to explain division suppression and minicell production in the *div IV-A1* system.

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