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Cell Wall Assembly During Inhibition of DNA Synthesis in *Streptococcus faecium*

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Growth sites which are bounded by raised wall bands can be observed in electron micrographs of replicas of *Streptococcus faecium*. When mitomycin C was added to an exponential-phase culture doubling in mass every 64 min, DNA synthesis was inhibited, and eventually cell division stopped. The growth sites formed before and after inhibition of DNA synthesis enlarged until they contained about 0.25 μm^3 of cell volume, at which point they ceased to increase in size. When these sites approached this 0.25-μm^3 limit, new sites were initiated; this result had also been observed in untreated cells undergoing a large range of exponential-phase mass doubling times. Thus, regardless of whether chromosome replication is inhibited or uninhibited, sites have the same finite capacity to enlarge to about 0.25 μm^3, and when this capacity is reached, new sites are initiated. Although initiation of new growth sites seems to be independent of normal chromosome replication, these results confirm previous studies showing that chromosome replication is necessary for the terminal events of growth site development which result in the division of a site into two separate poles. Two classes of models for the regulation of growth site initiation are discussed.

The surface of the gram-positive coccus *Streptococcus faecium* ATCC 9790 grows at discrete growth sites in which septa separate and expand into pairs of polar caps. The initial event in the formation of a new growth site is the synthesis of an annular ridge of material on the internal surface of the cell wall. This ridge forms a nascent septum which is usually assembled directly under a raised band found on the external surface of the cell wall (wall band; Fig. 1A). This is followed by the creation of a division furrow which cleaves the wall band and underlying septum and allows the septum to separate into two new layers of peripheral wall. The two bands formed by this initial cleavage serve to delineate the wall synthesis that occurs in a single site (Fig. 1A and B).

Recently, we have used these wall bands as markers to study the number and geometry of growth sites seen in platinum-carbon replicas of balanced exponential-phase cells which before fixation showed mass doubling times between 30 and 110 to 125 min. The results of these studies indicated that the volume of the two completed poles produced by a single site was constant and thus independent of growth rate (3) and that the initiation of new sites could not be related to the timing of rounds of chromosome synthesis but did occur at a constant cell volume (4).

A model was proposed in which a new site of surface growth would be produced when the volume contained within the two segments of wall joined by a single wall band reached a critical size. Depending on the growth rate, these two segments could be two poles (Fig. 1A; seen more frequently at slow growth rates) or a pole and a nascent pole (Fig. 1B; seen more frequently at rapid growth rates).

Here we have asked whether the three characteristics of envelope growth of *S. faecium* observed in exponential-phase cells (i.e., [i] the constant size of poles produced in growth sites; [ii] the independence of the initiation of new growth sites from the timing of DNA synthesis; and [iii] the constancy of the cell volume in the wall segments attached to newly split bands) can be observed in cultures treated with the inhibitor of DNA synthesis mitomycin C (MIT). MIT was chosen because it inhibits DNA synthesis rapidly with a minimal effect on the synthesis of other macromolecules (7).

MATERIALS AND METHODS

Cell growth and biochemical analysis. Cells of *S. faecium* ATCC 9790 were grown at 37°C as described previously (8). The 64-min mass doubling time was obtained by omitting L-glutamine from the chemically defined medium but supplying 28 μg of L-glutamic acid

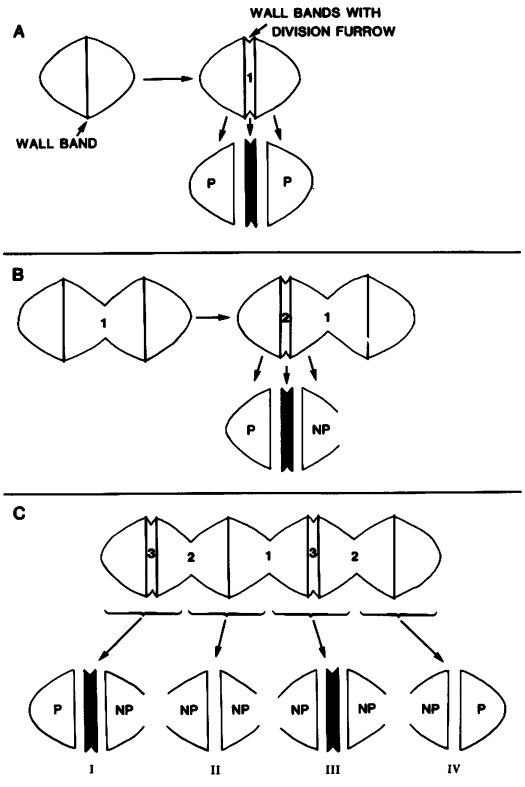

FIG. 1. Morphology of normal and MIT-treated cells. (A) The smallest cell in the population with one wall band, a unit cell. A division furrow will quickly form as the band is split in two, resulting in a central, primary growth site (site 1). Cells can be identified as having recently formed this site when the growth site (shaded) contains volume >0 and <0.06 μm^3. Completed cell poles (P). (B) An older cell with a large primary growth site (site 1) which can split either or both wall bands to form secondary growth sites (site 2). In the case shown, only the left band was split. Such a cell can be identified as having recently formed this secondary site because the site (shaded) contains a volume >0 and <0.06 μm^3. Each cell half can be analyzed separately; when this is done, half of the primary growth site is considered as a nascent pole (NP). (C) A cell treated with MIT for a long time which has developed tertiary growth sites (site 3). Each part of the cell can be analyzed separately. Part I contains a completed pole, a newly initiated growth site, and a nascent pole; II contains two nascent poles; III contains two nascent poles plus one new growth site; and IV contains one completed pole and one nascent pole.

per ml (13, 17). The medium contained 20 μg of L-tryptophan per ml to reduce chain formation (16).

Growth was monitored turbidimetrically at 675 nm in a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). Optical density readings were adjusted to agree with Beer's law (15) and expressed as adjusted optical density (AOD). One AOD unit is equivalent to 0.43 μg of exponential-phase cells (dry weight) per ml.

MIT (0.5 μg/ml; Calbiochem, La Jolla, Calif.) was added to exponentially growing cultures at a turbidity equivalent of 43 μg (dry weight) per ml after at least six to eight mass doublings. The effect of treatment on DNA synthesis was established by measuring the incorporation of well-equilibrated [*methyl*-^{3}H]thymidine (Amersham International, Ltd, Arlington Heights, Ill.; 1.5 μCi/ml with 15 μg of unlabeled thymidine per ml added to the medium) into cold trichloroacetic acid precipitates (7).

Particle counts. At intervals, 0.1-ml samples of cultures were added to 0.4 ml of 10.4% Formalin at room temperature. Samples were stored but then were counted immediately after an experiment was completed. After thorough agitation, samples of 0.05 or 0.10 ml were added to a 30-ml plastic cup, and a volume of 0.9% sterile saline was added to dilute the samples so that between 5,000 and 15,000 cells were analyzed per sample. At least three counts were made for each sample by using a Particle Data Celloscope (Particle Data Co., Chicago, Ill.), and the approximate number of cells per milliliter was calculated.

Electron microscopy. Preparation of platinum-carbon replicas and quantitation of electron micrographs were described previously (3, 4, 6). Micrography was done with a Hitachi model H-600 electron microscope (Hitachi, Ltd., Tokyo, Japan) at an instrumental magnification of ×6,000.

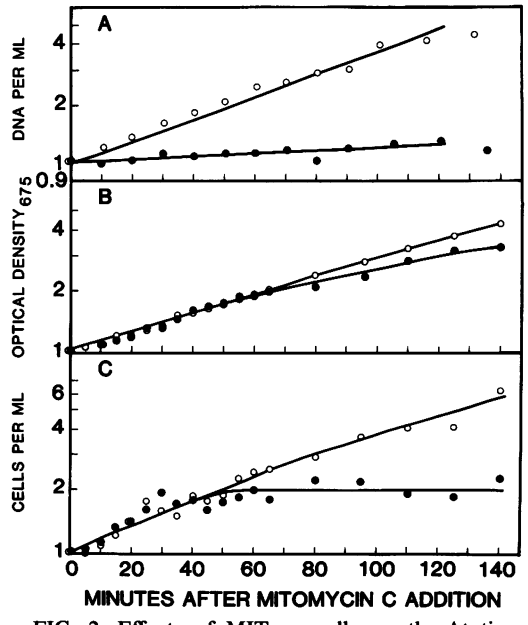

FIG. 2. Effects of MIT on cell growth. At time zero, the culture was split and MIT was added to half. Samples were taken from MIT-treated (●) and control (○) cultures. (A) DNA per milliliter when a relative value of 1 equals 0.52 μg/ml. (B) AOD at 675 nm, when a relative value of 1 equals 100 AOD. (C) Cells per milliliter when a relative value of 1 equals 9.5×10^7 cells.

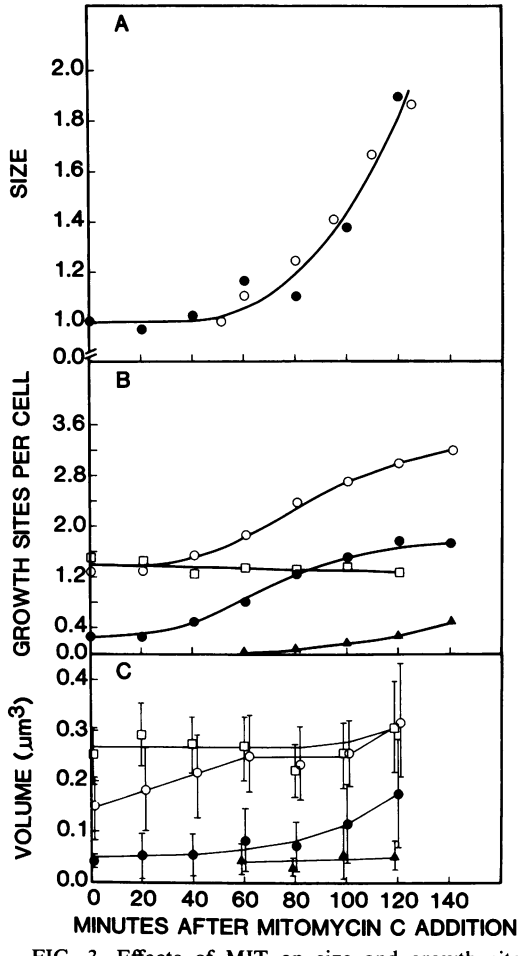

FIG. 3. Effects of MIT on size and growth site formation. (A) Mean cell volume calculated from computerized analysis of electron micrographs of replicas of MIT-treated cells (●), when a relative value of 1 equals 0.505 μm^3. Estimated mass per cell (○) based on increase in optical density per cell, when a relative value of 1 equals 155 AOD at 675 nm, and cell number equals 2.03×10^8 cells per ml from 48 min until the end of the experiment. (B) Mean growth sites per cell. Total sites in MIT cells (○); secondary sites in MIT cells (●); tertiary sites in MIT cells (▲); total sites in control cells (□). (C) Mean volume of completed poles and growth sites from computerized analysis of electron micrographs. Bars = ±1 standard deviation. Primary sites (○); secondary sites (●); tertiary sites (▲); sum of both completed poles (□).

RESULTS

Effects of MIT on cell growth. To simplify the interpretation as to the effect of DNA synthesis on envelope growth, MIT was added to cultures with a mass doubling time (64 min) which was slow enough to limit the number of replicating chromosomes to a maximum of one per cell (7, 9). At the concentration used (0.5 μg/ml), MIT inhibited DNA synthesis (Fig. 2A) while allowing the mass of the culture to increase at the pretreatment rate for about 60 min (Fig. 2B). Cells continued to divide for about 48 min after the addition of MIT (Fig. 2C). This 48-min period of residual cell division has been interpreted as the portion of the cell cycle that does not require DNA synthesis for division (D time) (1, 5). All of these findings are consistent with previous data from this laboratory.

Effects of MIT on volume and frequency of growth sites. As cell division was inhibited by MIT, the mean volume per cell increased as measured from electron micrographs of replicas of the cells taken from the treated cultures (Fig. 3A). The increase in mean mass per cell estimated from the AOD and the cell counts (Fig. 3A) was in agreement with these values. The primary question was how did cells whose DNA synthesis was inhibited by MIT accommodate the approximately twofold increases in volume shown in Fig. 3A. It was found (Fig. 3B and C) that this was done by an increase both in the number of growth sites observed per cell (Fig. 3B) and in the mean volume that these sites attained (Fig. 3C). To interpret the data in these figures, it must be understood that treated cells can have one additional category of growth site. In both treated and untreated cells, sites can be observed in a central, primary location and in flanking, secondary positions (Fig. 1A and B). After long periods of treatment with MIT, the third category of site was seen at tertiary locations (Fig. 1C).

When MIT inhibition of cell division started to be expressed, the frequency of secondary sites increased and began to plateau only after about 120 min (Fig. 3B). At about 60 min, the new class of cells with tertiary sites appeared. These tertiary sites showed the greatest rate of increase in the latter periods of treatment, when the rate of increase in production of secondary sites slowed. The summation of the frequencies for all growth sites (Fig. 3B) indicates that sites continued to be produced for more than 90 min after DNA synthesis had been inhibited by MIT.

During the first 60 min of treatment, the greatest increases were in the volumes associated with the primary sites (Fig. 3C). It is important to note that in the first 100 min of treatment the mean volume of these primary sites approached, but did not exceed, a constant volume range measured in the poles of these cells during this period. This range is illustrated by the standard deviation bars in Fig. 3C. That the maximum size of MIT-treated sites remained constant is quite remarkable, for it is known that by blocking cell division this drug leads to the formation of abnormally elongated sites (Fig. 4).

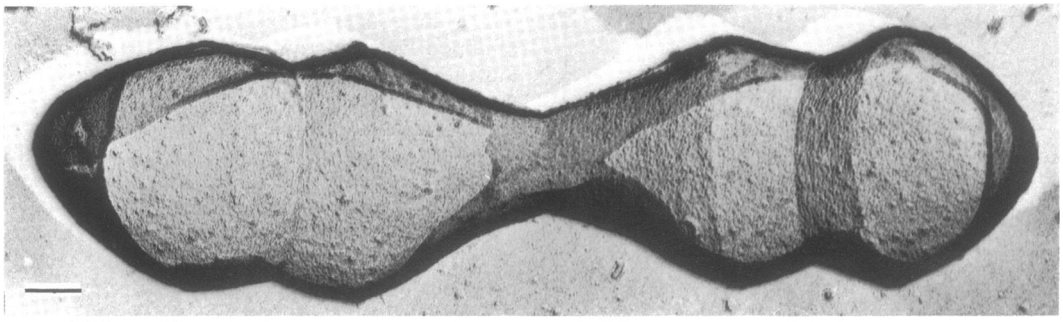

FIG. 4. Electron micrograph of a replica of a cell treated for 100 min with MIT. Bar = 0.2 μm.

As the average volume of primary sites in MIT-treated cells approached that of two completed poles (at about 60 min), the average volume of the secondary and tertiary sites increased. This increase in secondary and tertiary volume apparently compensated for the diminished capacity of primary sites to enlarge. After 120 min, the poles and primary sites showed a further increase in size which can be correlated with an increase in the radius measured along the wall band and axial height (tip to base at the band) in the poles of these cells (Fig. 5).

Although we have not analyzed the data in as much detail, cells treated with the inhibitor of DNA synthesis 6-(*p*-hydroxyphenylazo)-uracil, and thymine-requiring mutants of *S. faecium* starved for thymine, show qualitatively identical changes as MIT-treated cells (M. Higgins and L. Daneo-Moore, unpublished data).

Volume criterion for growth site formation. The above data indicate that in *S. faecium* the initiation of new, and the enlargement of new and old, envelope growth sites does not require ongoing DNA synthesis. Therefore, we explored the possibility that, like untreated exponential-phase cells, new growth sites might form when the volume associated with an unsplit wall band (Fig. 1A) reached a critical size (4). To study the initiation of growth sites, we selected cells with very small sites and assumed that such sites had just been "born." We have defined such "birth" sites as those whose volume is greater than 0 but less than 0.06 μm^3. This size criterion was set on empirical grounds, for it represents a very small site and yet yields enough cells in each population for analysis.

In the untreated cells, birth sites could be seen in cells at either a primary or secondary location (Fig. 1A and B). When birth sites were observed in the primary location in MIT-treated cells, the mean total cell volume of these cells was about 0.286 ± 0.066 μm^3 (Fig. 6), whereas the mean total volume of cells with secondary birth sites was almost exactly double that value (0.568 ± 0.111 μm^3). This is consistent with the idea that sites were introduced into untreated cells when a volume of about 0.286 μm^3 was associated with a wall band. The data in Fig. 6 indicate that the volume of cells with primary and secondary birth sites remained constant in the presence of MIT for about 100 min. Thereafter, an increase was noted in the volume of cells having secondary birth sites. No cells were observed with primary birth sites after 40 min because cell division was inhibited at that time and cells could not separate to yield cells with primary sites.

The mean volume of cells with tertiary birth sites was not a twofold incremental increase as seen in cells with primary and secondary birth sites during earlier periods of treatment. This is due to tertiary sites being introduced into cells in a very imprecise and asymmetric manner, with most cells having only one or two tertiary sites per cell even after 140 min of treatment (Fig. 1C), rather than the four sites one would predict if the number of sites doubled at a given time during treatment.

To compensate for the asymmetry with which sites were introduced into cells late in treatment, we developed a computerized technique for ex-

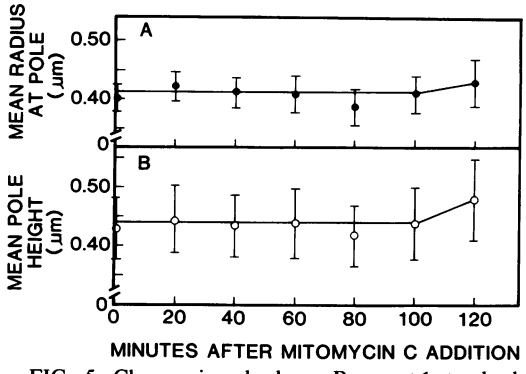

FIG. 5. Changes in pole shape. Bars = ±1 standard deviation. (A) Mean radius of cell measured along wall band at poles. (B) Mean pole height measured from wall band to tip of cell.

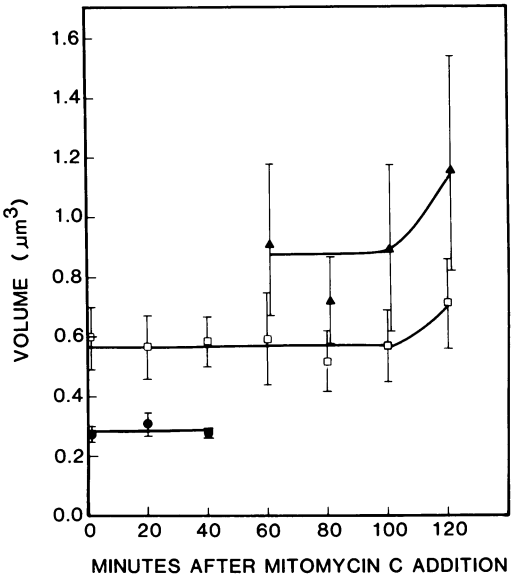

FIG. 6. Mean whole cell volume of cells with birth-size growth sites. Bars = ±1 standard deviation. Cells with primary birth sites (●); secondary birth sites (□); tertiary birth sites (▲).

amining the partial volume of the cell contained by the poles or nascent poles immediately attached to a birth site (Fig. 1C). Birth sites, regardless of their location, were attached to two wall segments (either two poles [Fig. 1A], a pole and nascent pole [Fig. 1B and C, I], or two nascent poles [Fig. 1C, III]). The average summed volume of the site plus adjoining segments equaled about 0.276 μm^3 (Fig. 7) and remained constant through the entire period of treatment. Moreover, when we examined the average volume of poles or nascent poles attached to unsplit wall bands (Fig. 1C, II and IV), we also obtained a constant, but somewhat smaller, volume through the entire period of treatment (0.245 μm^3).

These observations suggest that during MIT treatment, when the volume contained within wall segments connected by an equatorial wall band approached ca. 0.25 μm^3, a new site was formed.

DISCUSSION

Our previous studies have indicated that, in exponential-phase cells of *S. faecium*, sites of envelope synthesis produce pairs of poles whose size is constant (3) and that the initiation of these sites cannot be related to the timing of cycles of chromosome synthesis but can be related to the cytoplasmic volume contained by two wall segments joined by an equatorial wall band (4; A. L. Koch and M. L. Higgins, submitted for publication). Here we have shown that these characteristics of exponential-phase surface growth also can be observed in cells after DNA synthesis is inhibited by MIT.

The addition of MIT to an exponential-phase culture with a mass doubling time of 64 min was accompanied by inhibition of further cell division about 48 min later. Inhibition of further division led to the formation of elongated primary growth sites (Fig. 4) whose volume approached, but did not exceed, that of two completed poles measured in cells at the time the drug was added. Therefore, in treated and untreated cells, it appears that sites enlarge to the same maximum volume and then further enlargement stops. This maximum volume may be contained within two poles as in untreated exponential-phase cells or within elongated nonseparating sites as in MIT-treated cells. Thus, it seems that the maximum and constant output of a site is somewhat independent of the shape of the wall produced.

It had previously been shown that for small cells of *Escherichia coli* selected from sucrose gradients (2) and for exponential-phase cells of *Bacillus subtilis* (12), each subjected to a nutritional shift-up experiment, there is an increase in the rate of length extension during starvation for the required nutrient thymine. These findings suggest that there is an increase in the number of elongation sites which is not dependent on chromosome replication. On the other hand, the observation that in both organisms the linear

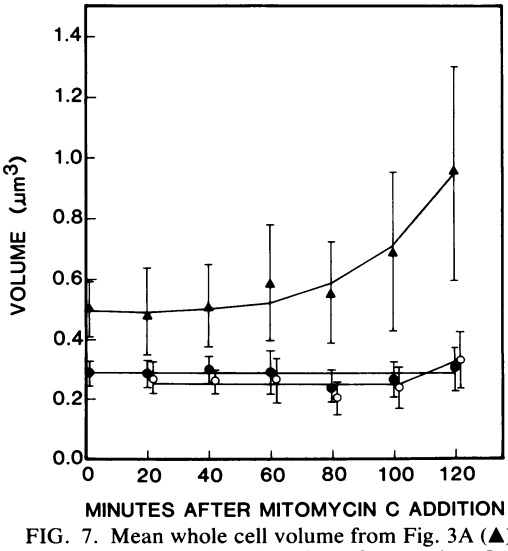

FIG. 7. Mean whole cell volume from Fig. 3A (▲). Split cell segments with birth sites (●; see Fig. 1C, I and III); split cell segments joined by a wall band but having no growth site (○; see Fig. 1C, II and IV).

rate of elongation does not change when DNA synthesis is inhibited (11, 12), which has been interpreted to mean there is no change in number of sites of elongation, is at variance with our findings. Obviously, the real problem in comparing these results to those obtained with *S. faecium* is that with rods, the pattern of envelope growth can only be studied indirectly by measuring rates of elongation, whereas with *S. faecium*, enumeration of growth sites can be achieved through unambiguous morphological measurements.

An early model of envelope growth in *S. faecium* suggested that ca. 30 min after chromosome replication began, a signal was generated to trigger growth site initiation, and completion of chromosome replication was prerequisite for septation (14). The results presented here suggest that it is fortuitous that wall sites initiate 30 min after the beginning of chromosome replication at some growth rates, but that termination of chromosome replication is required for normal pole formation and division. The observation that new wall growth sites are formed by MIT-treated cells as they increase in volume appears to confirm the conclusions of later studies which suggested that initiation of new sites of surface growth is independent of DNA replication (4; Koch and Higgins, submitted for publication).

To determine the mechanism that may regulate the initiation of new sites of envelope growth, two types of models are consistent with the results collected in this study. In one type of model, a band would split to form a new site when the cytoplasmic volume contained within the two segments of wall joined by a single band approaches 0.25 μm^3. The cell would turn on new sites (and also turn off old sites) by being able to account for the size (volume) of various portions of the cell. In a second type of model, sites are again turned on when the volume contained within two wall segments attached to a band approaches about 0.25 μm^3, not because the cell can measure volume but, rather, because at this size cells would experience an increase in turgor pressure (10). In this view, active transport and macromolecular synthesis would provide the turgor pressure necessary to split bands, and once a new site had been produced, the pressure would continue to split the septal wall into two layers of peripheral wall. As the septal radius decreased, the ability of a site to enlarge in surface area would become limited, and turgor pressure would increase owing to the continued synthesis of macromolecules. Growth would continue only if a new site(s) could be produced in response to this increase in pressure which would occur at a constant volume as mentioned above.

In conclusion, the results presented are consistent with a cell wall assembly mechanism in which (i) growth sites produce poles of constant size, (ii) initiation of new growth sites is independent of normal DNA synthesis, and (iii) newly initiated growth sites are found between wall segments containing a constant cell volume.

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