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Volume Growth, Murein Synthesis, and Murein Cross-Linkage During the Division Cycle of *Escherichia coli* PA3092

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Cells of *Escherichia coli* PA3092 were synchronized by centrifugal elutriation. The synchronously growing cells were double labeled with -^{3}H or DL-[*meso*-2,6-^{14}C]diaminopimelic acid (DAP) at different times. Cells incorporated [^{3}H]DAP at a continuously increasing rate during their cycle, with a maximum occurring at about 30 min before division for trichloroacetic acid-precipitated cells (whole cells) and about 10 min before division for sodium dodecyl sulfate-treated cells (sacculi). This was in good agreement with the observed kinetics of volume growth under these conditions. Furazlocillin, which preferentially interacts with penicillin-binding protein 3, modified the pattern of incorporation of [^{3}H]DAP. Electron microscopy indicated that furazlocillin did not inhibit the initiation of division but rather its completion. In addition, we measured the cross-linking of the murein inserted at different times during synchronous growth. The highest percentages were found to occur around division. At this same time, the cross-linking of old peptidoglycan was found to be decreased.

During the division cycle of rod-shaped organisms like *Escherichia coli*, cells double their length. Because of the rod-shaped cell, growth can be approximated as envelope growth. The envelope again can be considered as equivalent to the covalently closed sacculus. Envelope growth can be studied at various levels. One may study: (i) overall length, surface or volume extension, (ii) the synthesis or activities of envelope components during the division cycle, and (iii) the topographical distribution of labeled envelope components (for reviews, see references 14 and 16). This paper deals with points (i) and (ii).

Extensive analysis of the length and volume growth of *E. coli* B/r F26 synchronized with membrane elution and centrifugal elutriation suggested to us that *E. coli* B/r cells were growing at a continuously accelerating rate, with a maximum occurring near or at division (manuscript in preparation). Centrifugal elutriation (4, 11) enabled us to synchronize K-strains of *E. coli*, which are normally used in the study of wall growth and which are difficult, if not impossible, to synchronize with membrane elution.

The overall growth pattern that we found was compared with the incorporation of pulses of DL-[*meso*-2,6-^{3}H]diaminopimelic acid (DAP) during the division cycle.

Recently, it was found (3) that the cross-linking of murein in asynchronous cultures shows a stepwise increase during the process of insertion into the preexisting murein layer. This was interpreted as evidence for a maturation process of peptidoglycan occurring after its initial covalent attachment to the sacculus. Although many authors have tried to find a difference in structure for peptidoglycan in polar caps as compared with the structure elsewhere in the sacculus, this has never been clearly demonstrated (cf. reference 16). It therefore seemed relevant to us to study how the peptidoglycan maturation process takes place during different stages of the division cycle. We carried out experiments with synchronized cultures of *E. coli* PA3092, in which we looked at the cross-linking in both old and newly formed murein. These percentages showed different behavior during the division cycle, in the sense that the cross-linkage of newly inserted peptidoglycan was highest when that of old peptidoglycan was lowest and vice versa.

MATERIALS AND METHODS

Organisms and growth conditions. *E. coli* PA3092 (K-12, F$^-$ *lys thr leu trp his thyA argH thi lacY malA mtl mel tonA supE str*) was cultivated in minimal citrate medium (6) supplemented with 50 μg of thymine per ml, 0.04% Casamino Acids, 0.005 μg of $FeCl_3$ per ml, 1 μg of vitamin B_1 per ml, 1 mM $MgSO_4$, 0.04% glucose, 4 μg of DAP per ml, and 20 μg of each of the required amino acids per ml. The culture was grown with shaking at 30°C with a doubling time of 70 min.

Synchronization procedure. Synchronous cells were

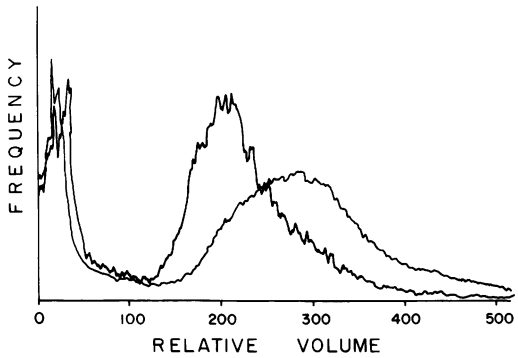

FIG. 1. Coulter counter distributions of exponentially growing cells of *E. coli* PA3092 (right) and of the fractions of small cells separated from this culture with centrifugal elutriation (left). The volume scale indicates relative volumes expressed in Coulter counter channel numbers.

obtained by an improved version of the method of centrifugal elutriation (4, 11). A short description of the procedure followed is given below. The modifications of the standard procedure (4) will be discussed elsewhere (manuscript in preparation). Of an exponentially growing culture, 50 ml was concentrated at an optical density of 0.45 (measured at 450 nm) by centrifugation in a Sorvall RC2B centrifuge for 2 min at 10,000 rpm. The cells were suspended in 2 ml of growth medium and loaded into the elutriator system at a rotor speed of 5,820 rpm and a flow rate of 1.8 ml/min with the aid of a syringe. Ten minutes thereafter, the flow rate was increased, with 0.1 ml/min every 30 s. At a flow rate of 3.2 ml/min, a total of 50 ml of cell suspension was collected in a sterile flask placed on ice. This cell suspension grew synchronously upon incubation at 30°C.

Determination of cell numbers and volume distributions. Cell numbers were determined with a Coulter counter (orifice, 30 μm) coupled to a multichannel analyzer. The volume distributions were plotted with an *x-y* plotter, and mean volumes were determined by the integration of these distributions. Total volumes after different times of synchronous growth were calculated by the multiplication of the values for cell numbers and mean volumes at each time.

Radioactive labeling. Uniformly labeled cultures were made by growing cells for three generations in the presence of *meso*-DL-2,6-DAP: [^{14}C]DAP (1 μCi/ml, 315 mCi/mmol; Commissariat à l'Energie Atomique, France) or [^{3}H]DAP (25 μCi/ml, 50 Ci/mmol; Commissariat à l'Energie Atomique, France). For pulse-labeling, 2 or 4 ml of the synchronously growing cultures was labeled for 8 min with [^{3}H]DAP at different times during the division cycle. After pulse-labeling, the samples were mixed with an equal volume of a solution of 8% sodium dodecyl sulfate (SDS) and boiled for 30 min. Duplicate samples were precipitated with 10% trichloroacetic acid TCA (see below). The incorporation of radioactivity into the sacculi was determined by the filtration of the SDS samples through 0.22-μm filters (Millipore Corp., Bedford, Mass.), followed by washing of the filters with a total of 100 ml of distilled water. The filters were then dried and counted in 5 ml of toluene-PPO (2,5-diphenyloxazole) in a liquid scintillation counter. The total incorporation of radioactivity into whole cells was determined by the precipitation of similar samples on glass-fiber filters (Whatman) with 10% TCA. These filters were washed, dried, and counted in the same way as described above.

Isolation of sacculi and chromatography of peptidoglycan fragment. The sacculi were purified by repeated centrifugation at 100,000 × *g*. Cold sacculi were added to all samples to avoid losses of radioactive material. The purified sacculi were then digested overnight in a buffer of ammonium acetate at pH 6.8 containing 200 μg of lysozyme per ml. The muropeptide fragments obtained in this way were separated by descending paper chromatography with a mixture of butanol-acetic acid-water (4:1:5) as a solvent.

After separation, the radioactive spots were cut out, dried, and counted after the addition of 0.2 ml of distilled water and 2 ml of a mixture of Triton X-100–toluene (1:4), containing 2 g of PPO per liter.

Electron microscopy. Cells were fixed in 0.1% OsO_4 and subjected to agar filtration (21), and the various samples were photographed with a Philips EM 300 electron microscope. Length distributions were made from projections of the cells onto a tablet digitizer (Summagraphics, Fairfield, Conn.). Total length at any time during synchronous growth was calculated by multiplying cell numbers at that time with the measured mean length of the cell population.

RESULTS

Total volume growth. To achieve synchronous growth, small cells were directly isolated from

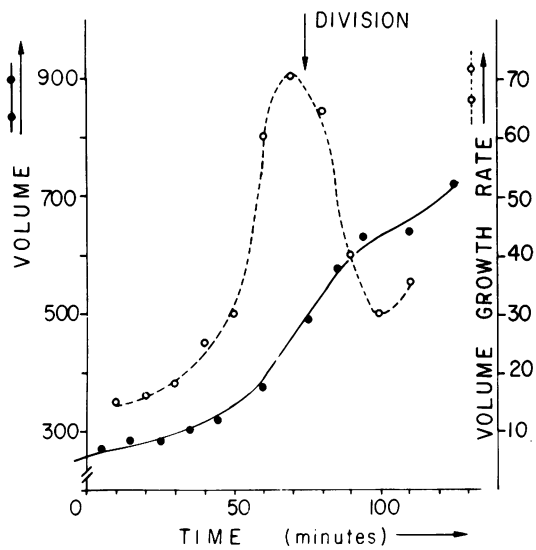

FIG. 2. Growth in total volume (●) and calculated growth rate (○) in *E. coli* PA3092 synchronized with centrifugal elutriation. The growth rate is seen to be continuously increasing, with a maximum occurring around the time of division. Total volume and growth rate are expressed in arbitrary units.

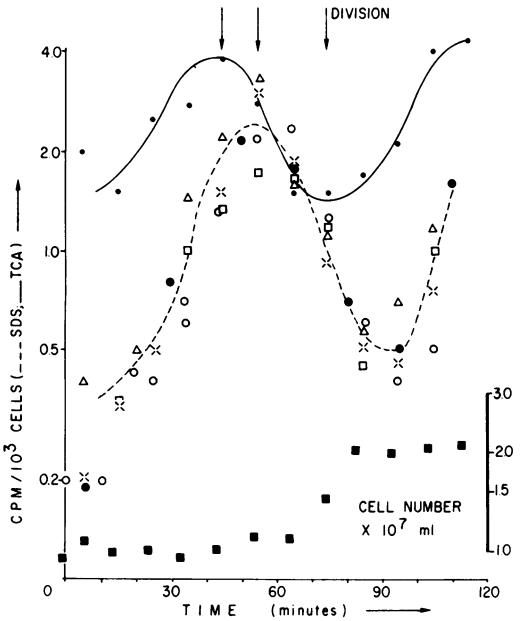

FIG. 3. Incorporation of [^{3}H]DAP into whole cells (upper curve) and into sacculi (curve in the middle). The increase in cell number with time is shown in the lower curve (■). The incorporation into sacculi was estimated from the results of five different experiments (○, ●, △, □, ×). For explanation, see the text.

their growth medium by centrifugal elutriation (Fig. 1). The fraction of small cells was inoculated in fresh growth medium at 30°C, and volume distributions and cell numbers were measured at subsequent times with a Coulter counter. The mean volume at each time point multiplied with cell number (total volume) is shown in Fig. 2. The relevant synchronization curve is depicted in Fig. 3. Volume growth (Fig. 2) can be seen to continuously increase during the division cycle. The maximal growth rate occurred just before or at division. This growth pattern has also been found for *E. coli* B/r F26 (manuscript in preparation), irrespective of whether small cells had been selected by membrane elution, Percoll centrifugation, or centrifugal elutriation. We have also shown that the collection of cells in the cold, which is needed to obtain a sufficient number of cells for biochemical determinations, does not affect the overall growth pattern (manuscript in preparation).

Incorporation of DAP. To check a possible effect of the above cold treatment on the incorporation, an asynchronous population of cells was cooled for 60 min. Upon rewarming to 30°C, cell number and DAP incorporation were measured (Fig. 4). Cell number and incorporation rate increased exponentially, with no sign of the oscillation as observed for the synchronized culture (Fig. 3). A cold effect might play a role during the first few minutes after inoculation for synchronous growth only.

With respect to DAP incorporation, we tried to distinguish between the incorporation into whole cells, on the one hand, and into sacculi, on the other. In the first case, cells were precipitated with TCA (7); in the second, cells were boiled in SDS to obtain the sacculi (13). Figure 3 shows the measured incorporation pattern. It was consistently found that maximal incorporation into whole cell precedes maximal incorporation into sacculi. Comparison with Fig. 2 shows that the latter preceded the time when the maximal growth rate occurred. It would seem that [^{3}H]DAP-containing material is first associated with the whole cell, presumably the cytoplasmic membrane, whereafter this material is (initially slowly) transferred to the sacculus (see below).

For *E. coli* PA3092, with a doubling time of 70 min, the sequence of events is thus as follows: (i) maximal incorporation of DAP into the whole cell about 30 min before division, (ii) maximal incorporation in sacculi about 10 min before division, and (iii) maximal growth just before or at division.

Inhibition of cell division with furazlocillin. The increased incorporation of DAP at the end of the division cycle (Fig. 3) could be interpreted as being needed to complete the division process. However, the increase in volume growth (Fig. 2)

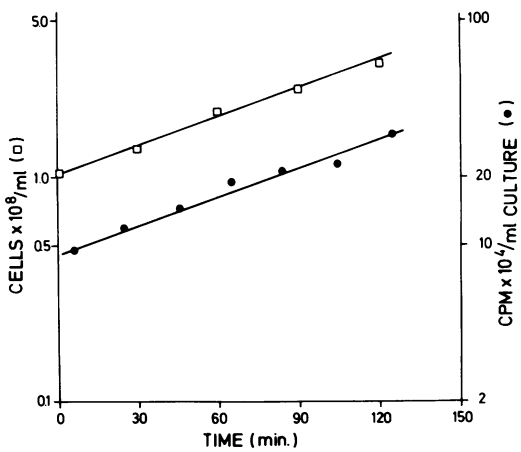

FIG. 4. Incorporation of [^{3}H]DAP (8-min pulse) into whole cells of *E. coli* PA3092. Shown is the incorporation into asynchronous cells, which had been submitted to cold shock for the same time period as the cells synchronized by centrifugal elutriation (Fig. 3). It also shows an exponential increase in incorporation rate with no apparent lag in the beginning. These results indicate that the variation in the rate of incorporation found in synchronous cultures (Fig. 3) is not the effect of the cold shock treatment.

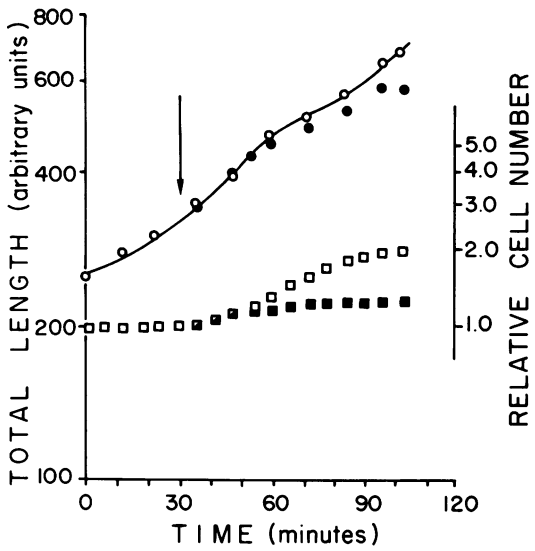

FIG. 5. Effect of furazlocillin on cell elongation calculated from length measurements from electron micrographs and on increases in cell numbers at different times during synchronous growth of *E. coli* PA3092. Symbols: ○, control culture; ●, culture to which 5 μg of furazlocillin per ml had been added at the time indicated by the arrow; □, cell numbers in control; ■, cell numbers in furazlocillin-treated culture.

just before division suggests that only part of the incorporated material is needed for division. In addition, autoradiographic evidence showed lateral incorporation zones at the end of the division cycle (10, 17, 20). We attempted to clarify this situation by adding the β-lactam antibiotic furazlocillin, which has been reported to specifically bind to penicillin-binding protein 3 (PBP-3; 2). PBP-3 has been found to be specifically involved in cell division (19). The effect of 1, 5, and 10 μg of furazlocillin per ml on growing *E. coli* PA3092 was followed by light microscopy. Small cells continued to appear at 1 μg/ml, and filamentation started at the higher concentrations. For this reason, we used 5 μg of furazlocillin per ml. (It seems that a thermosensitive division mutant like *E. coli* BUG 6 is somewhat more sensitive to this antibiotic [2].)

Furazlocillin was added to a synchronously growing culture at t = 30 min. Cell number was followed with a Coulter counter; cell length and the percentage of dividing cells were followed by electron microscopy of agar-filtrated cells. In addition, the incorporation of DAP into sacculi was determined at various times. The Coulter counter measurements (Fig. 5) show that cell division was effectively blocked. Cell elongation continued (Fig. 6a), and DAP incorporation (Fig. 6c) was markedly affected. Closer inspection, however, indicated that total cell length increase was influenced by the antibiotic (Fig. 5), as was part of the division process (Fig. 6b). Remarkably, cells continued to constrict but did not complete division. Morphologically, the division site was also changed (Fig. 7).

Cross-linkage during the division cycle. The cross-linkage of murein in the uniformly [^{14}C]- or [^{3}H]DAP-labeled synchronous culture of *E. coli* PA3092 that was additionally pulse-labeled

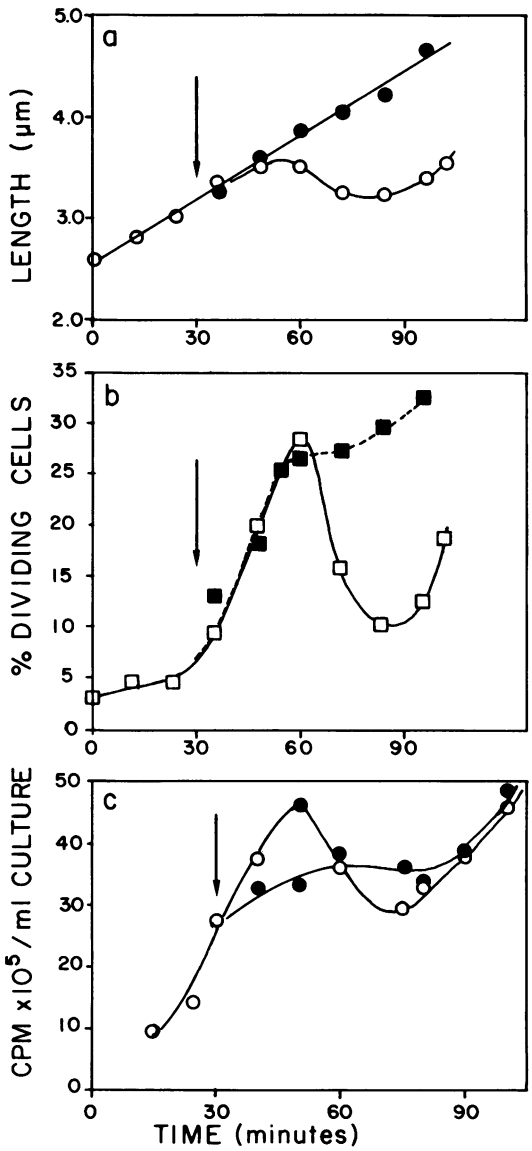

FIG. 6. Mean length (a), percentage of dividing cells (b), and incorporation of [^{3}H]DAP (c) in the same culture as described in the legend to Fig. 5. Open symbols indicate the results of measurements in the control culture; closed symbols indicate the results from the furazlocillin-treated culture. The arrows indicate the time of the addition of furazlocillin. For explanation, see the text.

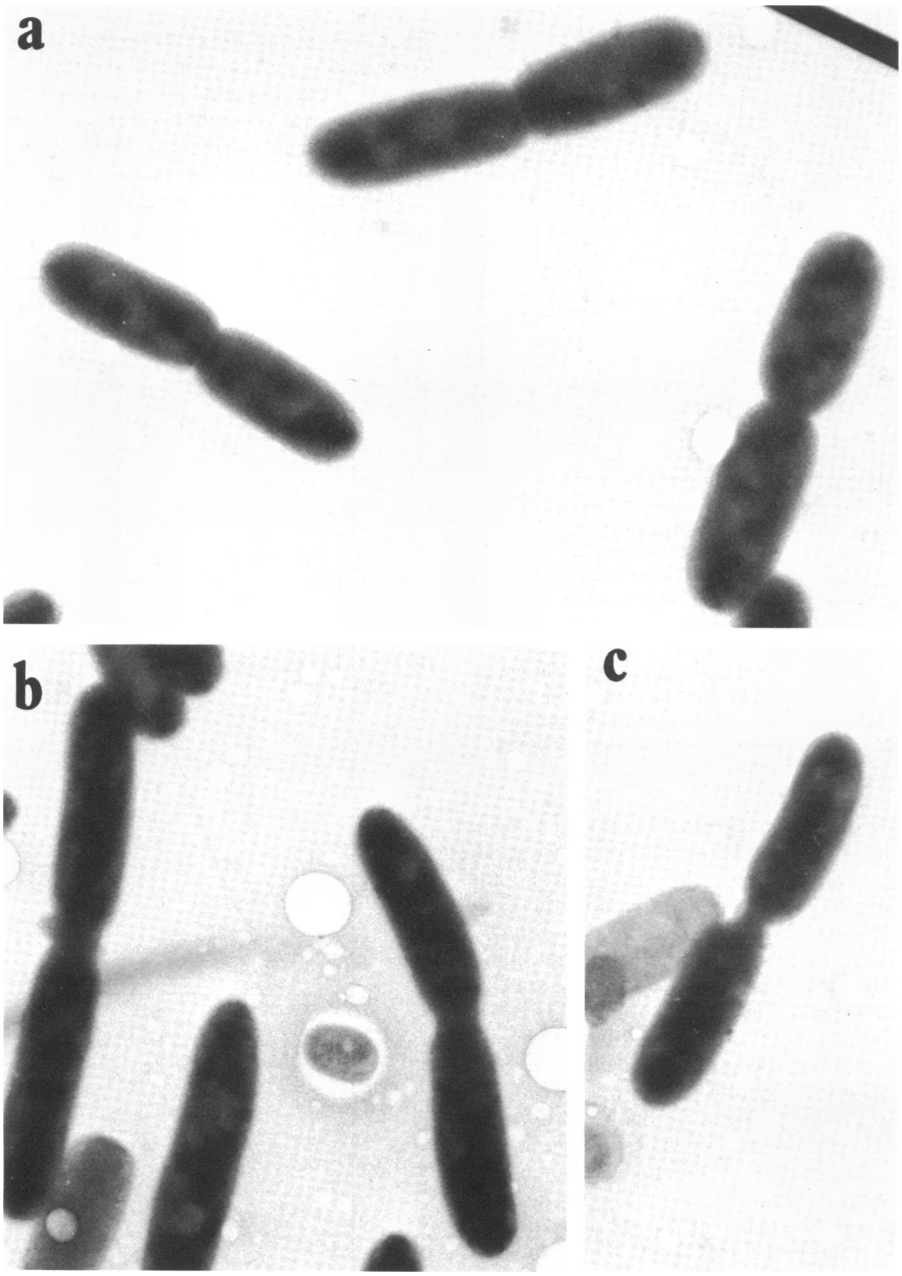

FIG. 7. Electron micrographs from the two cultures described in the legend to Fig. 5 show dividing cells from the control culture (a), and dividing cells from the furazlocillin-treated culture (b and c). The samples were taken 102 min after the start of synchronous growth. The appearance of blunt constrictions is clearly demonstrated (b and c).

with [^{3}H]DAP is shown in Fig. 8. It can be seen that the cross-linkage of the peptidoglycan inserted during the 8-min pulses was low in the beginning of the cell cycle and rose towards a maximum around division. The cross-linkage found in uniformly labeled material is seen to increase somewhat in the beginning of the cell cycle and to decrease around cell division at the time when newly formed murein is incorporated with highest cross-linkage.

The growth in total volume of the synchronized culture (Fig. 2) shows that maximal growth rate and maximal cross-linkage of newly inserted material more or less coincide.

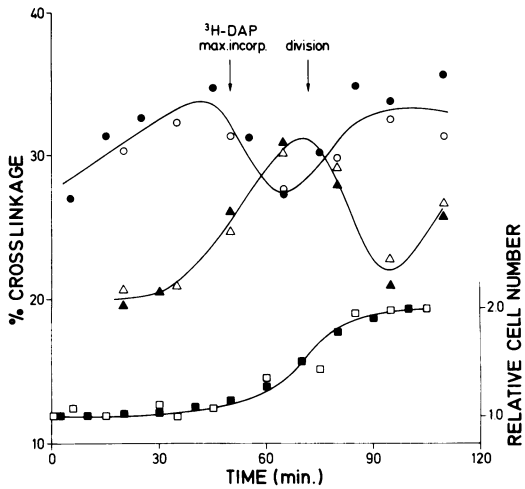

FIG. 8. Cross-linkage of peptidoglycan in sacculi of *E. coli* PA3092 synchronized with centrifugal elutriation. The percentages of cross-linkages were calculated as described in reference 17. The drawn line is the estimate of two different experiments. The increase in cell number is also shown. Symbols: ○ and ●, cross-linkage from cultures uniformly labeled with [^{14}C]DAP and [^{3}H]DAP, respectively; △ and ▲, cross-linkages of newly inserted peptidoglycan measured in 8-min pulses with [^{3}H]DAP; □ and ■, cell numbers. Arrows indicate the maximal incorporation of [^{3}H]DAP into sacculi (cf. Fig. 3) and the time of cell division.

DISCUSSION

Volume growth and DAP incorporation. The results presented in this paper indicate that after synchronization *E. coli* PA3092 grows at a continuously accelerating rate, with a maximum around division. The increase in growth rate appeared to be of the same order as the increase in the incorporation rate of [^{3}H]DAP into sacculi (Fig. 3). Early experiments on the incorporation of D-[^{14}C]glutamic acid into a B/r strain showed maximal incorporation about 15 min before division (7).

There was a discrepancy, however, with respect to the timing at which maximal growth and maximal DAP incorporation occurred. Volume and length seemed to grow at a maximal rate at or near division, whereas the cell wall showed a maximal rate of synthesis some time before division. These results are not necessarily in conflict with each other. We may assume that newly added material is at first becoming covalently attached onto the preexisting sacculus without increasing its surface area (9). In a later stage, these molecules are then drawn into the surface of the sacculus by osmotic forces as bonds in the old peptidoglycan are being broken by specific enzymes. This model, thus, implies a certain time difference between the actual attachment and the time at which the precursors contribute to surface enlargement.

Another conclusion one can draw from this investigation is that there seems to be a time difference of about 10 min between the incorporation of [^{3}H]DAP into sacculi and into whole cells (Fig. 3). A similar difference in incorporation was found for *E. coli* B that had been synchronized by amino acid starvation (13). These results suggest the existence of temporal assemblies of peptidoglycan precursors which are oscillating during the division cycle. Their amount would be high in the beginning of each cycle and decrease gradually as a cell proceeded towards division. Such a pool might exist in the form of high-molecular-weight murein precursors. There is one report in which the existence of soluble peptidoglycan precursors is described (12) and another where C55-isoprenol-linked compounds have been detected (1).

Inhibition of cell division. In the experiment in which furazlocillin was used, the normally found high incorporation rate of [^{3}H]DAP before division was suppressed, as was cell division. From the results of the electron microscopical measurements, it could, nevertheless, be seen that the percentage of constricting cells increased (Fig. 6). It has been proposed by some authors (15, 18) that the actual cell division process is composed of three different stages: (i) initiation of cell division, (ii) formation of a septum, and (iii) cell separation. Our results are in support of this idea. In mutants in which cell division was blocked in stage (ii), blunt constrictions can be seen (15) which bear a close resemblance to the constrictions that we observed in cells after treatment with furazlocillin. Blunt constrictions (Fig. 7) presumably arise when peptidoglycan components are inserted parallel, rather than vertical, to the length axis of the cell (15). It thus seems that furazlocillin affects stage (ii) rather than (i). As this antibiotic binds specifically to PBP-3 (2), a protein uniquely involved in the cell division process (19), we propose that furazlocillin in the concentration used (5 μg/ml) uncouples the first two stages of cell division.

Murein cross-linkage. We found reciprocal cross-linkage of old and newly inserted peptidoglycan during the division cycle of *E. coli* PA3092. Initially, existing murein showed a cross-link percentage around 30% in newborn cells, whereas newly inserted murein was cross-linked for about 20%. During synchronous growth, newly inserted peptidoglycan became more and more cross-linked until a maximum of about 30% was found around division (Fig. 8). The cross-linkage of existing peptidoglycan also varied. However, a maximum was reached about 30 min before cell division, whereas a minimum was observed around division. This

would mean that bonds in existing peptidoglycan are broken when the need for the incorporation of new material is highest. It should be recalled that murein hydrolases are also most active around division (5).

These observations on cross-linkages in synchronous cells agree with those made on asynchronous cells (3), in the sense that newly inserted peptidoglycan is more mature (cross-linked) in older cells than in younger cells. In addition, the maturation capability increased with cell age (Fig. 8).

The high cross-linkage of new murein at the end of the division cycle might have several reasons. First, this might be correlated with the accelerated volume growth just before division. Second, it might be related to the formation of polar caps. Support for the latter possibility can be found in the fact that filament-inducing antibiotics affect the completion of murein cross-linkage (3). Also, the overproduction of PBP-3 resulted in a higher rate of peptidoglycan synthesis and in an enhancement of cross-linkage (8).

Interpretation of the murein maturation process. As suggested by de Pedro and Schwarz (3), the maturation process for *E. coli* murein might be explained in terms of a remodeling and redistribution of peptidoglycan chains over the sacculus after the initial insertion into a growth zone. In this view, our results could mean that peptidoglycan chains inserted during the process of cell elongation are less cross-linked (less mature), whereas peptidoglycan chains inserted during the process of division do not undergo such a maturation and are therefore not expected to be redistributed over the sacculus. Maybe then the septum and the future cell poles are being synthesized in this way with a high cross-linkage and little or no further expansion, whereas the rest of the cell wall is formed with an initially lower cross-linkage. The maturation process in the latter case could be the result of the actual insertion of the peptidoglycan chains into the plane of the sacculus. Our results can be taken to be in support of the hypothesis of two different peptidoglycan-synthesizing systems in *E. coli* (2, 6, 19). One would serve the purpose of cell elongation, the other would be active during the formation of polar caps.

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