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Relationship Between Cellular Autolytic Activity, Peptidoglycan Synthesis, Septation, and the Cell Cycle in Synchronized Populations of *Streptococcus faecium*

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Synchronized, slowly growing (T_D = 70 to 80 min) cultures were used to study several wall-associated parameters during the cell cycle: rate of peptidoglycan synthesis, septation, and cellular autolytic activity. The rate of peptidoglycan synthesis per cell declined during most of the period of chromosome replication (C), but increased during the latter part of C and into the period between chromosome termination and cell division (D). An increase in cellular septation was correlated with the increased rate of peptidoglycan synthesis. Cellular autolytic capacity increased during the early portion of C, reached a maximum late in C or early in D, and declined during D. Inhibition of DNA synthesis during C prevented the decline in autolytic capacity at the end of the cell cycle, caused a slight reduction in the rate of peptidoglycan synthesis, delayed but did not prevent septation, and prevented the impending cell division by inhibiting cell separation. Inhibition of DNA synthesis during D did not prevent the increase in autolytic capacity during the next C phase, but, once again, prevented the decline at the end of the subsequent cycle. Thus, increased autolytic capacity at the beginning of the cell cycle did not seem to be related to chromosome initiation, whereas decreased autolytic capacity at the end of the cell cycle seemed to be related to chromosome termination. The data presented are consistent with the role of autolytic enzyme activity in the previously proposed model for cell division of *S. faecium* (G. D. Shockman et al., Ann. N.Y. Acad. Sci. **235**:161-197, 1974).

A critical link between chromosome replication and cell division has been shown to exist in several bacterial species (4, 14, 32). This link could be mediated through a direct association between the chromosome and the cell surface (20, 27, 33, 34). In some organisms, inhibition of chromosome replication appears to prevent cell division by specifically stopping septation but allowing cellular elongation to continue (14, 32). Such an alteration of the mode of surface synthesis probably reflects changes in the synthetic pattern of peptidoglycan (PG), the shape-maintaining component of bacterial walls (6a). PG hydrolase activities, which may play a role in the wall assembly process (6a), could be influenced by changes in the wall synthetic pattern that accompany inhibition of chromosome replication.

Previous studies in this laboratory have shown that in *Streptococcus faecium* (*S. faecalis* ATCC 9790) the pattern of wall synthesis is influenced by DNA replication. More specifically, in asynchronous, exponential-phase cultures, inhibition of DNA replication by mitomycin C (MIT C) allowed wall elongation to continue at the expense of septation, resulting in elongated cells (16, 21). In addition, cellular autolytic capacity was not appreciably affected by inhibition of DNA synthesis (28). These and many other data were used as a basis for a model in which maximal cellular autolytic activity is responsible for the splitting of nascent cross walls into peripheral wall during the period of chromosome replication (29). Chromosome termination is followed by decreased wall splitting, cross wall closure, and cell division.

The experiments presented here were intended to examine some aspects of this model in slowly dividing synchronized populations of *S. faecium*. The patterns of wall PG synthesis, cellular autolytic capacity, and septation in relationship to the chromosome replication and cell division cycles were examined. Furthermore, the effect of inhibition of DNA synthesis at specific times in the cell cycle on these parameters was used as an additional probe of the validity of the model.

MATERIALS AND METHODS

Growth conditions, sucrose gradient selection,

determinations of cell numbers and rates of cellular autolysis, steady-state labeling techniques, and approximation of the cell cycle. These methods are all described in the two previous papers (22, 23).

Determination of PG synthesis by using pulse and continuous labeling. At 5-min intervals during growth of synchronized cultures, 0.15-ml samples were pipetted into 0.15 ml of growth medium containing L-[4,5-^{3}H]lysine (30 μg/ml, 5.0 μCi/ml, 0.167 μCi/μg; Amersham/Searle, Arlington Heights, Ill.) in a 37°C water bath. Each 4-min pulse was stopped by the addition of 2 ml of 10% trichloroacetic acid containing 100 μg of unlabeled lysine per ml. Incorporation of [^{3}H]lysine (30 μg/ml, 1.0 μCi/ml, 0.033 μCi/μg) into PG was also measured by using fully equilibrated labeling conditions (22). Cold trichloroacetic acid precipitates were stored on ice for at least 1 h (usually overnight). Unlabeled carrier cells (0.1 to 0.2 mg) were added. The samples were then treated by the method of Boothby et al. (3) and counted (22).

Determination of fraction of cells containing septa. Visualization of septa was enhanced by using UV microscopy of acridine orange-stained cells by a modification of the method of Siccardi et al. (31). A drop of cell suspension was fixed in 8.3% Formalin and allowed to dry on a marked area on a clean slide. The slides were soaked in McIlvane buffer (0.06 M citric acid–0.06 M sodium dibasic phosphate, adjusted to pH 3.8 with sodium hydroxide) for 20 min, drained, briefly washed in 95% ethanol and then McIlvane buffer, drained again, placed in 0.1% acridine orange, and protected from the light. After 20 min, the slide was removed, drained, washed twice in McIlvane buffer, dried, and kept in the dark until used. Slides were examined under oil immersion (×800) with a Zeiss UV microscope for the relative percentages of single (unseptated) cells, double (completely septated, but not separated) cells, triple cells, and chains. At least 200 cells from several fields were counted for each determination.

Inhibition of DNA synthesis with MIT C. Solutions of MIT C (200 μg/ml; ICN Pharmaceuticals) were stored at −20°C in the dark and used at a final concentration of 0.5 μg/ml. This concentration was previously shown (28) to inhibit DNA synthesis without significantly affecting the rate of RNA or protein synthesis for periods of 45 min or more.

RESULTS

Rate of PG synthesis during the cell division cycle. The rates at which 4-min pulses of [^{3}H]lysine were incorporated into insoluble PG changed during the cell division cycle (Fig. 1). In the two experiments shown (Fig. 1), the rate of incorporation of lysine into PG was relatively constant for intervals of 10 to 20 min at the end of the initial plateau in cell numbers. Five to fifteen minutes after cell numbers began to increase (cell division and separation), the rate of incorporation accelerated to reach a new constant rate, which was approximately twice the previous rate, early to midway in the second plateau in cell numbers. This process was repeated in the second cell division cycle, again with nearly a doubling in rate of incorporation. Results of the two separate experiments (Fig. 1) are presented to convey an impression of the degree of variability encountered.

To more easily relate these data to the cell division cycle, rates of incorporation of pulses into PG and PG accumulation (determined by continuous labeling) per cell unit were calculated and plotted (Fig. 2A and B, respectively). In general, periods of maximum accumulation followed slightly behind periods of maximum rate of incorporation of pulses, and the maxima for both measures of PG synthesis peaked during plateaus in cell number (corresponding to the middle to the end of the period between chromosome termination and cell division [D]). Since cell division synchrony was less than perfect, expression of these parameters on a per-cell basis may accurately reflect only cell cycle events which occur during the plateaus in cell numbers.

Comparisons of the rate of PG synthesis per cell during each sequential cycle showed a decreasing rate, particularly notable in two of the three experiments (Fig. 2A). A similar decline

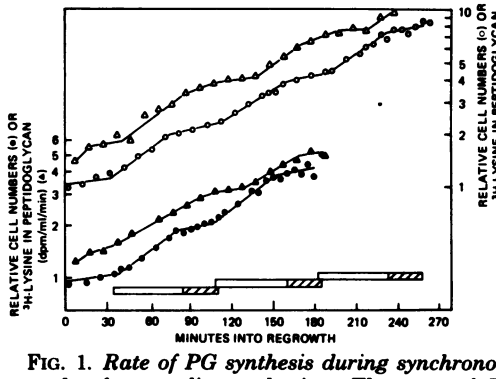

FIG. 1. *Rate of PG synthesis during synchronous growth after gradient selection. The rate of PG synthesis was determined by the incorporation of [^{3}H]lysine into PG during 4-min pulses. For purposes of clarity, the data are plotted as "relative increase" of an arbitrary value at time zero. Symbols: [^{3}H]-lysine (dpm/ml per min) in PG (△, ▲); cell numbers, (○, ●). The open and closed symbols represent two separate experiments; the open triangles are the average of three pulses, and the closed triangles are the average of two pulses. In a typical experiment (▲), the mean zero-time value for the PG pulse is 9,600 dpm/ml per min. The bar diagrams at the base of the graph depict the cell cycle as estimated from the pattern of DNA synthesis described elsewhere (22). The unshaded portion of the bar represents the period of chromosome replication (C ≅ 50 min), and the shaded portion indicates the period after chromosome termination before division (D ≅ 25 min).*

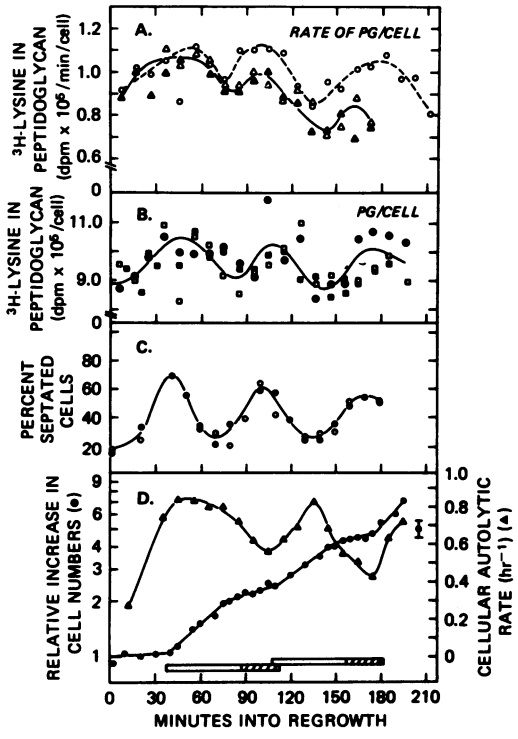

FIG. 2. *Comparison of (A) rate of PG synthesis per cell; (B) PG accumulation per cell; (C) percentage of septated cells; and (D) cellular autolytic capacity during synchronous growth of gradient-selected cells. Symbols: (A) Rate of PG synthesis per cell* (○---○, △—△, ▲—▲, *for three separate experiments); (B) PG accumulation per cell* (●, □, ■, *for three separate experiments); (C) percentage of septated cells* (○, ●, *for two separate experiments); (D) cellular autolytic rate* (hr^{-1}) (△) *and cell numbers* (●) *from a single representative experiment.*

was also observed by Hoffman et al. (24) for the incorporation of D-glutamic acid into *Escherichia coli* B/r and remains unexplained.

Septation. Cyclic fluctuations in the fraction of cells containing visible septa were also observed (Fig. 2C). The fraction of cells containing septa increased from 15 to 20% in the initial gradient-selected population to maxima of 60 to 70%, 5 to 7 min before the rapid rise in cell number (cell separation). Thus, septation began 10 min before the end of the period of chromosome replication [C] and continued, to reach a maximum 15 to 20 min into D. The time intervals between maxima (and minima) in percentage of septated cells were the same as the doubling time for cell numbers.

Cellular autolytic capacity. Comparison of the cyclic variations in cellular autolytic capacity (23) with the rates of PG synthesis and accumulation per cell and with the fraction of septated cells (Fig. 2) showed a clearly inverse relationship. Maximum capacity to autolyze (Fig. 2D) occurred at about the time that the fraction of septated cells (Fig. 2C) as well as the rate and accumulation of PG per cell (Fig. 2A and B) were at their respective minima (about the middle of C). Similarly, minimum capacity to autolyze occurred when the fraction of septated cells was largest and the rate of PG synthesis was at its maximum (toward the end of D).

Effects of inhibition of DNA synthesis on cell wall-related parameters. The relationship of the chromosome cycle to cell surface growth and division was further investigated, using MIT C (0.5 μg/ml) to inhibit DNA synthesis. Previous studies (28) with rapidly growing exponential-phase cultures showed that this concentration of MIT C rapidly stopped the incorporation of thymidine into DNA but permitted normal rates of incorporation of uracil into RNA and leucine into protein for at least 50 min. Similarly, with slowly dividing, gradient-synchronized populations (Fig. 3), MIT C added at 70 min rapidly inhibited further incorporation of [^{3}H]thymidine (into DNA), but had little

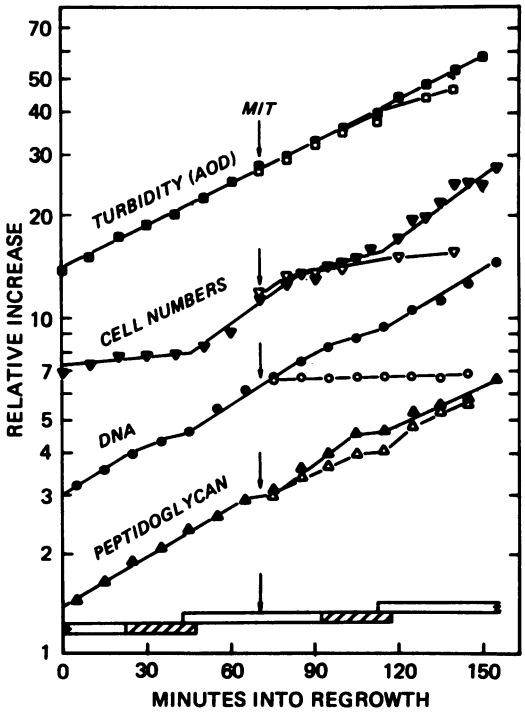

FIG. 3. *Relative increases in several culture parameters after the addition of MIT C (0.5 μg/ml) at 70 min into regrowth of gradient selected cells. The solid symbols are for untreated controls. The open symbols are for the cultures treated with MIT C. Symbols: turbidity (T_D = 73 min)* (□, ■); *cell numbers (T_D = 70 min)* (▽, ▼); *DNA* (○, ●); *PG accumulation* (△, ▲).

effect on the rate of turbidity increase until 60 or more min later. All observations of MIT C-treated culture were made within 60 to 70 min after MIT C addition. A relatively small reduction in the rate of PG accumulation and an increase in cell number corresponding only to that expected for cells that had completed their chromosomes (cells already in D at the time of MIT C addition) were also observed.

After addition of MIT C at 70 min (about the middle of C), the rate of incorporation of 4-min pulses of [^{3}H]lysine into PG was only slightly lower than that of the control culture (Fig. 4A). This rate of PG synthesis continued in parallel with the control for the next 70 min. Only after 140 min was a decline in rate of PG synthesis, accompanied by a decreased rate of turbidity increase, seen.

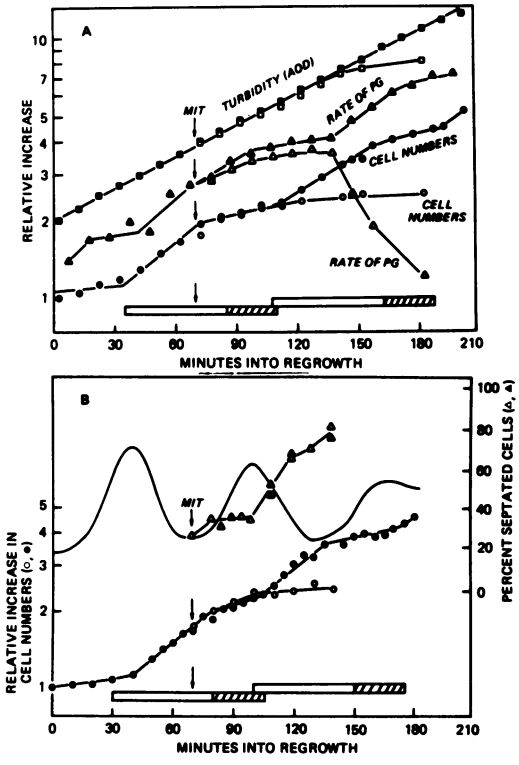

FIG. 4. *Effect of MIT C (0.5 μg/ml) addition at 70 min into regrowth of gradient-selected cells on (A) the rate of PG synthesis and (B) septation of cells. The solid symbols are for untreated control cultures. The open symbols are for cultures treated with MIT C. Symbols in A: Turbidity (T_D = 78 min)* (□, ■); *rate of PG synthesis* (△, ▲); *cell numbers (T_D = 75 min)* (○, ●). *Symbols in B: Cell numbers (T_D = 70 min)* (○, ●); *percentage of septated cells* (△, ▲) *for MIT C-treated cultures in two different experiments in which the cell number data were nearly identical. The solid line without symbols represents the untreated control for septation taken from Fig. 2C.*

MIT C treatment of synchronized cultures at 70 min also affected septation (Fig. 4B). Although 75 to 80% of the cells eventually went on to complete septa, the process appeared to be delayed by 20 to 25 min. The maximum fraction of cells with septa occurred just before the decline in rate of PG synthesis, and, as shown by the constancy of cell numbers, septated cells failed to separate. Inhibition of DNA synthesis followed by delayed septation and failure to separate suggests that chromosome replication may be tied to cell separation as well as to the timing of septation.

MIT C treatment at 70 min also prevented the decline in capacity of cells to autolyze normally observed in such cultures during D (Fig. 5). This observation was repeated when MIT C was added at 143 min after regrowth, in the second cell cycle (Fig. 5). The slight stimulation of autolytic capacity observed immediately after MIT C was added at 70 min (Fig. 5) was not obtained when MIT C was added during the second cycle, at 143 min (Fig. 5) or at 70 min in a separate experiment (not shown).

Although inhibition of DNA synthesis about the middle of C, and presumably prevention of chromosome termination, was not accompanied by a decline in autolytic capacity, MIT C addition at about the time of chromosome initiation (105 min) failed to significantly affect the rise in autolytic capacity (Fig. 6). However, even when added at this time, the expected decline in autolytic capacity, about 35 to 40 min later, was not observed. In this experiment, after MIT C addition, cell numbers increased by 75 to 80% rather than the full doubling in numbers expected.

DISCUSSION

Previous studies established that assembly of new wall at septally localized sites is largely responsible for surface enlargement in *S. faecium* (18, 19). In addition to septally associated wall assembly primarily directed toward surface enlargement, wall assembly primarily directed toward wall thickening was shown to occur continuously over essentially the entire coccal surface in growing and dividing cells (15, 16, 19, 21, 29).

We believe that PG accumulation measured by the fully equilibrated labeling technique is a measure of overall wall PG assembly, including both wall surface expansion and wall thickening. In contrast to continuous labels, pulse labels measure rates of synthesis from exogenous precursors over short intervals. The relatively slow increase in degree of peptide cross-linking of already assembled insoluble PG (7), plus kinetic and other evidence for the assembly of two PG

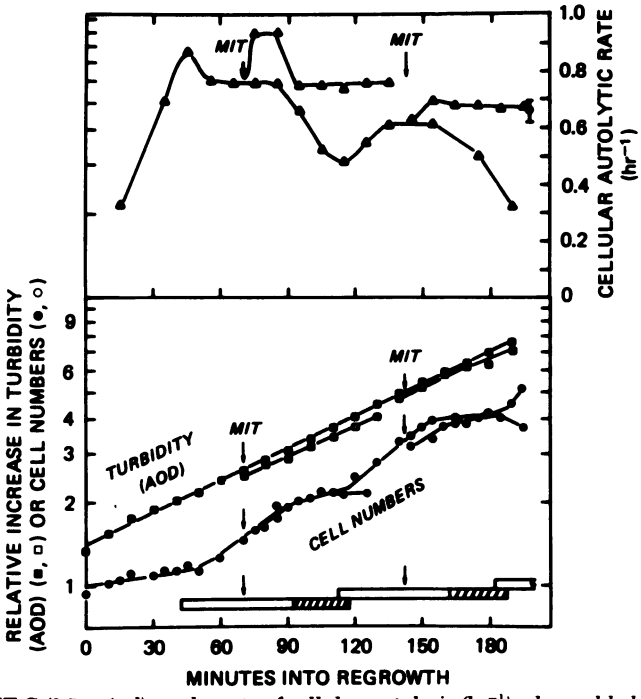

FIG. 5. *Effect of MIT C (0.5 μg/ml) on the rate of cellular autolysis (hr^{-1}) when added at 70 or 143 min into regrowth of gradient-selected cells. Symbols: Turbidity (T_D = 77 min) (□, ■); cellular autolytic rate (△, ▲); cell numbers (T_D = 72 min) (○, ●). The solid symbols represent untreated controls, and the open symbols are for the MIT C-treated cultures.*

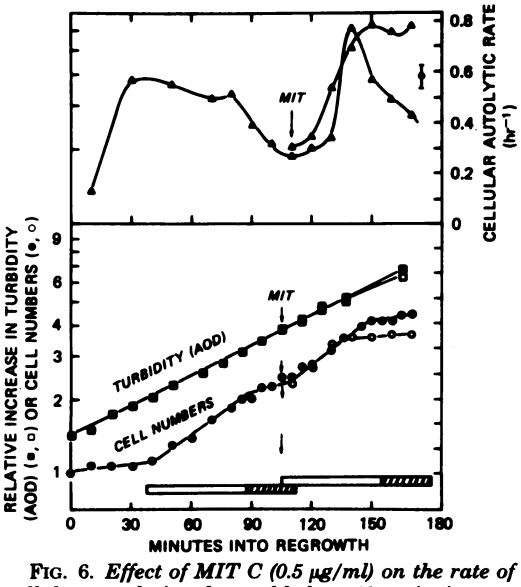

FIG. 6. *Effect of MIT C (0.5 μg/ml) on the rate of cellular autolysis when added at 105 min into regrowth of gradient-selected cells. Symbols and conditions are the same as in Fig. 5.*

products in *Lactobacillus acidophilus* and *S. faecium* (2, 6, 7), suggest that the observed changes in rates of PG assembly may be a reflection of changes in the relative balance between wall enlargement and wall thickening during the cell cycle. Thus, an increase in rate of wall assembly directed toward surface enlargement would occur above a background of a consistent and slower rate of wall thickening over the entire coccal surface. If this is the case, then the rate changes detected by pulse labeling could reflect changes in the mode of wall assembly during the cell cycle. The increasing rate of incorporation, which began 10 to 20 min before the end of C and reached a maximum constant rate about 10 to 15 min into D (Fig. 1), could reflect a shift from peripheral wall elongation to centripetal septation. This interpretation is supported by the concurrent increase in the fraction of cells containing septa (Fig. 2C). Also, this timing sequence agrees with the observations that the D period in rapidly dividing cells of *S. faecium* (T_D = 31 to 34 min) can be divided into two segments, a D1 period of about 20 min, during which RNA and protein synthesis continue, and a D2 period of about 5 min, which can continue after inhibition of RNA and/or protein synthesis (16, 29). The final stages of septation and cell separation probably occur during D2, which represents a phase of the cell cycle after completion of the cross wall and physiological separation of daughter cell cytoplasms (4).

The studies using MIT C to inhibit DNA synthesis at selected times in the cell cycle (Fig. 4–6) provide some information on the relationship of the chromosome replication cycle to surface growth and autolytic capacity. Inhibition of chromosome replication during C did not substantially affect the rate of PG synthesis until much later (Fig. 4A), but did: (i) prevent completion of division and cell separation (Fig. 4B); (ii) delay but not prevent septation (Fig. 4B); and (iii) prevent the expected decline in autolytic capacity at the end of the cell cycle, during D (Fig. 5).

Inhibition of DNA synthesis later in the cell cycle (Fig. 6; during D and very near the beginning of the next C) permitted the usual rise in autolytic capacity at the beginning of the next cycle, but again prevented its subsequent decline. These observations are consistent with the view that the normally occurring increase in autolytic capacity near the beginning of the cell cycle is not coupled to chromosome initiation, but that the fall in lytic activity toward the end of the cycle (during D) is coupled to some event that occurs close to chromosome termination.

Addition of MIT C during D (Fig. 6) was accompanied by a 75 to 80% increase in cell numbers rather than the expected full doubling. Lack of division of all cells could have been due to one or more factors. For example, because of the less than perfect synchrony obtained, a fraction of cells may not have fully completed the previous round of chromosome replication or may have failed to "process" or segregate their chromosome, as is thought to occur during the early portion of D in *E. coli* (8). In view of the 50 min required for all cells to complete division (Fig. 6) and assuming that the observed heterogeneity in division times is distributed normally, then addition of MIT C 5 min before the expected end of D should permit division of only 80% of the cells. In fact, the results of this experiment support the proposed timing for completion of D. If the average chromosome replication cycle were placed between the midpoints of the rapid increases in cell numbers, as is usually assumed for *E. coli* (12, 13, 25), then after MIT C addition only 16% of the cells could be expected to complete division.

Studies using synchronized cultures of *E. coli* also demonstrated changing rates of cell surface synthesis during the division cycle, with maxima occurring presumably during the D phase for that organism (9, 10). However, in contrast to *S. faecium*, total PG hydrolase activity increased to a maximum during D (10, 11). On the other hand, the cell cycle-dependent endopeptidase activity increased to its maximal level but did not fluctuate after inhibition of chromosome completion (11). Although the two systems are very difficult to compare for numerous technical reasons, in both organism regulation of PG hydrolase activities appear to be intimately tied to the DNA replication and cell division cycles.

The data presented here are consistent with the role of autolytic activity in the previously proposed model for cell division in this species (29). The model proposed that PG hydrolase activity (5, 30), localized at nascent cross walls (16), increased during the phase of initiation of new septal sites and elongation of peripheral wall about 30 min into the C phase. Later in the cycle, after chromosome termination, a decline in autolytic activity permitted centripetal penetration and closure of the cross wall. Although impossible to rule out, it seems unlikely that the consistently observed cyclic fluctuations in autolytic capacity are fortuitous. Enzymes involved in PG assembly or modification, which were not measured in this study but whose activities have been observed to change during division of *E. coli* (1, 9–11, 26), may also be involved in the complex morphogenetic sequence leading to cell division.

ACKNOWLEDGMENTS

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