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# Analysis of the Multiseptate Potential of *Bacillus subtilis*

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*Bacillus subtilis* was grown at 30 C in 15 media which produced generation times of between 200 and 40 min. A correlation was observed between the growth rate and the number of septa per cell. The time between the production of a septum and its involvement in cell division was not related to the growth rate, but, for the 15 populations, had a mean value of 138 min. Multiseptate cells became progressively evident when the organism was grown at rates in excess of a 138-min generation time.

A characteristic of *Bacillus* sp. is that they are frequently multiseptate (*Bergey's Manual*, 7th ed.). One explanation for this phenomenon is that, although the production of cell septa might be in accord with the overall growth of the cell, the timing of cell division is a particularly variable event (9). There have, however, been no studies to determine the effect of the growth medium on this character. This approach would seem appropriate since it has been well established that the size, composition, and growth rate of bacterial cells are controlled by the nutritional constitution of the growth medium (8, 10). This paper describes the application of this technique to study the production of multiseptate cells by *B. subtilis*. It was observed that both cell division and the production of multiseptate cells were ordered events in the cell cycle.

## MATERIALS AND METHODS

The strain of *B. subtilis* used in this investigation was obtained from The Imperial College, University of London, England. The organism was grown in media containing ammonium sulfate (0.1%), magnesium sulfate (0.02%), dipotassium hydrogen phosphate (0.7%), potassium dihydrogen phosphate (0.3%), and the following additions (numbers refer to media designations): 1, succinate; 2, alanine; 3, arabinose; 4, histidine; 5, xylose; 6, citrate; 7, proline; 8, fructose; 9, aspartate; 10, glycerol; 11, 10 + histidine and proline; 12, 11 + alanine; 13, 12 + aspartate; 14, 13 + arginine; and 15, glucose + Casamino Acids (Difco). The final concentrations were 2 mg/ml for each principal carbon source, 200 μg/ml for each amino acid in media 11, 12, 13 and 14, and 25 mg/ml of Casamino Acids in medium 15.

The organism was grown in 100-ml batch quantities of each of the 15 media over a 7-day period. When a population reached a concentration of $10^7$ cells/ml, cells were reincubated in fresh medium; after 16 hr of further growth, the concentration was approximately $10^6$ cells/ml. During growth from $10^6$ to $10^7$ cells/ml, numbers were determined by using a model B Coulter Counter, and samples were removed for cell wall staining. The latter was accomplished by mordanting cells in 10% tannic acid for 20 min, washing in distilled water, and finally staining with 0.02% crystal violet for 20 sec (4).

For each medium, the growth rate was measured on at least three cultures, and the pattern of septation was ascertained by microscopic examination of not less than 2,000 cells.

## RESULTS

The growth rate, expressed as the number of divisions per hour, and the patterns of cell septation obtained for each medium are shown in Table 1. A fivefold variation in growth rate was obtained and, as this increased from 0.30 to 1.50 divisions/hr, the observed average number of septa per cell changed from 0.55 to 5.30. Thus, the production of multiseptate cells was related to the growth rate. For the 15 populations, the number of septa in any cell was found to be contained predominantly within the mathematical series: 0, 1, 3, 7. In general, at the slower growth rates (e.g., 0.30 division/hr), cell populations contained cells with none or one septum. At intermediate rates (e.g., 0.52 division/hr), cells with one or three were evident. At the highest growth rate (1.50 division/hr), cells with three and seven septa were predominant. In these three situations, the maximum number of septa observed, i.e., one, three and seven, show that the populations were involved in one, two, and three successive divisions, respectively. The relationship between the average number of successive division sites per

TABLE 1. *Growth rate and septation pattern of Bacillus subtilis obtained in different media*

| Medium[a] | Growth rate (divisions/hr) | Percentage of each population containing the following no. of septa | | | | | Avg no. of septa per cell |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 7 | |
| 1 | 0.30 | 45 | 55 | 0 | 0 | 0 | 0.55 |
| 2 | 0.36 | 24 | 75 | 0 | 1 | 0 | 0.78 |
| 3 | 0.40 | 17 | 79 | 0 | 4 | 0 | 0.91 |
| 4 | 0.42 | 16 | 73 | 1 | 10 | 0 | 1.05 |
| 5 | 0.48 | 8 | 82 | 0 | 10 | 0 | 1.12 |
| 6 | 0.50 | 0 | 75 | 1 | 24 | 0 | 1.49 |
| 7 | 0.52 | 0 | 81 | 0 | 19 | 0 | 1.38 |
| 8 | 0.52 | 0 | 77 | 2 | 21 | 0 | 1.44 |
| 9 | 0.58 | 1 | 70 | 4 | 25 | 0 | 1.53 |
| 10 | 0.63 | 0 | 75 | 0 | 25 | 0 | 1.50 |
| 11 | 0.67 | 0 | 54 | 0 | 42 | 4 | 2.08 |
| 12 | 0.75 | 0 | 52 | 0 | 43 | 5 | 2.16 |
| 13 | 0.91 | 0 | 32 | 0 | 53 | 15 | 2.96 |
| 14 | 1.10 | 0 | 17 | 0 | 53 | 30 | 3.86 |
| 15 | 1.50 | 2 | 2 | 2 | 35 | 53 | 5.30[b] |

[a] Composition of each medium is described in the text.

[b] In medium 15, approximately 6% of the population contained cells with 4, 5, 9 and 11 septa.

cell ($y$) and the growth rate is shown in Fig. 1. The values for $y$ were obtained from the average number of septa per cell ($x$), where

$$x = 2^y - 1 \qquad (1)$$

The relationship in Fig. 1 was shown by regression analysis (11) to be highly significant when represented by the equation

$$y = 2.1 + 2.9\,z \qquad (2)$$

where $z$ is the logarithm of the number of divisions per hour.

By using equation 2, it was determined that the average cell contained none or one septum when the growth rate was between 0.30 and 0.42 division/hr; one and three septa between 0.42 and 0.93 division/hr; three and seven septa between growth rates of 0.93 and 1.50 divisions/hr.

Two particularly interesting situations arose when the growth rate was 0.42 or 0.93 division/hr. (i) When $y = 1$, the average cell contained one septum throughout its life cycle, and the growth rate was 0.42 division/hr which is a generation time of 144 min. In this situation, cell division coincided with the production of a new septum which became the site of cell separation after 144 min. (ii) When $y = 2$, the average cell contained three septa during the cell cycle, and the growth rate was 0.93 division/hr, a generation time of 65 min. In this case, cell division coincided with the production of two new septa, and these became the sites for cell division after two generations or 130 min.

Therefore, when the growth rate was increased from a 144-min to a 65-min generation time, the time between the appearance of cell septa and their involvement in cell division changed only from 144 to 130 min.

The values of 144 in (i) and 130 in (ii) were readily determined since the populations contained only one cell type in terms of septation pattern, and the production of new septa must have coincided with cell division. In the 15 media employed in this study, cell populations always contained more than one cell type. However, it is possible to determine from asynchronous populations the timing of septum production in the cell cycle by knowing (i) the percentage of a population which has attained a particular septation pattern and (ii) the growth rate of the culture.

If $P$ stands for the proportion of a cell population in which septum production has occurred, and $t/G_t$ the fraction of the generation time at which the average cell formed septa, the relationship between $P$ and $t/G_t$ is given by

$$\log (P + 1) = \log 2\,(1 - t/G_t) \qquad (3)$$

To derive equation 3, use was made of the function which describes the age distribution of cells in an exponentially dividing culture (1). This function is represented graphically in Fig. 2,

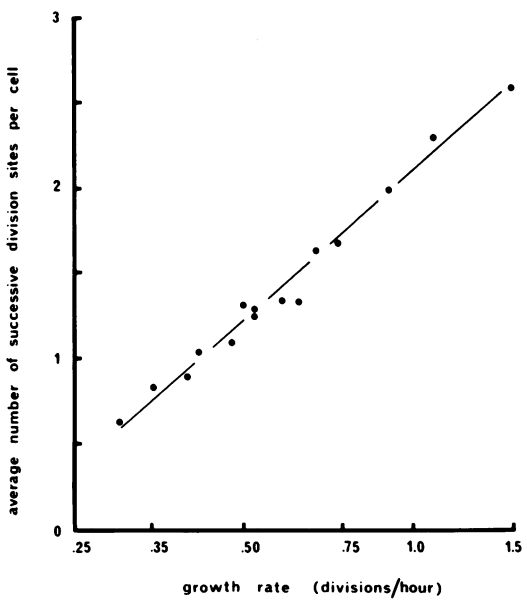


FIG. 1. *Relationship between the average number of successive division sites per cell and the growth rate.*

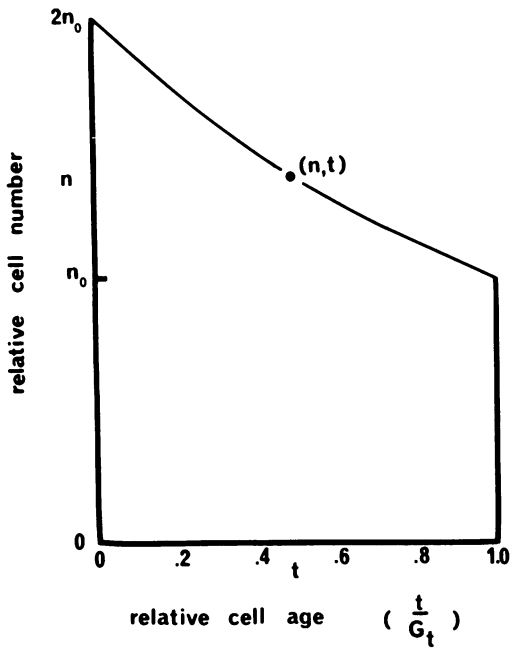


FIG. 2. *Idealized distribution of cell ages, relative to division, of an exponentially dividing culture in which two cells are produced at each division.*

in which cell age ($t/G_t$) is shown on the abscissa, cell frequency on the ordinate, and the total number of cells in the population is represented by the area under the curve. Since two daughter cells are produced by a single division, the number ($N_o$) of cells at division ($t = G_t$) will be one-half the number of newly formed cells ($t = 0$). Because the culture is increasing in number exponentially, the number ($N$) of dividing cells must similarly increase. Thus, the relative frequency of cells in each age group increases exponentially from $N_o$ to $2N_o$. The number of cells at any time ($t$) is $N = N_o\, 2^{-t/G_t}$.

The proportion ($P$) of the total population of ages between $t$ and $G_t$ is:

$$P = \frac{\int_t^{G_t} N_0\, 2^{-t/G_t}\, dt}{\int_0^{G_t} N_0\, 2^{-t/G_t}\, dt}$$

Therefore

$$P = \frac{N_0 G_t/\ln 2(2^{-t/G_t} - 1/2)}{N_0 G_t/\ln 2(1/2)}$$

And rearranging

$$\log(P + 1) = \log 2(1 - t/G_t) \qquad (3)$$

By using the graphical relationship between $P$ and $t/G_t$ in Fig. 3 and the values in Table 1, the time between the production of cell septa and their involvement in cell division was calculated for each of the fifteen populations. The results of these calculations are shown in Fig. 4, in which growth rate appears on the abscissa and "elapsed time" on the ordinate. Regression analysis (11) demonstrated that there was no significant relationship between "elapsed time" and growth rate, instead the former were found to be from a normal population with a mean value of 138 min.

It appears that, over the fivefold variation in growth rate, from 0.30 to 1.50 divisions/hr, the time between appearance of septa and use in cell division has a mean value of 138 min. A model can therefore be constructed which predicts the number of septa contained by a cell at particular growth rates. Accordingly, Fig. 5 shows the change in septation which occurs over a 200-min time period during the cycle of three cells A, B, and C which have generation times of 200, 100, and 50 min, respectively.

For cell A, cell division occurs at zero time, and immediately before this division the cell must

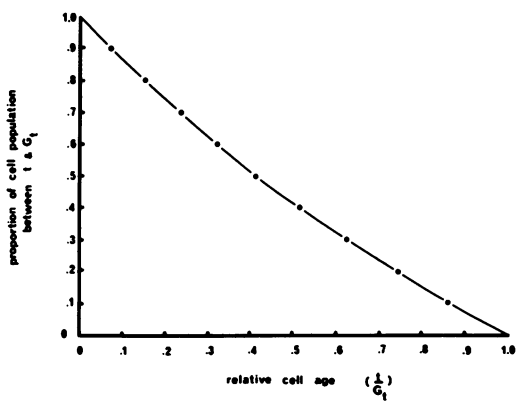


FIG. 3. *Relation of cell age ($t/G_t$) to the proportion of the population between t and $G_t$.*

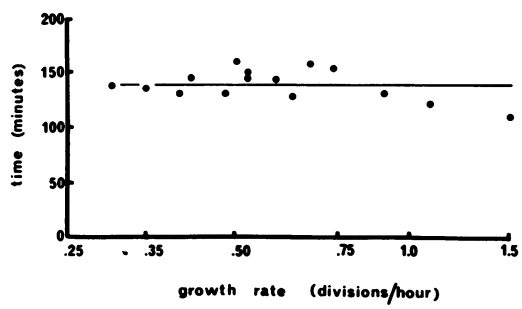


FIG. 4. *Time between the appearance of a septum and its use in cell division in relation to the growth rate.*

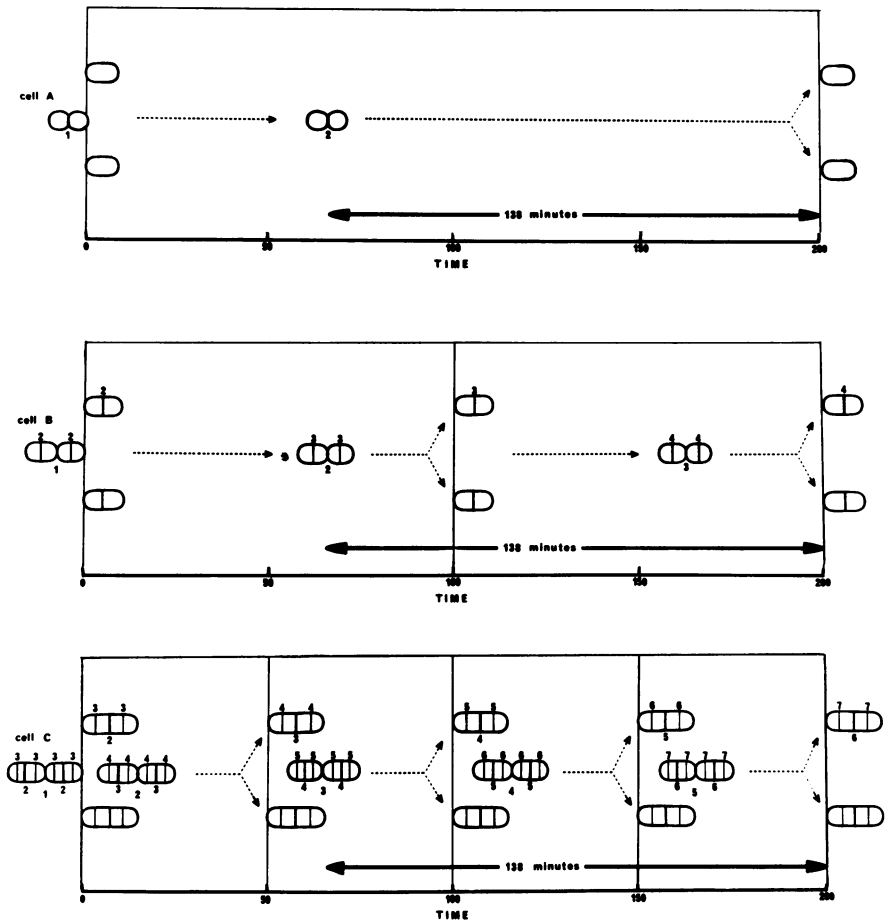


FIG. 5. *Change, during a 200-min growth period, in the septation patterns of three cells, A, B, and C, with generation times of 200, 100 and 50 min, respectively, when the time between the appearance of a septum and its use in cell division is a constant 138 min.*

contain one septum (no. 1). The two daughter cells produced by this division divide again after 200 min; however, 138 min before this, each forms a septum (no. 2). Therefore, a cell grown with a 200-min generation time contains no septum for 62 min and one for the remaining 138 min.

Cell B has a generation time of 100 min and, at division (zero time), contains not only one septum (no. 1) for the imminent division but also two additional septa (no. 2) for the next division. The septa for any division are made in a previous cycle, and the cell contains one septum for 62 min of its cycle and three for the remaining 38 min.

At zero time, cell C contains one septum (no. 1) for the imminent division, two septa (no. 2) for those divisions which occur after 50 min, and four (no. 3) for those occurring after 100 min, i.e., a total of seven septa. Because the generation time is 50 min, the septa for any division are produced two cycles before, and the cell contains three septa for 12 min and seven for 38 min of each cycle.

By using this model, it can be predicted that, at a generation time of 46 min, the average cell should contain seven septa throughout the cell cycle. However, by using equation 2, this septation pattern does not occur until a generation time of approximately 30 min. It can be seen from Fig. 1 that only two populations were considered which had generation times of less than 60 min, and the slope of the line is considerably influenced by these two values. It would have been desirable to have obtained more populations with generation times less than 60 min. This was attempted by using media in which glucose was supplemented with combinations of amino acids (2). Surprisingly, growth rates and septation patterns,

in excess of that provided by glycerol and amino acids (medium 14), were not obtained. Additionally, when glucose was supplemented by Nutrient Broth (Oxoid), although generation times of between 30 and 35 min were obtained, balanced growth was not. The latter was evident by determining the cell size distribution with a Coulter Counter, when it was observed that at no time could a constant cell size be maintained during the batch culture conditions used in this study. This consistency was found, however, in all other media employed. It is possible, therefore, that in a complex medium (e.g., glucose and Nutrient Broth) a departure from the model presented here may exist.

## DISCUSSION

The model to explain the production of multiseptate cells by this strain of *B. subtilis* is an extention of the concept that the size, composition, and growth rate of bacterial cells can be controlled, at a particular temperature, by the nutritional constitution of the environment (8, 10). Since it was established that faster growing cells are larger and contain higher amounts of macromolecules, the explanations offered by different workers have shown a certain consistency.

It is of a selective advantage for a bacterium to grow at the fastest rate possible, but the final growth rate would be restricted by the slowest individual synthesizing system, and this in turn could be inhibited by the growth temperature. The rate of total synthesis can then be increased only by the operation of a greater number of synthesizing units. In accord with this hypothesis, it has been reported that, over a wide range of growth rates, the ratio of protein to ribonucleic acid does not change substantially and that the amounts of each are exponential functions of the growth rate (8).

A particularly interesting situation exists with regard to the deoxyribonucleic acid content of bacterial cells, which is also a function of the growth rate (2, 5–8, 10). It has been observed that in *Escherichia coli*, growing at 37 C between one and three divisions per hour, the time between the initiation of chromosome replication and the separation of the replicated chromosomes at division is approximately 1 hr (2, 4). The ability of this organism to grow at rates in excess of a 60-min generation time was explained on the basis of multiple replication forks and overlap in synthesis (2, 5, 6). Thus, as the growth rate was increased, the initiation of replication occurred at a progressively earlier time even though this involved synthesis of the chromosome for one division to occur in a previous cycle. It was also observed that synthesis for one division may be initiated before a previous one had been completed.

The observations reported here on septation show a similarity to that system describing chromosome synthesis in *E. coli* (2, 5, 6). As the growth rate was increased, cell wall synthesis was progressively directed towards the production of septa at earlier times in the cell cycle. This process ultimately produced multiseptate cells. It seems apparent that some association may exist between cell division and the timing of initiation of chromosome replication and septum formation, as suggested in the case of *E. coli* and *B. subtilis*, respectively. However, at present there can only be speculation concerning the events occurring between septum production and use in cell division. The production of a septum is unlikely to trigger chromosome replication for the forthcoming division since chromosomes must be separate before the formation of a septum divides the cell.

A further problem, accentuated by this work, is not only integrating cell wall synthesis with that of other parts of the cell but also explaining multiseptation production itself. Thus, in becoming multiseptate, a number of septa are produced simultaneously in different parts of the cell. This implies that, not only are there specific areas of the cell surface at which formation can occur, but a control system must exist for the simultaneous expression of these areas in accord with the growth rate. A hypothetical model has been presented to explain the number of replication forks on the chromosome in terms of the growth rate and corresponding cell size (3). A similar mechanism may be applicable to the production of multiseptate cells.

It is not known whether this model explaining multiseptation as a function of the growth rate can be applied to other species of *Bacillus* which exhibit a considerable variation in degree of septation (*Bergey's Manual*, 7th ed.). However, it seems possible that the former approach of considering multiseptation as a delay in cell separation at division may be replaced by an alternative concept regarding septation as a controlled process in the cell cycle.

### ACKNOWLEDGMENTS

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