Effect of Inhibition of Deoxyribonucleic Acid and Protein Synthesis on the Direction of Cell Wall Growth in *Streptococcus faecalis*

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Selective inhibition of protein synthesis in *Streptococcus faecalis* (ATCC 9790) was accompanied by a rapid and severe inhibition of cell division and a reduction of enlargement of cellular surface area. Continued synthesis of cell wall polymers resulted in rapid thickening of the wall to an extent not seen in exponential-phase populations. Thus, the normal direction of wall growth was changed from a preferential feeding out of new wall surface to that of thickening existing cell surfaces. However, the overall manner in which the wall thickened, from nascent septa toward polar regions, was the same in both exponential-phase and inhibited populations. In contrast, selective inhibition of deoxyribonucleic acid (DNA) synthesis using mitomycin C was accompanied by an increase in cellular surface area and by division of about 80% of the cells in random populations. Little or no wall thickening was observed until the synthesis of macromolecules other than DNA was impaired and further cell division ceased. Concomitant inhibition of both DNA and protein synthesis inhibited cell division but permitted an increase in average cell volume. In such doubly inhibited cells, walls thickened less than in cells inhibited for protein synthesis only. On the basis of the results obtained, a model for cell surface enlargement and cell division is presented. The model proposes that: (i) each wall enlargement site is influenced by an individual chromosome replication cycle; (ii) during chromosome replication peripheral surface enlargement would be favored over thickening (or septation); (iii) a signal associated with chromosome termination would favor thickening (and septation) at the expense of surface enlargement; and (iv) a factor or signal related to protein synthesis would be required for one or more of the near terminal stages of cell division or cell separation, or both.

Considerable information has accumulated concerning the chemical structure and the enzymatic mechanisms for the biosynthesis of bacterial cell wall peptidoglycans and of the “accessory” wall polymers (20, 25). As yet little information is available concerning the regulation of wall biosynthesis or the integration of wall biosynthetic reactions with the many other processes which occur during cellular growth and division. Obviously an increase in volume of the protoplast requires an enlargement of the surface area of the cell which, in bacteria, includes the protective and relatively inflexible cell wall. The relationship between increases in volume and surface area is not a simple one, even in bacterial species of relatively simple shape (e.g., cocci and rods), since the geometry of the cell and its wall surface change during the cell division cycle (14, 22, 32).

The process of bacterial cell surface growth can be divided into at least four distinct and partially overlapping stages. These are: (i) enlargement of the cell wall surface; (ii) septation (or cross wall formation); (iii) daughter cell separation (after completion of the cross wall); and (iv) wall thickening. This last process, wall thickening, occurs in a variety of gram-positive species (14), most obviously under conditions of unbalanced growth (12, 17, 29). However, at least in *Streptococcus faecalis* (ATCC 9790), wall thickening has been shown to occur during, and to be a part of, the normal surface growth and division process in balanced, exponentially growing cultures (13).

In exponential-phase cultures of *S. faecalis*, wall thickening appears to (i) result from wall synthesis at a large number of synthetic sites on the entire coccal surface; (ii) occur more rapidly...
near the septal sites engaged in wall surface enlargement; and (iii) to be a self-limiting process, since greatly thickened walls are rarely seen (11, 12).

For some time it has been apparent that peptidoglycan and wall biosynthesis could be uncoupled from the biosynthesis of other cellular macromolecules (29). For example, in S. faecalis, cell wall synthesis continued after deprivation of a required amino acid that is not a component of the peptidoglycan, such as valine or threonine. After long periods of starvation, cells contained a higher percentage of wall substance per unit of cellular mass (29). The increase in cell wall substance was shown to be due to an increase in wall thickness over the entire coccal surface rather than to an increase in the cellular surface area-to-volume ratio. Subsequent studies showed that much of the wall thickening occurred within the first 2 to 3 h of valine or threonine starvation (12).

Since the most direct and obvious consequence of amino acid deprivation is termination of a further net increase in cellular protein, it has been assumed that wall thickening is directly related to the absence of protein synthesis. This interpretation is in agreement with results obtained using antibiotics to inhibit protein synthesis (5, 6). However, at the high concentrations commonly used, most of these antibiotics can affect more than one biosynthetic process (4, 10, 27). Furthermore, shortly after exposure to an antibiotic, secondary effects on other macromolecular and morphological parameters can be frequently observed.

We have now examined the effect of selective inhibition of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein synthesis on (i) various parameters of wall biosynthesis (peptidoglycan and rhamnose polysaccharide accumulation); (ii) wall surface growth (wall thickening and surface enlargement); and (iii) cell division (increase in number of cell units). Balanced, exponentially growing cultures were exposed to concentrations of inhibitors which relatively selectively inhibit DNA, RNA, or protein synthesis (4, 10, 27). Since selectivity of inhibition is frequently a matter of degree or time of exposure, or both, the kinetics of inhibition of accumulation of the various informational macromolecules was carefully monitored. With the biosynthetic parameters, determinations of cell numbers via a particle counter permitted the direct comparison of ultrastructural measurements (e.g., wall thickness) made on individual cells. Many of the changes observed appear to be exaggerated manifestations of events which occur during the normal cell division cycle. Therefore, we have also attempted to derive information concerning the regulatory influences of informational macromolecules on processes involved in normal surface enlargement and cell division. In fact, the data have been used to derive a tentative scheme for some of the events occurring during the surface growth and division cycle in S. faecalis.

MATERIALS AND METHODS

Cell growth and biochemical analyses. Cells were grown as described previously in a chemically defined medium modified from that of Gallant and Toennies (28). Increase in mass was monitored turbidimetrically (28). The relative amount of DNA, RNA, and protein synthesis was determined by the rates of incorporation of 14C- or 3H-labeled thymidine, uracil, and leucine into trichloroacetic acid-precipitable products. Peptidoglycan synthesis was determined similarly by using 14C-[l-lysine and a recently described method (1, 2). To use incorporation of radioactive isotopes as a measure of net macromolecular biosynthesis, cells were grown in the presence of each precursor for six to seven mass doublings. Under these conditions, the rate of isotope incorporation into trichloroacetic acid-precipitable material is directly proportional to the growth rate of the culture (26). The amounts and specific activities of the various macromolecular precursors used were as follows: for DNA, [3H]-thymidine (1.5 μCi/ml, 0.05 μCi/μg); for RNA, [14C]-uracil (0.1 μCi/ml, 0.005 μCi/μg); for protein, L-14C-leucine (0.2 μCi/ml, 0.005 μCi/μg); for peptidoglycan, L-[14C]-lysine (0.125 μCi/ml, 0.005 μCi/μg). Rhamnose was determined by using a semi-micro modification of the procedure of Dische-Shetlles (8).

In some instances, the data obtained from incorporation of precursors by antibiotic-treated cells are expressed as "percent inhibited" as compared with synthesis in an untreated control (4, 27). All precursor incorporation values were related to the amount of precursor incorporated at the time of antibiotic addition, which was given a relative value of 1.0. In an untreated control culture (log phase) with a doubling time of 30 min, the amount incorporated at 30 min would be 2.0. A treated culture exhibiting at 30 min a relative incorporation value of 1.75 would be considered to be inhibited by 25% with respect to the control, or [(1.75-1.0)/(2.0-1.0)] × 100 = 75% of the control, or 25% inhibited. This treatment was necessary in order to compare nonexponential increase in cellular parameters. This expression of the biosynthetic data is useful in defining selective rates of inhibition (4, 27). A linear, rather than exponential, treatment was used, in view of the lack of exponential increase in the antibiotic-treated cultures.

Antibiotics were added to exponentially growing (log) cultures at a turbidity equivalent of 136 μg/ml (dry weight) after six to eight mass doublings in the exponential phase. The antibiotics were chloramphenicol (50 μg/ml; CAP; Parke, Davis and Co.); 5-azacytidine, (5 μg/ml; AZAC; Calbiochem); actinomycin D (0.25 to 5.0 μg/ml; ACT; Merck, Sharp
and Dohme); rifampin (0.1 µg/ml; RIF; Dow Chemical Co.); and mitomycin C (0.5 µg/ml; MIT; Nutritional Biochemicals Corp.).

Samples for cell counting were fixed in Formalin (final concentration, 10%) for 1 h on ice, diluted to between 0.08 and 0.24 µg (dry weight) of bacterial substance per ml in 0.85% NaCl, and counted as soon as possible in a model B Coulter counter (Coulter Electronics, Chicago, Ill.) equipped with a 30-µm diameter orifice. For cell volume determinations, the Coulter counter was calibrated with Dow latex beads (1.171 µm in diameter).

The glutaraldehyde-osmium tetroxide fixation, Epon 812 embedding, and uranyl acetate-lead citrate staining have been described (9, 12).

**Measurements of cell wall thickness.** Measurements were taken from cells showing a tri-banded profile around virtually the entire cell perimeter. Such cells have been observed to be central and longitudinal (12). Figure 1A shows a density scan of an exponential-phase cell envelope (the dashed lines in Fig. 2A show the limits of the area scanned to produce Fig. 1A). The cell wall thickness was defined as the distance between the outer limits of the outer (C1, Fig. 1A) and inner (C3, Fig. 1A) electron-dense bands of the cell wall. An equal distribution of measurements of older, thicker poles and the newer, thinner equatorial regions were obtained by (i) visually separating cells showing a nascent cross wall into two daughter cells (Fig. 3A, B), and (ii) taking a single measurement of thickness of both the old and new wall portion of each daughter cell.

The measurements were made directly from electron image plates exposed at about 30,000 (microscope calibrated daily with a carbon grating replica) with a Bausch and Lomb dissection microscope at ×10 equipped with an ocular micrometer. The measurements were repeated at least twice on separate days, usually by two observers, on a minimum of 40 cells per experimental point.

**RESULTS**

Effects of the selected concentrations of inhibitors on increase in cell numbers and on the synthesis of cellular mass, DNA, RNA, protein, peptidoglycan, and rhamnose-containing polysaccharide. The effects of the antibiotics on the incorporation of the appropriate precursor into DNA, RNA, and protein are shown in the graphs on the left in Fig. 4. With CAP (50 µg/ml) and AZAC (5 µg/ml), further incorporation of leucine ceased immediately, whereas the incorporation of uracil and thymidine continued at the exponential rate for 15 min or more. These two compounds were thus considered to be relatively specific inhibitors of protein synthesis. At this concentration, AZAC has no effect on RNA synthesis for 60 min (31) and the inhibition of protein synthesis reverses itself 20 to 30 min after exposure, consistent with other reports (4, 27).

Both RIF (0.1 µg/ml) and ACT (0.25 µg/ml) inhibited RNA accumulation extremely rapidly, but also inhibited protein synthesis with equal speed. This was probably due to the close coupling of these two syntheses in this species. Secondary to these effects was a delayed and
Fig. 2. Typical thin section views of normal and antibiotic-treated envelopes. Electron micrographs were selected to show the morphology of normal and treated envelopes and do not reflect the average wall thickness values shown in Fig. 5. Dashed lines indicate scanned areas shown in Fig. 1A and B. (A) Log = a normal exponentially dividing cell; (B) CAP 40' = a cell treated with 50 μg of CAP per ml for 40 min; (C) AZAC 40' = a cell treated with 5 μg of AZAC per ml for 40 min; (D) ACT 30' = a cell treated with 0.25 μg of ACT per ml for 30 min; (E) RIF 30' = a cell treated with 0.1 μg of RIF per ml for 30 min; and (F) MIT 45' = a cell treated with 0.5 μg of MIT per ml for 45 min. Bar in (A) applies to all micrographs and equals 100 nm.

Fig. 3. Diagrammatic representation of a central longitudinal section of a typical exponential phase cell. The new cell wall being produced in the current generation is shown unstippled, and the old polar wall produced in past generations is stippled. Wall bands ring the outer cell wall surface of the organism and serve as lines of demarcation between new equatorial wall and old polar wall. The mesosome (M) of this organism is usually attached to the septum by a stalk. For purposes of quantitating the average cell wall thickness, cells were visually divided into two daughter cells, A and B, and measurements were drawn equally from old and new regions. Measurements were only taken from cells with a tri-banded wall profile around virtually the entire cell perimeter.

less extreme inhibition of the rates of DNA and mass increases. Therefore, RIF and ACT were considered to be inhibitors of both RNA and protein synthesis.

MIT (0.5 μg/ml) inhibited DNA accumulation and had only relatively little and delayed effects on RNA and protein synthesis. MIT was thus considered to be a relatively specific inhibitor of DNA synthesis.

Since none of these inhibitors was selective in an absolute sense (as expected), the data were interpreted quantitatively in terms of relative speed and extent of inhibition.

The graphs on the right in Fig. 4 show the
kinetics of the effects of each treatment on increase in cell number and accumulation of cell wall substance (both peptidoglycan and rhamnose-containing wall polysaccharides). All of the compounds which rapidly and severely inhibited further protein synthesis (CAP, AZAC, ACT, and RIF), irrespective of their inhibitory effect on RNA synthesis, had nearly the same effects on both cell wall synthesis and cell division. All four substances permitted (i) only a small (5 to 15%) increase in cell numbers, and (ii) continued synthesis of both wall polymers, but at rates somewhat reduced from that of untreated controls. The combination of little increase in cell number with a substantial increase in cell wall polymers resulted in an overall increase in amount of wall substance per cell (see below).

In contrast, inhibition of further DNA synthesis by MIT permitted cell division at the control rate, resulting in an 80% increase in cell number. MIT treatment also permitted the two wall polymers to continue to accumulate at nearly the control rate for about 40 min, at which time
their rates of accumulation slowed. In this case, continued cell division in parallel with wall polymer accumulation did not result in an increase in wall substance per cell, at least during the first 20 min (see below).

Correlation of wall thickening with inhibition of protein synthesis and not with inhibition of DNA, RNA, or continued peptidoglycan synthesis. A striking correlation between the kinetics of the inhibition of protein synthesis and the increase in wall thickness was observed (Fig. 5). For example, CAP and ACT treatments inhibited protein synthesis most rapidly, and 10 or 15 min after treatment the walls of these cells were substantially thicker than cells from AZAC- or RIF-treated cultures.

In contrast, 15 min after MIT addition, an increase in average wall thickness was not observed (Fig. 5). Only later, at 45 min after treatment, was a relatively small increase in average wall thickness seen, concomitant with the observed secondary effects of this antibiotic on protein (and RNA) synthesis (Fig. 4, MIT A]).

The speed or extent of inhibition of RNA synthesis did not correlate with wall thickening. Some of the agents used inhibited RNA synthesis rapidly (e.g., ACT and RIF), whereas others caused little (e.g., CAP) or no (e.g., AZAC) inhibition of RNA synthesis (Fig. 4A). Outstanding in this respect was AZAC, which failed to affect the rate of RNA synthesis (4, 27) but which resulted in substantial wall thickening.

Wall thickening also appeared not to be related to the rate of peptidoglycan (or rhamnose-containing polysaccharide) synthesis. With all of the inhibitors used, peptidoglycan synthesis continued at virtually the same rate as that measured for log-phase cells for a period of 10 to 15 min and, in the case of MIT treatment, even longer (Fig. 4B).

Ultrastructure and analysis of the frequency distribution of wall thickness measurements. Thickened walls retained the tri-banded profile of log-phase cells (Fig. 2). Comparison of density scans of the wall profiles (Fig. 1) showed that increased wall thickness was primarily accounted for by thickening of the middle, less dense portion of the wall (C2 in Fig. 1).

As observed previously (12), the distribution of wall thickness measurements of log-phase cells was skewed toward the thinner values (Fig. 6, log; Table 1). Increases in average wall thickness were accompanied by (Fig. 5): (i) a substantial decrease in frequency of walls in the thinner wall classes (e.g., classes 1 and 2 in Table 1); (ii) an increase in frequency of walls in

![Figure 5](http://jb.asm.org/) Comparison of the kinetics of inhibition of protein synthesis with the kinetics of increase in average cell wall thickness. Cell wall thicknesses were measured on central, longitudinal sections as described in Materials and Methods and are expressed as increases or decreases (in nanometers) relative to the average wall thickness of exponential-phase cells (27.8 nm). The method for determining and comparing protein synthesis on a percent basis is described in Materials and Methods.
the intermediate class (class 3 in Table 1), and very thick wall (classes 4 and 5 in Table 1); and (iii) an increase in symmetry of distribution for the thickness measurements (Fig. 6).

Upon inhibition of protein synthesis, all of the above changes were seen to occur at a speed commensurate with the speed of inhibition of protein synthesis (Fig. 5). Since the measurements were equally distributed between the equatorial and polar regions of the cell (see Materials and Methods), the rapid decrease in the thinner wall classes and the increase in symmetry of the frequency distribution measurements are consistent with a more rapid wall thickening of thinner and newer wall (i.e., near nascent septal sites primarily engaged in wall enlargement). Increases in frequency of the thicker wall classes and the appearance of a class of thick-walled cells not found in log-phase cells (12) (striking in the case of ACT treatment for 70 min) appeared relatively slowly. Such a pattern is consistent with wall thickening occurring over the entire wall surface.

MIT treatment for 15 min resulted in a small increase in the thinner wall classes and reduction in the average wall thickness measurements (Table 1 and Fig. 5). At 45 min after MIT addition, when other parameters of growth such as rates of increase in cell numbers and RNA and protein synthesis were significantly affected (Fig. 4) and a small increase in average wall thickness was observed (Fig. 5), a shift in frequency distribution was also seen (Table 1 and Fig. 6). At this late time, the frequency of walls of intermediate thickness (class 3) was increased at the expense of the thinner-walled classes.

**Correlation of wall thickening with inhibition of cell division.** As discussed above, treatment with four drugs, CAP, RIF, ACT, and AZAC, was almost immediately accompanied by a severe inhibition of further cell division after an increase in cell numbers of 5 to 15% (Fig. 4B). In contrast, MIT permitted cell division to occur for a longer time interval and to a much greater extent. In all five cases, synthesis of wall polymers continued, but only with the first four drugs was significant wall thickening observed. On the basis of these observations, the simple hypothesis was made that wall thickening is a consequence of continued wall polymer accumulation in cells in which cell division (and perhaps also surface enlarge-

**Table 1.** Frequency distribution of wall thickness measurements in cultures treated with chloramphenicol, 5-azacytidine, and mitomycin C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time of exposure (min)</th>
<th>Wall thickness measurements (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Class 1 (23.8 to 27.2 nm)</td>
<td>Class 2 (27.2 to 30.6 nm)</td>
</tr>
<tr>
<td>LOG</td>
<td>2.9</td>
<td>56.1</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>10</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.4</td>
</tr>
<tr>
<td>5-Azacytidine</td>
<td>10</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.0</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>15</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>4.6</td>
</tr>
</tbody>
</table>

**Fig. 6.** Histogram analysis of cell wall thickness measurements of normal and antibiotic-treated cells. The dashed line indicates the average thickness of the normal exponentially dividing cell population (log). The symbols indicating the type and time of antibiotic treatment are as in Fig. 2. The method of measuring cell wall thicknesses is given in Materials and Methods.
ment) was inhibited. If this was the case, then the kinetics of increase in amounts of wall polymers accumulated per cell should agree with the observed increase in wall thickness (already on a per-cell basis), regardless of the specific effect of each treatment on either cell division or wall polymer biosynthesis.

Comparison of these two parameters (wall thickness and amount of peptidoglycan per cell) on the same basis (Fig. 7) showed that the kinetics of increases in amounts of peptidoglycan per cell were in good agreement with the kinetics of increases in relative cell wall thickness (Fig. 7A). The largest and most rapid increases in amounts of peptidoglycan per cell (Fig. 7B) were observed for those treatments (CAP, ACT, RIF) which resulted in only a small increase in cell numbers. MIT treatment was not accompanied by an increase in peptidoglycan per cell for over 30 min (Fig. 7B), nor was an increase in average wall thickness observed until quite late after treatment (Fig. 7A; 45 min). This correlation was observed even though synthesis of wall polymers occurred at a rate very close to that for log-phase cells for at least the first 40 min (Fig. 4, MIT [B]).

Perhaps the most convincing is the case of the AZAC-treated cultures, where a superficial inspection of Fig. 7A and B fails to reveal a correlation. However, AZAC transiently and reversibly inhibited protein synthesis, mass increase, and cell division (Fig. 4). Thus, during the 20-min period of inhibition, the relative amount of peptidoglycan per cell and average wall thickness increased. Resumption of cell division, about 20 min after exposure, resulted in a decrease in amount of peptidoglycan per cell (Fig. 7B), which at later times (e.g., at 40 min) approached the amounts seen in log cultures (i.e., 1.0). Between 20 and 40 min, a corresponding decrease in average wall thickness was not seen (Fig. 7A). Since polar (and in this case, thickened) wall is conserved, the continued presence of thickened polar wall would contribute to the measurements and would require several generations of recovery from AZAC to approach the distribution of thickness measurements of the untreated controls.

Role of surface enlargement in wall thickening. The unexpectedly good correlation between relative amounts of peptidoglycan per cell and relative wall thickness suggested that not only was the presence or absence of continued cell division a factor in the occurrence of wall thickening, but the presence or absence of an increase in surface area per cell may also play a role. Surface enlargement and cell division may not always be closely coupled since, under some circumstances, nondividing cells can increase in volume and in the surface area required to enclose the increased volume (for a recent review see ref. 14). Therefore, the effects of inhibition of DNA and protein synthesis on the relationship between surface area enlargement and wall thickening were examined. For these experiments, ACT was used at a concentration of 5 μg/ml to rapidly and severely inhibit DNA synthesis. ACT rather than MIT was used since the latter, even at high concentrations, failed to act as rapidly on DNA synthesis (L. Daneo-Moore, unpublished observations).

Treatment with 5 or even 1 μg of ACT per ml
Table 2. Effects of 40 min of treatment with chloramphenicol and actinomycin D, alone and in combination, on cell wall thickening, increase in cell numbers, and protein and DNA synthesis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WALL THICKNESS INCREASE (nm ± SD)</th>
<th>Relative increase in*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Increase</td>
</tr>
<tr>
<td>None</td>
<td>27.8 ± 2.2</td>
<td>2.32</td>
</tr>
<tr>
<td>Chloramphenicol (50 μg/ml)</td>
<td>32.5 ± 3.0</td>
<td>4.7</td>
</tr>
<tr>
<td>Actinomycin D (5 μg/ml)</td>
<td>31.1 ± 2.1</td>
<td>3.3</td>
</tr>
<tr>
<td>Chloramphenicol plus Actinomycin D (5 μg/ml)</td>
<td>31.5 ± 2.0</td>
<td>3.7</td>
</tr>
</tbody>
</table>

*Calculated as relative increases from zero time values of 1.0.

Figure 8. Effect of various concentrations of actinomycin D on cell wall thickness and amount of peptidoglycan per milliliter of culture with time. Actinomycin concentrations, in μg/ml, are: (O) 0.25, (△) 1.0, and (▼) 5.0. (A) Increases in wall thickness; (B) increase in peptidoglycan per milliliter of culture.

Table 3. Effects of exposure of exponential-phase cells to various concentrations of actinomycin D for 15 min on increases in cell volume, cell numbers, and DNA synthesis

<table>
<thead>
<tr>
<th>Actinomycin D (μg/ml)</th>
<th>Mean cell volume</th>
<th>Cell no.</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.00</td>
<td>1.35</td>
<td>1.35</td>
</tr>
<tr>
<td>0.25</td>
<td>1.09</td>
<td>1.14</td>
<td>1.20</td>
</tr>
<tr>
<td>1.0</td>
<td>1.17</td>
<td>1.18</td>
<td>1.06</td>
</tr>
<tr>
<td>5.0</td>
<td>1.19</td>
<td>1.12</td>
<td>1.01</td>
</tr>
</tbody>
</table>

*Calculated as relative increases from zero time values of 1.0.

For exponential-phase cells.

In contrast to cells in balanced exponential growth, wall thickening after antibiotic treatment does not seem to be self-limiting but does appear to proceed as seen in log cells; that is, those regions associated with the septum appear to thicken more rapidly than the wall found in the poles (13, 14). Since in both cases thickening occurs along this same gradient, it seems attractive to consider the antibiotic-induced process as the rapid and concerted expression of potential synthetic sites normally engaged in conditions there is either a decrease in rate of wall biosynthesis or an increase in cellular surface area. These two possibilities were investigated further by comparing the results of treatment with ACT concentrations of 0.25, 1.0, and 5.0 μg/ml on peptidoglycan synthesis and average cell volume.

The effect of ACT on peptidoglycan synthesis was almost the same at three concentrations tested (Fig. 8B). That is, peptidoglycan synthesis continued at nearly the control rate for about 10 min before synthesis slowed. At 15 min the amount of peptidoglycan made was only 10% less than in the untreated control. Also, all three concentrations of ACT inhibited cell division to about the same extent (Table 3). However, only the two higher concentrations of ACT (1 and 5 μg/ml) rapidly halted DNA synthesis (Table 3). The two higher ACT concentrations also permitted a greater increase in average cellular volume than did the lower (0.25 μg/ml) concentration. It seems that superimposing a rapid and severe inhibition of DNA synthesis on cells in which protein synthesis and cell division are inhibited permits an increase in cell volume but permits less wall thickening. Since the rates of peptidoglycan synthesis were virtually identical, inhibition of DNA synthesis appears to favor surface enlargement at the expense of wall thickening.

DISCUSSION

In contrast to cells in balanced exponential growth, wall thickening after antibiotic treatment does not seem to be self-limiting but does appear to proceed as seen in log cells; that is, those regions associated with the septum appear to thicken more rapidly than the wall found in the poles (13, 14). Since in both cases thickening occurs along this same gradient, it seems attractive to consider the antibiotic-induced process as the rapid and concerted expression of potential synthetic sites normally engaged in
the slower thickening of walls of log cells. Factors other than availability of precursor are probably involved in limiting ultimate wall thickness, since the extent of thickening of the two polar caps of log cells appears to be regulated. A decreased efficiency of interaction of membrane-bound enzymes with precursors, carriers, and/or wall acceptor could provide a surface age-dependent decrease in rate of thickening. A preferential thickening of newer, equatorial wall after starvation for valine or threonine was observed previously (12).

In antibiotic-treated cells, it seems clear that both peptidoglycan and wall polysaccharides containing rhamnose continue to accumulate even after substantial inhibition of either DNA, RNA, or protein synthesis (Fig. 4). In all cases, except perhaps for ACT treatment (Fig. 4, ACT [B]), the kinetics of increase in the two types of wall polymers examined were nearly the same, indicating the absence of a change in overall composition of the wall during treatment, and strongly suggesting that the synthesis of the two covalently linked polymers are closely coordinated. The addition of new teichoic and teichuronic acid moieties to only concurrently synthesized peptidoglycan (19), and the absence of appreciable dissociation of teichoic acid from peptidoglycan synthesis after chloramphenicol treatment (16), both occurring in Bacillus subtilis, also support the idea that the biosynthesis of covalently linked wall polymers is coordinated.

In the interpretation of the thickening observed after inhibition of protein synthesis, three presumably related features seem to be of importance. First is the severe inhibition of further cell division observed 5 to 10 min after inhibition of protein synthesis, but not observed very soon after inhibition of DNA synthesis (Fig. 4B). Second is the relatively rapid inhibition of an increase in cellular surface area to enclose the increased cell volume. The close agreement between the kinetics of wall thickening with peptidoglycan accumulation per cell (Fig. 7) indicated a lack of increase in surface area. The data from the culture treated so that both protein and DNA synthesis were concomitantly and severely inhibited confirmed it (Tables 2 and 3; Fig. 8). Third is the similarity between the kinetics of inhibition of protein synthesis and the kinetics of wall thickening (Fig. 5).

The extent to which newly synthesized wall polymers are incorporated into enlarged or thickened wall could depend on the rate of surface enlargement and on the stage of the cell division cycle at that instant. Integration of the analysis of morphological and biochemical alteration observed upon treatment of exponential-phase populations with appropriate inhibitors (21) permitted us to derive a model for the regulation of cell surface growth and division.

The chromosome replication time (C time) can be calculated from the relative amount of DNA synthesized after inhibition of protein synthesis (39%; see Fig. 4, CAP) (23). Calculated in this manner, the C time for S. faecalis is approximately 50 to 52 min, only a little longer than the C time of Escherichia coli.

The fraction of the population which goes on to divide after inhibition of DNA synthesis (80%; see Fig. 4, MIT [B]) can be used to derive the time between the termination of a round of chromosome replication and the subsequent cell division (D time of Helmstetter [7]). This value was found to be 25 to 28 min for S. faecalis. This value is similar to a D time of 20 to 25 min found for E. coli (3, 18) and Salmonella (30). Cell division therefore appears to be related to DNA synthesis through a signal associated with termination of chromosome replication.

Finally, from the number of cells in a random exponential-phase population which goes on to divide after inhibition of protein synthesis (5 to 16%; see Fig. 4 and Table 2) and the generalized growth equation, it can be determined that protein synthesis is not required for about 5 min before final daughter cell separation. This time must be at the end of the cell division cycle and must be an integral part of the D time as defined by Helmstetter (7). The overlapping sequences of C and D times in an S. faecalis culture dividing every 30 min are shown in Fig. 9.

On the basis of the observation that protein synthesis is not required for the last 5 min before cell separation, we have divided the D time into two components: D1 (20 min in Fig. 9), during which protein synthesis may be partially or totally required, and D2 (5 min in Fig. 9), during which protein synthesis is no longer required. Therefore, the S. faecalis regulatory scheme is similar but not identical to that proposed by Zusman et al. (33) for E. coli. They

\[ N(t) = N_0 \times e^{r \times t} \]

where \( N \) is the final number of cells observed after antibiotic addition at a cell density of \( N_0 \) and \( t \) (in minutes) is the time in the cell division cycle (actually before final cell separation) of the event crucial to cell division which is affected by the antibiotic. In this discussion, the D time is the time in the cell division cycle obtained after inhibition of DNA synthesis. The D1 time is the time in the cell division cycle obtained in the same manner, but from residual division after inhibition of protein synthesis. The \( D_2 \) time is defined as \( D_1 \) - \( D_2 \), and therefore represents a time between completion of chromosome replication and that later division event which is prevented by inhibition of protein synthesis.

\[ D = D_1 - D_2 \]

\[ N(t) = N_0 \times e^{r \times t} \]
propose a scheme for the cell division process where \( \alpha \), which is related to termination of chromosome replication, interacts with \( \beta \), which is related to protein synthesis.

The relationship observed among chromosome replication, surface enlargement, and cell division must be site specific. This requirement comes from the observation of the initiation of secondary, subequatorial wall growth sites (nascent septa) well before the completion of the primary central cross wall, at least in rapidly growing cultures (13, 14; Fig. 9). At certain times in the cell cycle these secondary sites are primarily engaged in peripheral wall synthesis (and surface enlargement), whereas the primary site is septating the protoplasm. Thus, whereas the primary equatorial site would be under the control of the replication cycle of one chromosome, the secondary sites would be under the control of the daughter cell’s replication cycle. In this way, regulation of surface enlargement and division at each site would occur independently. Communication between a chromosome and a septal wall growth site could be mediated via septally associated mesosomes, as proposed previously (10). In S. faecalis, selective inhibition of DNA synthesis was accompanied by a decrease in mesosome size, whereas inhibition of protein synthesis (and cell division) was accompanied by an increase in mesosome size.

Prevention of termination of chromosome replication, by means of inhibition of DNA synthesis in the middle of a round of replication, permits increases in cellular mass (Fig. 4) and volume (Table 3). Similar observations have been made in other species (3, 15, 24). Thus, during rounds of chromosome replication, surface enlargement can and does occur.

In the present study, wall thickening was least in cells in which chromosome replication had not been completed, i.e., in populations blocked during C time (see Fig. 9). In contrast, wall thickening was most pronounced in cells which failed to exhibit surface enlargement and division after inhibition of protein synthesis. These cells were blocked primarily in the Dz stage of the cell cycle (Fig. 9); therefore, cell surface enlargement and wall thickening appeared to be temporally separable during the cell cycle.

Our observations are most consistent with a model in which each chromosome replication cycle controls one surface enlargement site, with cessation of surface enlargement occurring after completion of chromosome replication. Cell wall thickening during the normal cell division cycle would occur primarily during the D time, secondarily to cessation of surface expansion. Although it remains possible that wall thickening is merely a consequence of decreased competition for wall precursors and is unrelated to events in the cell division cycle, this seems unlikely for several reasons. First is the occurrence of a defined pattern of wall thickening as a normal part of surface growth in exponentially growing and dividing cells (13). Second is the close correlation of the kinetics of wall thickening with the kinetics of inhibition of protein synthesis and the observed absence of thickening shortly after the inhibition of DNA synthesis. Last are the observations made on doubly inhibited cells. Because of possible unknown secondary effects and decreased specificity upon exposure to higher concentrations of ACT, this last set of observations is open to some question. However, the effect of the concentrations of ACT which inhibited DNA as well as protein synthesis on wall thickening was very similar to that of MIT inhibition of DNA synthesis. These effects differed in that ACT inhibited cell division and an increase in average cell volume was observed, whereas MIT permitted substantial cell division. Both increased average cell volume and cell division require more surface.

The model presented here is purely descriptive. Elsewhere, a mechanism has been proposed to account for the morphological and regulatory features of the cell surface expansion process in S. faecalis (G. D. Shockman, L. Daneo-Moore, and M. L. Higgins, Ann. N.Y. Acad. Sci., in press).
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LITERATURE CITED