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Initiation of Wall Assembly Sites in *Streptococcus faecium*

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In electron micrographs of replicas of *Streptococcus faecium*, sites of wall growth are located between pairs of raised equatorial bands. Analysis of cells taken from cultures with mass doubling times between 30 and 125 min indicates that rounds of wall synthesis are initiated at a time close to division, which is temporally unrelated to the initiation or termination of chromosome replication. Growth sites are initiated at a relatively constant volume independent of growth rate when the volume contained within the two segments of wall adjoining an equatorial band marker approaches ca. 0.26 μm^3.

Previously, we presented evidence that the envelope of *Streptococcus faecium* (ATCC 9790) grows by the separation and expansion of its nascent cross walls into pairs of polar caps. This process occurs in discrete growth sites which are found between pairs of raised bands of wall material (8). By using electron microscopy to study replicas of exponential-phase cells which had doubled in mass between 30 and 110 min, Edelstein et al. showed that both the average surface area and volume of the poles of these cells are constant and invariant with growth rate (4). These results suggest that, similar to chromosome replication, a round of envelope synthesis in *S. faecium* produces a constant amount of new product (i.e., two new poles) which is invariant with growth rate.

We further explored this process by estimating the time at which a round of envelope synthesis is initiated within the cell cycle in cultures with different mass doubling times. Our results indicate that (i) the initiation of a round of envelope synthesis cannot be related to the timing of a round of chromosome replication, (ii) the initiation of a round of envelope synthesis occurs at a relatively constant volume at all growth rates, and (iii) the constant volume is that volume contained within the two segments of wall adjoining an equatorial band rather than the volume of the whole cell.

MATERIALS AND METHODS

Cell growth. Cells of *S. faecium* ATCC 9790 were grown at 37°C, as described previously (10), in a chemically defined medium (23) in which the L-tryptophan concentration was lowered to 20 μg/ml to reduce chain formation (24). Specific growth rates were obtained by adjusting the glutamic acid concentration in the absence of glutamine (25).

Electron microscopy. Sample preparation was previously described for existing cell populations used in this analysis (4). Additional samples were fixed by the same method; however, they were suspended in distilled water and placed on polylysine-coated mica to minimize cell aggregation. The cells were then dehydrated in ethanol, dried in a critical point apparatus (1) (Polaron Instruments, Hatfield, Pa.), and stored under vacuum in a desiccator. Platinum-carbon replicas of these cells were made in a Balzers high-vacuum unit (model BA-360M; Balzers, Liechtenstein). Replicas were cleared with 30% (wt/vol) chromic trioxide solution (16 h at 25°C), washed 10 times with distilled water, and mounted on 400-mesh copper grids. All cells contained within sets of grid bars were counted for morphological analyses or photographed for volume determinations.

Cells of *S. faecium* frequently adhere to the mica substrate used in the replica process in such a manner that the major axis is not parallel with the support (i.e., the cells are tilted). In untilted cells, the wall bands (see Fig. 1) appear as straight lines in electron micrographs, whereas in tilted cells the bands appear as ellipses. The angle of tilt (θ) can be calculated with a digitizer to measure the diameter (D) of the cell at the band and the maximum distance that the ellipse deviates from a line passing through the major axis of the ellipse (B) ($\cos \theta = B/D \times 0.5$). Fortunately, most of the cells photographed for this study were untilted before photography, with a rotating-tilting stage (used with a Hitachi model H-600 electron microscope [Hitachi, Tokyo, Japan]). However, for cells photographed at an earlier date with a Siemens Elmiskop 1A electron microscope, we used θ to compensate for the shortening of the major axis of the cell which occurs upon tilting. The efficacy of the procedure was studied by photographing a series of cells tilted at 10° increments from 0° to 60°, using the tilting stage. Comparison of the volumes of cells with 0° of tilt with those volumes calculated by using θ to correct volumes of tilted cells showed that, through 30°, the results differed by <5%. However, at angles >30°, an additional empirical correction had to be introduced to compensate for the loss of view of the apex of the poles. The error in the empirical procedure increases from about 5 to 7% as the angle of tilt increases from 30 to 60°; however, less

than 5% of the cells used in this study were tilted at angles greater than 30°.

Photography was done with a Hitachi model H-600 electron microscope at an instrumental magnification of ×6,000. Quantitation of electron micrographs was described previously (4, 6).

RESULTS

Cells of exponential-phase *S. faecium* had between zero and three growth sites, located at a central (primary) or peripheral (secondary) location (Fig. 1B, C, D, and D'). Cells could initiate new sites of envelope growth at secondary sites before, (Fig. 1C → D) or at primary sites after (Fig. 1A → B), division. In cases in which initiation occurred before division, one secondary growth site often formed in one side of the cell well in advance of the other side (cf. Fig. 1D and D'). Such asymmetry occurred in cells at all growth rates examined but was more common in slowly growing cultures, resulting in an incidence rate of >0.25 cells with secondary sites (Fig. 2). The relative frequency of cells with one or two secondary sites was a function of growth rate (Fig. 3A) and reached a maximum of almost 0.3 of the population in the culture with a mass doubling time (t_D) of 30 min, but decreased to about 0.04 at t_Ds greater than about 85 min.

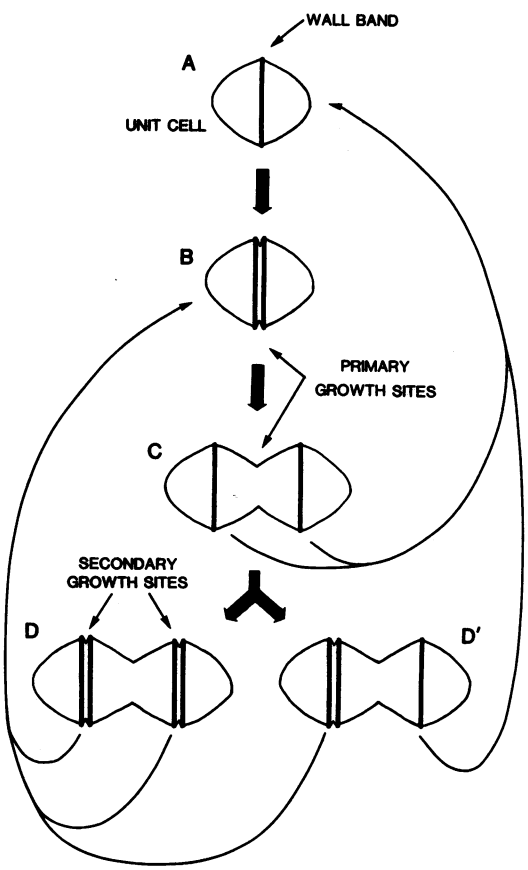

FIG. 1. Cell division cycle of *S. faecium*. A, A unit cell is composed of two poles joined at a wall band. B, As the wall band splits, a primary growth site is formed. C, The primary site enlarges and the cell may divide, or form one (D') or two (D) secondary growth sites. D, A cell with two secondary sites will form two cells with small primary sites at division (B). D', An asymmetrical cell with one secondary site will form one cell with a small primary site (B) plus one unit cell (A) at division.

In the other case in which initiation of new sites occurred after division (Fig. 1A → B), the site had to be established in a so-called unit cell which, in *S. faecium*, consists of two poles joined by a single equatorial wall band (4) (Fig. 1A). The relative frequency of unit cells observed was also a function of growth rate (Fig. 3B), being essentially zero at t_Ds <60 min and reaching a plateau of about 0.075 at t_Ds greater than about 85 min.

These results indicate that, as the growth rate of the culture increases, there is an increasing tendency for secondary sites to be initiated before the primary growth site is completed (i.e., the pathway C → D or C → D' in Fig. 1 becomes more likely). Also, as growth rate decreases, sites are introduced in a more asymmetric manner (i.e., the pathway C → D' is observed more frequently).

The data in Fig. 3A have been used to estimate the time in the cell cycle of each culture at which a growth site was initiated. For calculating the average time before division during which new secondary sites were produced (Fig. 1C → D or D'), the relative frequency data in Fig. 3A were introduced into the following form (14) of the age distribution equation (18):

$$t_X = \frac{\ln (1 + y)}{\ln 2} \times t_D \qquad (1)$$

where t_X is the time (minutes) before division that a growth site is initiated, y is the relative frequency of cells with secondary sites at a particular growth rate (Fig. 3A), and t_D is the mass doubling time in minutes.

For slower t_Ds (>60 min) when sites were initiated in unit cells (Fig. 1A → B), the time (minutes) after division at which primary sites were initiated (t_X') was estimated by a variant of the same equation, for which y' is the relative frequency of cells with a primary site (1 minus the relative frequency of unit cells), and the time (minutes) after division equals t_D minus t_X, so that

$$t_X' = t_D - \frac{\ln (1 + y')}{\ln 2} \times t_D \qquad (2)$$

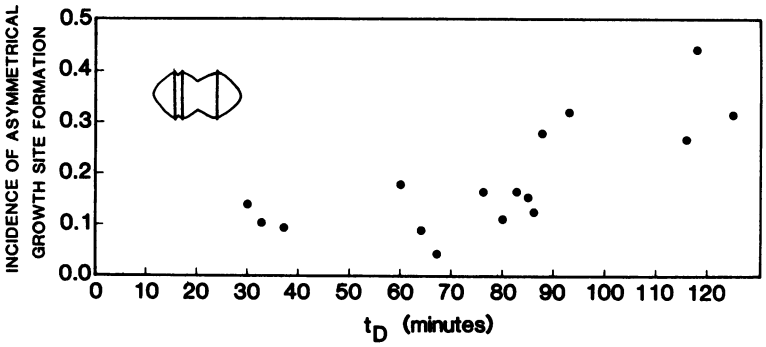

FIG. 2. Incidence rate of asymmetrical growth site formation. The number of cells with one secondary growth site divided by the number of all cells with secondary growth sites for each population of cells. The denominator in this fraction ranged from 8 to 115 cells.

The results of these calculations are shown in Fig. 4, in which the horizontal line at 0 min represents the theoretical time for division for each culture and the solid circles indicate the average time before division that secondary sites were initiated. The open circles represent the calculated time after division when those cultures with unit cells initiated primary sites. These calculations indicate that secondary sites formed ca. 11 min before division for t_Ds of 30 to 75 min, and the time was reduced to 6.5 min before division for t_Ds of 85 to 125 min. The inflection in the line drawn through these points reflects a similar deviation from linearity shown by the data in Fig. 3. For the slowly growing cultures with t_Ds exceeding 85 min, the primary sites appeared in the unit cells about 5.5 min after division. These data suggest that at t_Ds greater than 75 min, while many cells initiate new sites shortly before division, there is an

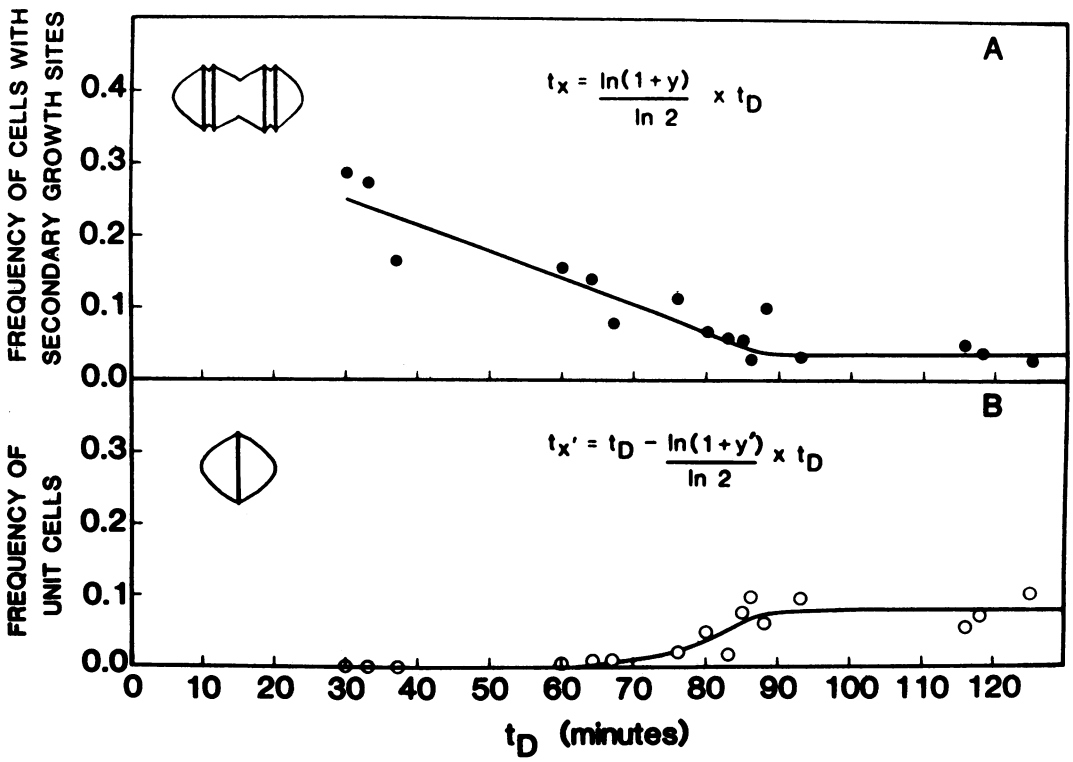

FIG. 3. A, Relative frequency of cells with one or two secondary growth sites (regardless of size of the growth site) for each population of cells. B, Relative frequency of unit cells in each population. Population size range was from 232 to 727 cells.

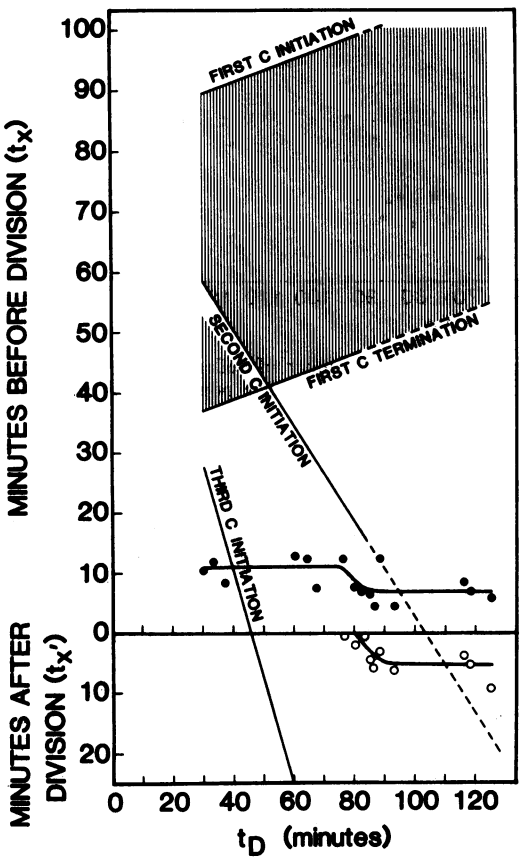

FIG. 4. Estimation of timing of cell cycle events at mass doubling times between 30 and 125 min. Equation 1 was used as described in the text to calculate minutes before division that new secondary growth sites initiate (●). Equation 2 allowed calculation of minutes after division that central sites initiate in slowly growing cultures (○). Note that $t_X = 0$ represents time of cell division. Chromosome replication, which requires 50 to 52 min [7, 9], is referred to as C time, and the time between termination of chromosome replication and cell division is D time [2], which increases proportionally with t_D [L. Daneo-Moore, P. Bourbeau, and D. Carson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, I66, p. 95; and manuscript in preparation] in *S. faecium*. C + D minutes was added to zero time [time of cell division] to draw the line labeled "first C initiation," whose slope is a function of D increasing linearly as t_D increases. The shaded area indicates chromosome replication, which lasts 52 min. Lines were drawn so that the next two C initiations occur t_D min after the previous ones. As an example, at t_D = 30 min, C [52 min] + D [37 min] = time of first C initiation. 89 min − 30 min = time of second C initiation and 59 min − 30 min = time of third C initiation in minutes before division. C time for this organism was determined by residual DNA synthesis after chloramphenicol treatment [7] and by [^{3}H]thymidine incorporation into DNA of synchronously growing cells [9]. These references describe cells grown with t_Ds between 30 and 83 min; to complete Fig. 4, the assumption was made that C time remains at 52 min for cells growing at rates slower than 83 min, and this was illustrated by a thin broken line. The increase in D time with increasing t_D was determined by regression analysis of the residual divisions observed after mitomycin C was added to multiple cultures showing large ranges of mass doubling times [L. Daneo-Moore, P. Bourbeau, and D. Carson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, I66, p. 95] D time may vary by 5 min, depending on culture density at all doubling times [L. Daneo-Moore, unpublished data].)

increase in the relative frequency of this event taking place after division. The asymmetry in the slowly growing cultures was apparently not resolved before division, resulting in production of unit cells. The average time of initiation of new sites fell somewhere between 6.5 min before and 5.5 min after division.

The use of equation 1 or 2 to estimate the time of an event occurring during the cell cycle requires that the event under study be related to, although not necessarily required for, cell division. In other words, the equations could not be used if the event under study were to occur randomly with respect to cell age. For cells with a mass doubling time of 30 min, the percentage of cells with secondary growth sites increased as a function of cell volume (Fig. 5A). (Unit cells are rarely observed at this growth rate.) Similarly, for cells with a mass doubling time of 83 min, the percentage of cells with both primary and secondary growth sites increased as a function of cell volume (Fig. 5B). If the assumption is made that cell volume increases as a function of cell age, then data obtained from equations 1 and 2 should provide a reasonable estimate of the time of secondary and primary growth site initiations during the cell cycle at various growth rates (Fig. 4). The data in Figure 5 suggest that the sites that are not initiated before division are initiated shortly after division. These results support the conclusion that, in slow cultures, initiations occur at some time around division.

An inaccuracy in the estimation of the time of initiation obtained from equations 1 and 2 arises from the fact that site initiation did not occur in all dividing cells (for equation 1) but did occur in a fraction of newly divided cells (for equation 2). This inaccuracy was not significant in rapidly dividing cells, in which >96% initiated sites before division (Fig. 5A). However, in slowly dividing cells, the actual t_x and t_x' times are likely to be somewhat longer than estimated by this approach.

Also shown in Fig. 4 are our best estimates for the timing of initiation and termination of the chromosome replication cycle (see legend to Fig. 4 for methods by which C, the time for a replication point to traverse the chromosome, and D, the time between termination of a round

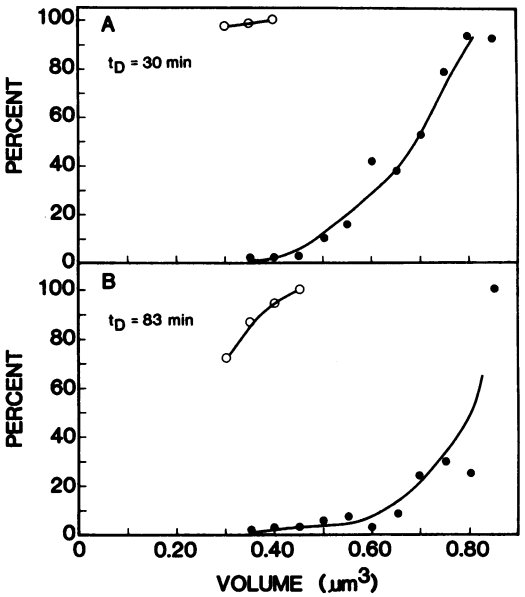

FIG. 5. Percentage of cells in each volume class that have one primary growth site (○) or one or two secondary growth sites (●). A, t_D = 30 min. B, t_D = 83 min.

of chromosome replication and cell division [2], were estimated). No constant relationship could be found between the C or D times in the cell division cycle and the cell age at initiation of growth sites.

Since the data in Fig. 4 do not support a model in which there is a temporal relationship between the initiation of rounds of chromosome and envelope synthesis, replicas of these cells were studied to determine whether an alternate model could be proposed in which sites of envelope growth would be initiated at a constant cell volume. Figure 6A shows that the mean volume of all cells in each population decreased as t_D increased from 30 to 88 min. To estimate cell volume of only those cells involved in growth site initiation, cells were selected on the criterion that they contained growth sites that were >0 but <0.06 μm³. This volume range for what we will call birth sites was chosen empirically on the basis that such sites were quite small but at the same time would give enough cells for analysis. The data in Fig. 6B indicate that the mean volume of cells with birth sites remained relatively constant (the slope of the linear regression line with weighted means is not [statistically] significantly different from zero [$P > 0.20$]) and support a model in which primary envelope growth sites are initiated in cells of a constant volume which appears to be independent of growth rate.

The same question was asked with respect to the initiation of envelope sites at secondary locations, where the mean volume of cells containing one secondary site in the birth-size range (>0 and <0.06 μm³) is given as a function of t_D in Fig. 6C. The observed scatter probably is due to the asymmetry with which secondary sites were introduced into cells plus the small number of cells available for analysis in some populations; it seems that the total mean volume of cells in which secondary birth sites were observed was not constant over the range of growth rates studied. However, when we compensated for this asymmetry by measuring the volume in only the cell half bearing a birth-size (>0 and <0.06 μm³) secondary site, we found the mean volume in this cell half to be relatively constant (Fig. 6D) and only slightly smaller than the mean volume of whole cells having primary sites in this same birth-size range (Fig. 6B).

The argument that sites were formed at primary or secondary locations when their respec-

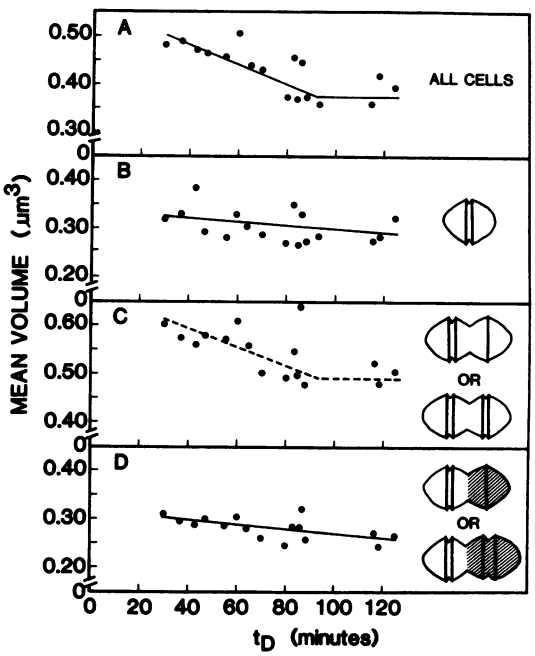

FIG. 6. Mean volume for each growth rate. A, All cells in each population in which the population size range was 77 to 1,563 cells. B, Cells with birth-size primary growth sites in which sample size range was from 3 to 81 cells. C, Cells with birth-size secondary growth sites in which sample size was 1 to 216 cells. D, Half-cells with birth-size growth sites (shaded part not included) in which sample size was 1 to 315 cells. Samples in B, C, and D are from populations in A. Lines were drawn by linear regression in A, B, and D; broken line in C was drawn through points but with slope of line in A. Point at which regression line in A changes slope was chosen by eye.

tive whole-cell or half-cell volume reached a critical, growth rate-independent volume (0.261 μm^3, the mean birth volume, minus 0.03 μm^3, the mean birth-size growth site volume) is supported by the statistical test mentioned above for whole cells (Fig. 6B). The slight slope of the linear regression line with weighted means in Fig. 6D is not (statistically) significantly different from zero ($P > 0.10$) when combined with data from Fig. 6B to increase the number of observations for each data point.

DISCUSSION

On the basis of the freqency of primary and secondary growth sites observed in replicas of cells, it was calculated that new sites are introduced into cells at about 11 min before division in cells with t_Ds less than 75 min; whereas, sites are initiated over a range of times that extends from ca. 6.5 min before to 5.5 min after division in cells with t_Ds greater than 85 min. The two equations used to calculate initiation times before and after division (equations 1 and 2) are forms of the general growth equation and carry with them the assumption that all cells in the population have the same generation time (14, 18). An independently derived, more precise estimation of the time for initiation will be given in a study in which the coefficient of variation of size of cells in the terminal stages of division is used to estimate the size of cells at the beginning and end of this phase (A. L. Koch and M. L. Higgins, manuscript in preparation).

By the latter analysis, growth sites appear to be initiated at about 6.8 and 6.4 min before division for cells with t_Ds of 30 and 83 min, respectively (in contrast to the 11- and 6.5-min estimates derived from the present work for the same cells). Thus, both studies give similar qualitative results: namely, that the initiation of sites of envelope growth occurs at a relatively constant time with regard to cell division and cannot be related to the initiation or termination of rounds of chromosome replication.

It should be emphasized that, although no correlation can be observed between the timing of rounds of chromosome replication and envelope synthesis, past work has shown that the completion of envelope growth sites in *S. faecium* is dependent on DNA synthesis. In these studies, the addition of the specific inhibitor of DNA synthesis, mitomycin C, to exponential-phase cultures prevented growth sites from separating, although these sites do continue to increase in size (7). Thus, it seems that in *S. faecium*, although the initiation of envelope growth sites is not directly related to the timing of chromosome replication, the completion of such sites is dependent on the completion of rounds of DNA replication. This would be in keeping with the "veto" regulatory mechanism postulated by Helmstetter et al. (5), in which the terminal stages of the cell cycle are delayed until the chromosome is fully replicated and segregated.

Various models of cell growth in rod-shaped bacteria have proposed that the number of envelope elongation sites increases at a given time in the cell cycle corresponding to the time at which the cell reaches a critical age (11, 13, 15, 16, 26), size (3), or density (20). Some studies have suggested that this increase in the number of sites is timed by chromosome replication or segregation (19, 21, 22), whereas others assert that this event is regulated independent of chromosome replication (3, 15, 17, 26). The diversity in these models may be due to the fact that in experiments with rod-shaped bacteria, there is no easily identifiable marker by which growth sites can be evaluated, and methods of observation are therefore indirect.

The results obtained with *S. faecium* are inconsistent with chromosome replication regulating the initiation of growth sites but do not allow any of the common models (size, time, density-pressure) proposed for rods to be favored over another. At present, the data collected for *S. faecium* can be used to create quite plausible models in which size, time, or pressure would be the primary determinant in the initiation of new growth sites. For example, a new site might form when (i) the cell volume contained by two segments of wall joined by a single band reaches about 0.25 μm^3 (a size model in agreement with Fig. 6), (ii) a cell reaches a given time in the cell cycle which varies slightly with growth rate, ranging on the average between 6 and 11 min before division (a time model consistent with the calculations presented in Fig. 4), or (iii) the pressure in a cell begins to increase in the latter stages of development of a growth site when the surface area of this site would be increasing more slowly than the synthesis of cytoplasmic macromolecules contained within the cell (a pressure model based on geometric studies of envelope site growth in this organism [8] and another model [12]).

Recently this pressure model for the envelope growth of *S. faecium* was given in detail (12). The argument was proffered that the turgor pressure developed within the cell as a result of active transport and macromolecular synthesis provides the energy needed to split wall bands to create new sites and, once a site is created, to separate the septal wall into two layers of peripheral wall. As the division furrow continuously reduces the diameter of the septum, less and less new peripheral wall is made (8). If the cytoplasm continues to be synthesized, an increase in pressure will occur as the output of the

site decreases. This increase in pressure would trigger the formation of new sites at the points on the surface under the greatest strain, the discontinuities in curvature of the wall segments located at the wall bands.

In summary, the versions of the size-time-pressure models for *S. faecium* are not mutually exclusive, and it is likely that further investigations will evolve a model that rests on the central aspects of each of these schemes. An additional component of this model will address the problem of the regulation of the synthetic and autolytic enzymes which must carry out the assembly and remodeling of the envelope.

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