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Evidence for Diffuse Growth of the Cylindrical Portion of the *Escherichia coli* Murein Sacculus

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High-resolution autoradiography of thin sections of *Escherichia coli* cells whose murein was pulse-labeled with [^{3}H]diaminopimelic acid after a period of diaminopimelic acid deprivation indicated that elongation of the murein sacculus occurs by a multisite (diffuse) process. Upon chasing, radioactivity in polar murein was stable, whereas radioactivity in cylindrical murein was reduced, indicating that diffuse intercalation of new murein occurred during cell elongation. Elongation and septation were shown to be overlapping processes.

Murein (bacterial cell wall peptidoglycan) metabolism in bacteria is the subject of much study because this process is the target of the most widely used and most rapidly growing class of clinically useful antibacterial agents. The process also offers a unique opportunity to study an example of procaryotic morphogenesis (cell elongation, septation, and cell separation) at the molecular level. Fundamental to such studies is a knowledge of the supramolecular growth pattern of septal and cylindrical murein.

It is well established that synthesis of new murein in streptococci is initiated at an equatorial ring (5, 6) and then proceeds centrally to provide a double-layered cross wall which forms the new halves of the two daughter cells. The hemispherical shape of the new halves may then be determined passively in response to the internal osmotic pressure of the cell (7). In *Escherichia coli*, however, the topography of murein sacculus growth is unclear. High-resolution autoradiographic studies, using [^{3}H]diaminopimelic acid (DAP) labeling of murein, provide conflicting results. An often quoted study reported the surprising result that incorporation of new material occurs primarily in a central zone (12) and that subsequently, during chasing, the radiolabeled midzonal material is rapidly redistributed throughout the whole cylinder (12). This is consistent with the even distribution of label among progeny cells observed in earlier experiments (8, 19). More recent results (15, 20) indicate that there is a broad zone of incorporation of DAP into the sides of the sacculus during elongation of the cell. They support the notion that growth is multisite without addressing the question of the redistribution of central zone material to other sites. Because knowledge of the sites of insertion of new murein into the sacculus is crucial to the development of models describing the processes of elongation and septation in *E. coli*, we repeated the autoradiographic study. We employed the same *E. coli* strain (W7) and growth conditions as did Ryter et al. (12) and Schwarz and co-workers (15) but used somewhat different conditions for pulse-chase and processing of the cells. Our results clearly indicated a multisite insertion of new murein during elongation of the cell, with no evidence of redistribution from a central zone.

MATERIALS AND METHODS

Organism and growth conditions. *E. coli* W7 (*dap lysA*) was used throughout. The bacteria were cultivated at 37°C in medium E (21) supplemented with glucose (0.2%), L-lysine (50 μg/ml), and unlabeled DAP (5 μg/ml in overnight cultures and 2 μg/ml in experimental cultures). To ensure that the initial concentration of DAP in the experimental medium was 2.0 μg/ml, the overnight starter bacteria were collected on a membrane filter (see below) before transfer to the experimental medium. The optical density of log-phase cultures was followed with a Klett-Summerson colorimeter equipped with a W66 filter. The doubling time of strain W7 at 37°C in minimal-glucose medium aerated in a rotary shaker was 50 min.

Pulse-labeling of murein and sampling procedure. Incorporation by strain W7 of the [^{3}H]DAP preparation was highly specific for murein. Less than 1% of the incorporated radioactivity was lysine; the remainder was DAP. To obtain cells suitable for pulse-labeling and chasing (see below), log-phase bacteria (100 Klett units) were collected on a 47-mm membrane filter (pore size, 0.45 μm; Millipore Corp.), washed three times with 10 ml of 22°C water, transferred to prewarmed medium lacking DAP, and incubated for 25 min with normal aeration. At that point, preexisting pools of DAP and murein precursors were exhausted,

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enabling rapid incorporation of added [^{3}H]DAP and later effective chasing by unlabeled DAP (see below). Continued DAP starvation resulted in abrupt lysis of the bacteria after about 55 min.

The growth and DAP starvation conditions outlined were essential for the reproducibility of the labeling experiments. Too high concentrations of unlabeled DAP in the prestarvation medium, inefficient washing, and starvation periods of less than 20 min were associated with persisting intracellular pools of unlabeled DAP and nucleotide- and lipid-bound precursors and therefore with less rapid incorporation of added [^{3}H]DAP and inefficient chasing. On the other hand, exhaustion of DAP in the prestarvation medium (e.g., by allowing cultures to exceed an optical density of 120 Klett units) led to premature lysis of bacteria.

Specific labeling of murein was obtained by adding [^{3}H]DAP (40 μCi/ml = 0.152 μg/ml, experiment A; or 30 μCi/ml = 0.114 μg/ml experiment B) to cultures of prestarved bacteria (120 Klett units). After 7 min, by which time essentially all of the [^{3}H]DAP had been utilized, chasing was done by diluting the pulse-labeled cultures into prewarmed medium containing unlabeled DAP (10 μg/ml). Samples of radiolabeled bacteria were withdrawn at intervals, added to 2 volumes of water at 100°C, and kept for 30 min at 100°C before washing them twice with water with the aid of a centrifuge (Beckman microfuge B, 3 min at 10,000 × *g*). By this procedure, autolytic enzymes were inactivated, and free and nucleotide-bound DAP was efficiently removed. In cells labeled for 5 min, only about 1% of the [^{3}H]DAP in the cells was bactoprenol linked, and the remainder of the DAP was in the murein sacculus.

Autoradiography. After being washed, the pellets of bacteria were treated with 4% glutaraldehyde for 2 h and 2% OsO_4 for 2 h, dehydrated using 70 to 90 to 100% ethanol before treatment with propylene oxide, and embedded in an Epon 812-based mixture. The embedded bacteria were sectioned at the thickness of 60 nm with an LKB III ultraMicrotome. The sections were placed on grids and covered with a film of Ilford L4 nuclear emulsion of appropriate thickness in the dark, as described by Caro (4). The grids were stored at 4°C in the dark and inspected at intervals for grain density. For this purpose, the films on the grids were developed with the physical developer of Caro (4), stained with uranyl acetate in 50% ethanol and then with 2% lead citrate, and examined with a JEOL 100S electron microscope. The desired grain density was usually achieved after 2 to 4 weeks of exposure. To simplify comparisons of grain densities in pulsed and chased cells, the same exposure time was applied to all samples from a given experiment. Grains were studied by using photographs at a final magnification of ×20,000, except for the samples taken after prolonged periods of chase which were examined at ×5,000. Except for grids containing extensively chased cells, the areas photographed were randomly chosen. Since cells without grains were predominant, grain-free areas were avoided, thereby introducing a bias toward higher numbers of grains per cell.

Only longitudinally sectioned cells were included in the study. The lengths of all cells that contained grains and the distances of the grains from the nearest cell pole were measured with a ruler. For plotting, these distances were expressed as percent (0 to 50) cell length. Grains located on a visible septum or centrally (0 to 2 mm from the center of cells that were twice the length of daughter cells at a ×20,000 magnification) were defined as septal and potentially septal, respectively. The precaution taken in defining centrally located grains was adopted to avoid underestimation of septal grains. We observed no complete septa, and constrictions were seen in 8% of the cells. Either septation was suppressed when cells were pulse-labeled after a starvation period, or boiling of samples and further processing destroyed nascent septa by "unfolding" them (2). Grains located at 0 to 3 mm (at ×20,000) or 0 to 1 mm (at ×5,000) from a pole were defined as polar.

Chemicals. [^{3}H]DAP (a mixture of DD-, LL-, and *meso*-DAP; 50 Ci/mmol) came from Service des Molecules Marquees, Commissariat à l'Energie Atomique, Gif sur Yvette, France.

RESULTS

Methodological considerations. The intracellular pools of DAP and DAP-containing precursors in strain W7 were greatly influenced by the concentration of DAP and the cell density in the pregrowth and experimental medium, by the washing procedure, and by the length of the DAP starvation period. The pool size, of course, affected the [^{3}H]DAP incorporation rate in pulses and the efficiency of chases. Therefore, it was essential to establish an optimal and reproducible pulse-chase protocol (see above). Furthermore, with the protocol adopted, abrupt medium shifts and washings during the pulse-chase period were avoided.

In previous pulse-chase experiments (12), the cells were not depleted of DAP precursor pools. When we repeated an experiment exactly as described previously (12), the amount of radioactive DAP in the sacculus more than doubled during the chase (Fig. 1). Figure 1 also shows the much more rapid chase and smaller incorporation during the chase that occurs in cells starved for DAP under the conditions used in the present experiments.

Location of newly inserted murein. The distribution of grains in thin sections of longitudinally cut cells labeled with [^{3}H]DAP for 5 min is shown in Fig. 2. The grains show no strongly preferred localization(s). In fact, when grains associated with visible septa are excluded (crosshatched area in Fig. 2), the density of the remaining grains is nearly uniform except for a higher density in the poles, which may reflect the higher specific activity to be expected in poles formed during pulse-labeling.

With the higher concentration of [^{3}H]DAP used in experiment A (Table 1), some radioactive DAP remained in intracellular pools so that chasing did not stop the incorporation of [^{3}H]DAP until after 25 min (see above). However, the continued incorporation thus obtained yielded the diffuse distribution of grains seen in

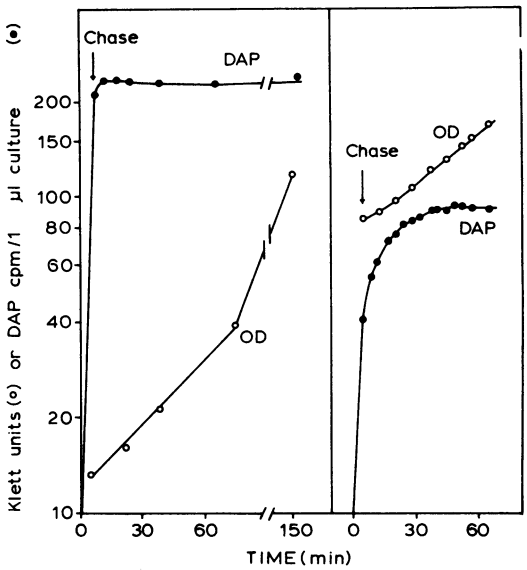

FIG. 1. Pulse-chase experiments: comparison of nonstarved and prestarved *E. coli* W7. (Left) Starvation procedure and [^{3}H]DAP labeling and chasing as in experiment B (see text); (right) pregrowth, labeling, and chasing procedure as in reference 9 except that an equimolar amount of [^{14}C]DAP was used instead of [^{3}H]DAP. OD, Optical density.

5-min pulses (data not shown). The ratio of cylindrical grains to polar and septal grains was lower after 25 min of incorporation (2.3) than after a 5-min pulse (3.1), possibly because of increasing dilution of the cylindrical radioactivity (see below) by nonradioactive murein during the later part of the 25-min pulse (Table 1, experiment A).

Fate of pulse-labeled murein during a chase. The distribution of grains after a chase of 1.25 generations is shown in Fig. 3. There was no dramatic change during the chase. Grains were still randomly distributed along the cylindrical portion of the cell. However, there were relatively more grains present in the cell poles after a 1.25-generation chase. A similar result was obtained after a three-generation chase. These data are summarized in Table 1. Since much less than 5% of the [^{3}H]DAP was lost from the cells during the chase (Fig. 1), the relative increase in polar grains cannot have been caused simply by the selective loss of DAP from cylindrical murein. A diffuse intercalation of new nonradioactive material would reduce the amount of radioactive murein per unit of surface area of the cylindrical portion of the sacculus without affecting the polar radioactivity of the prelabeled cells. In fact, after chasing, the number of polar grains was actually higher than that predicted for the completely stable poles (Table 1). This was probably because only fields containing grains were included in the count of total cells.

Thus, the chase experiments, as well as the pulse experiments, support the notion that DAP is incorporated at many sites rather than in a central zone during elongation of *E. coli* cells.

DISCUSSION

The present experiments show that elongation of the murein sacculus in *E. coli* is a multisite process. Multiple growth sites in *E. coli* were originally suggested by Van Tubergen and Setlow (19) and later by Lin and co-workers (8). The situation in *E. coli*, therefore, seems to be similar to that in another rod-shaped bacterium, *Bacillus megaterium* (11). Although our data do not give information on the total number of murein growth sites in *E. coli*, four or five sites per cell would be the minimum estimate based on the limited resolution of grains produced from [^{3}H]DAP. The actual number of growth sites could be as high as 200 if each penicillin-binding protein with murein transglycosylase and transpeptidase activity (10, 16) functions separately.

It has been suggested, on the basis of studies of a morphologically conditional mutant of *Klebsiella pneumoniae* (13), that elongation and septation of a cell do not occur simultaneously but rather take place as alternating processes. Analysis of the grain distribution in cells pulse-labeled for 1/10 of a generation (Table 1, experiment A) revealed that 12% of the cells contained both cylindrical grains and either polar or septal grains. If the distribution of grains had been completely random, about 23% of the cells should have contained cylindrical grains together with either polar or septal grains. Thus, our results indicate that in *E. coli*, elongation and

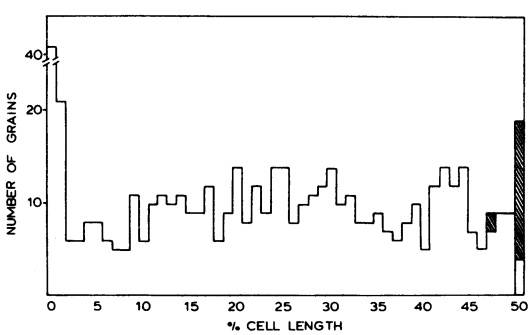

FIG. 2. Location of the grains in [^{3}H]DAP pulse-labeled and sectioned *E. coli*. The distance of each grain relative to the nearest cell pole was expressed as percent (0 to 50) cell length (see text). Crosshatched areas indicate grains associated with a visible septum. Data are from a 5-min pulse (Table 1, experiments A and B).

TABLE 1. Distribution and density of grains in [^{3}H]DAP-labeled and sectioned *E. coli* cells

Expt	Sample (generation)	No. of cells	Total no. of grains			Polar and septal grains per cell	Cylindrical grains/ polar and septal grains
			Polar	Septal[a]	Cylindrical		
A	Pulse (0.1)	212	68	14	254	0.39	3.10
	Pulse (0.5)	164	70	14	195	0.51	2.32
	Chase (1.25)	230	57	0	83	0.25 (0.20)[b]	1.46
B	Pulse (0.1)	92	34	13	142	0.51	3.02
	Chase (3.0)	532	66	0	95	0.12 (0.07)[b]	1.44

[a] Grains associated with visible septa plus midcylindrical grains in cells twice the length of newborn cells (potential septal grains [see text]).

[b] Values predicted if polar grains were fully stable during the chase (see text).

septation can occur simultaneously and that if the processes tend to alternate there must be considerable overlap.

In contrast to the earlier report (12), we did not observe an initial central zone of incorporation followed by a redistribution of grains. In the study cited, washing the bacteria on a membrane filter before pulse-labeling and before chasing was used to effect rapid medium shifts. The observed central zone of grains (12) may actually represent newly formed or nascent septa which would contribute a great deal of [^{3}H]DAP to the central zone relative to what would be expected to be present in the flattened cylindrical portion of intact sacculi. These authors used whole sacculi, where the contribution from septa may be exaggerated. In the thin sections of cells examined here, this problem is avoided. The "reshuffling" reported by Ryter et al. (12) may be an artifact caused by continued labeling of the sacculi during the chase (Fig. 1) combined with cell division, which has the effect of converting centrally located grains to polar grains. This is clearly not a satisfactory explanation as the central grains were said to be dispersed randomly rather than preferentially showing up in the polar rather than the cylindrical portions of the sacculi. An alternative rationalization of the result is that during filtration and washing, autolysis of septal murein occurs and that during the subsequent chase recovery of septum formation is delayed while the cylindrical wall continues to be labeled. We have not examined this possibility, although it is well established that the septum is the region of the sacculus most susceptible to autolysis (2, 3, 14, 17, 18), and we have on occasion observed a loss of label from pulse-labeled cells after filtration and washing. Since it is known that the seemingly innocuous act of filtration and resuspension in fresh medium can induce an overproduction of outer membrane proteins involved in iron transport (1), filtration may have other secondary effects related to leakage of metabolites, such as triggering the autolysis of septa.

In our experiments the cells tended to decrease in length during chasing, indicating that septation was suppressed during DAP starvation. The low incidence of cells showing constrictions (8 versus 11% observed by Verwer and Nanninga [20]) would seem to corroborate this conclusion. This may also explain why the percentage of polar grains (Table 1) always was 20 to 30% lower than expected from the actual contour lengths of poles and cylinders in the thin sections studied (calculations not shown). In other words, before pulse-labeling, starvation had decreased the initiation of septation, and, consequently, the percentage of old (unlabeled) poles became higher than normal in the pulse-labeled cells studied. Thus, our modified procedure produced artifacts too.

In these experiments we used a starvation period of 25 min. For most of this period, the intracellular pool of DAP was adequate for continued murein synthesis. We estimate that DAP becomes limiting and that murein synthesis stops after about 20 to 23 min of DAP deprivation. The principal effect of a few-minute interruption of murein synthesis, as judged by our results, is that fewer septa were formed during the starvation period and reinitiation of septation upon the addition of DAP may have been delayed. Although this effect of starvation is a cause for concern, it may have had one positive aspect. It revealed more clearly, than would otherwise have been the case, that multiple growth sites were randomly distributed along the cylindrical portion of the sacculus.

In the thin sections of cells of all size classes pulse-labeled after a starvation period, we saw little evidence of a central zone of synthesis. In fact, the near-random grain distribution observed is similar to that shown in plate II of Schwarz and co-workers (15) for temperature-conditional division mutants of *E. coli* growing at the restrictive temperature. Both results seem to indicate clearly that during elongation of the cell, new murein is incorporated into the murein sacculus at multiple sites. However, such data

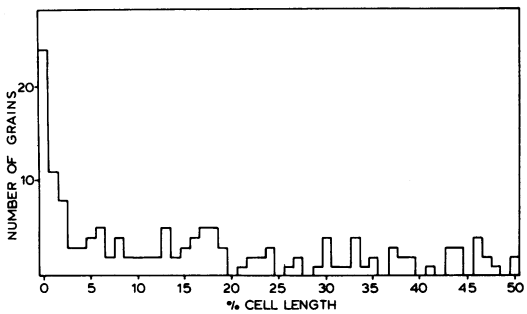

FIG. 3. Location of the grains in thin-sectioned *E. coli* cells after being pulse-labeled with [^{3}H]DAP for 7 min followed by a chase of 1.25 generations (data summarized in Table 1). The distance of each grain relative to the nearest cell pole was expressed as percent (0 to 50) cell length.

would be compatible with the experiments showing a relative increase of centrally located grains (12, 20) if septation is started long before visible septa occur, e.g., by formation of an initiation ring only doubling the thickness of the single-layered sacculus. Candidates for sites of early synthesis of septal murein could be the centrally located nascent periseptal annuli described by MacAlister and co-workers (9). These structures seem to represent an early stage of septation preceding septal ingrowth of the cytoplasmic membrane.

Nevertheless, the present results, as well as earlier data, show that elongation of an *E. coli* cell occurs by insertion of new murein into the preexisting sacculus at multiple sites rather than in a single central zone. In addition, the general loss of grains from the sides of the sacculus but not from the poles during a chase of over three generations is also consistent with a multisite process for insertion of new cylindrical murein. It also emphasizes the eternal nature of the poles of the sacculus.

The concept of elongation of the cell by intercalation of new strands at multiple sites along the cylindrical portion of the murein sacculus is consistent with the enzymological evidence (10) demonstrating that single polypeptides catalyze both the polymerization of glycan strands and cross-linking of new strands to other glycan chains. This applies, e.g., to penicillin-binding proteins 1A and 1B, which seems to be primarily responsible for elongation of the sacculus (10). It seems likely to us that as such an enzyme polymerizes a glycan strand the same polypeptide would immediately and permanently cross-link the strand to the sacculus. A reshuffling process whereby new glycan strands inserted into the sacculus are released and transported a long distance from a central zone and then permanently fixed in the sacculus by a second transpeptidation would seem highly unlikely considering the known properties of the enzymes involved.

With this in mind, we are focusing our attention on the processes of elongation of the murein sacculus and cell septation in an attempt to describe more clearly in biochemical terms the macromolecular events which take place.

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