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Structural Modifications in the Peptidoglycan of *Escherichia coli* Associated with Changes in the State of Growth of the Culture

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By determining the composition in muropeptides of the murein of a number of strains of *Escherichia coli*, purified from cells at various states of growth, the sacculus was found to be considerably modified when cells stop active growth. Murein from resting cells becomes hypercross-linked and richer in covalently bound lipoprotein, whereas the mean length of the glycan chains is considerably reduced. The alteration of the sacculus occurs progressively during the transitions from active growth to stationary phase and vice versa.

During the last few years a considerable amount of evidence has accumulated supporting the idea that the bacterial sacculus is subjected to a fairly dynamic metabolism by postinsertional modification of its structure and chemical composition (6–9, 13, 16). In exponentially growing *Escherichia coli*, the peptidoglycan newly inserted into the growing sacculus acquires its characteristic final composition by a maturation process in which it becomes further cross-linked and richer in covalently bound lipoprotein (6). The maturation of murein in *E. coli* occurs on a time scale similar to that of the cell cycle; therefore, the mean composition of the murein remains constant as long as the cells continue growing exponentially.

Whereas aging of the peptidoglycan during the cell cycle has considerable experimental support, there is little information on possible alterations in the structure of the sacculus during transitions between states of growth. The recent demonstration in *E. coli* and other microorganisms of dramatic changes in the levels of some penicillin-binding proteins during the transitions from active growth into stationary phase (and vice versa [2–4]) provided a further reason for us to investigate the problem in detail.

Our task has been made considerably easier by the timely development by Schwarz et al. of a method that applies the powerful techniques of high-pressure liquid chromatography (H.P.L.C.) to the analysis of the peptidoglycan (7, 11). The method revealed the presence of at least 31 different muropeptides in the peptidoglycan of *E. coli*. Some had a rather surprising structure (dimeric muropeptides cross-linked via a diaminopimelyl-diaminopimelic acid direct bridge [cross-linked trimers]) which may play important structural roles in the murein sacculus.

Our results indicate that, in *E. coli*, the murein undergoes considerable changes during the transition from the exponential phase of growth into stationary phase, and vice versa.

MATERIALS AND METHODS

Organisms and growth conditions. The following strains of *E. coli* were used in this study: *E. coli* W7 *dapA lysA* (10), KN126 K12 F$^-$ *ilv trpA9*(Am) *tyr*(Am) *sup-126*(Ts) (17), and MC6 K12 F$^-$ *proA leuA thr thyA thi dra drm* (5). Bacteria were cultivated at 37°C with shaking in L broth (14) supplemented with 10 mg of *meso*-diaminopimelic acid and per liter 50 mg of thymine per liter.

Radiochemicals. *meso*-3,4,5,[^{3}H]diaminopimelic acid (35 Ci/mmol; 2.5 mCi/ml) was purchased from Commisariat à l'Energie Atomique, Service des Molécules Marquées, Gif-sur-Yvette, France.

Purification of peptidoglycan. Peptidoglycan was prepared as described by Höltje et al. (12). A volume of culture was mixed to a dilution of 1:1 with 8% boiling sodium dodecyl sulfate (SDS) and incubated for 50 to 60 min in a boiling-water bath. After cooling to 40°C, SDS-insoluble material was recovered by centrifugation (100,000 × *g*; 45 min), suspended in warm water (35 to 40°C), and centrifuged again under the same conditions. The last step was repeated twice to get rid of SDS. The last pellet was resuspended in 10 mM Tris–10 mM NaCl (pH 7.2) and digested with α-amilase (100 μg/ml; 1 h at 37°C) and with pronase E (100 μg/ml; 1 h at 60°C). Enzyme action was stopped by diluting the sample 1:1 in boiling 4% SDS; 30 min later, peptidoglycan was recovered by centrifugation (100,000 × *g*; 45 min), washed three times by centrifugation, and resuspended in distilled water. The last pellet was resuspended in 50 mM phosphate buffer (pH 4.9) at a concentration of 2 to 5 mg of murein per ml, digested with muramidase from "*Chalaropsis*" sp. (20 μg of enzyme per ml; 12 h at 37°C) purified as described by Hash and Rothlauf (11). The digests were used for H.P.L.C. immediately or were stored frozen at −70°C until used.

Analysis of purified peptidoglycan by H.P.L.C. Fractionation of the muropeptide mixtures obtained as indicated above was performed by the method of Glauner et al. (7). The muropeptide mixtures were diluted 1:1 with 0.5 M borate buffer (pH 9) and reduced with $NaBH_4$ (8 mg/ml; 30 min at 20°C); after adjusting the pH to 4 with *ortho*-phosphoric acid, the material was applied to a reverse-phase H.P.L.C. column (LiCrosorb RP-18 [250 by 4 mm]; 5-μm particle size; E. Merck AG, Darmstadt, Federal Republic of Germany). The column was eluted with a linear gradient of methanol (0 to 15% [vol/vol]) in 50 mM phosphate buffer (pH 4.69) at a flow rate of 0.5 ml/min. The resolved muropeptides were detected by monitoring the UV absorbance of the eluent at 202 nm. When radioactive samples were analyzed, the outlet of the optical detector was connected to a fraction collector, and the eluent was collected in 0.4-min fractions.

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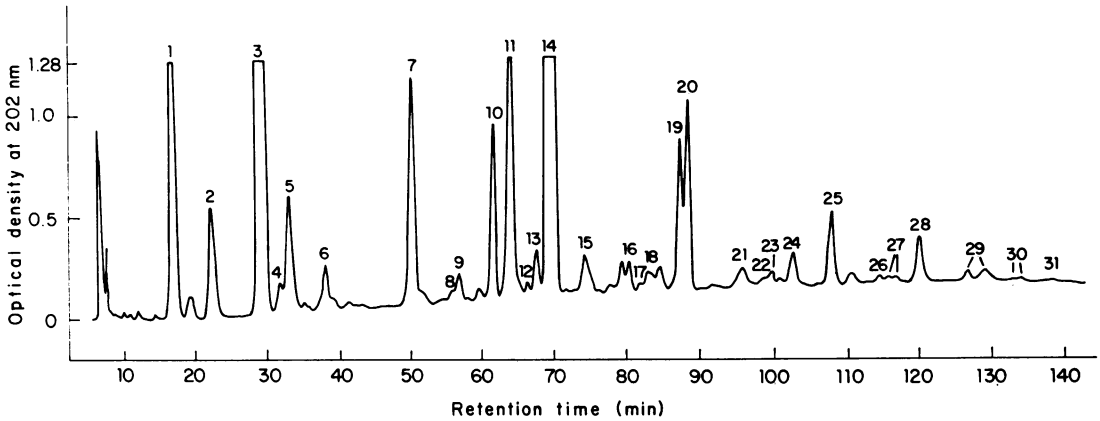

FIG. 1. Elution profile of muropeptides from *E. coli* W7 growing exponentially obtained by HPLC monitoring of the UV absorbance (at 202 nm) of the eluent. Relevant peaks are numbered and listed together with the composition of the compounds as described previously (7). To facilitate nomenclature, the following abbreviations were used: gly 4 (5), glycine residue at position 4 (or 5) in the peptide side chain of the muropeptide; lys-arg, lysine-arginine dipeptide from a molecule of covalently bound lipoprotein at the diaminopimelic acid residue of the peptide side chain of the muropeptide; anhydro, (1-6)-anhydromuramyl residue in the disaccharide moiety of the muropeptide; (dap-dap), muropeptide cross-linked by a diaminopimelyl-diaminopimelic acid peptide bridge. penta, pentapeptide side chain; tetra, tetrapeptide side chain; and tri, tripeptide side chain. Peaks: 1, disaccharide-tri; 2, disaccharide-tetra-gly 4; 3, disaccharide-tetra; 4, disaccharide-penta-gly 5; 5, disaccharide-dipeptide; 6, disaccharide-penta; 7, disaccharide-tri-lys-arg; 8, bisdisaccharide-tetra-tri-gly 4; 9, bisdisaccharide-tri-tri-(dap-dap); 10, bisdisaccharide-tetra-tri; 11, bisdisaccharide-tetra-tri-(dap-dap) plus bisdisaccharide-tetra-tetra-gly 4; 12, disaccharide-tri-anhydro; 13, bisdisaccharide-tetra-penta-gly 5; 14, bisdisaccharide-tetra-tetra; 15, bisdisaccharide-tetra-penta; 16, bisdisaccharide-tri-tri-(dap-dap)-lys-arg; 17, disaccharide-tetra-anhydro; 18, trisdisaccharide-tetra-tetra-tri-(dap-dap); 19, trisdisaccharide-tetra-tetra-tetra; 20, bisdisaccharide-tetra-tri-(dap-dap)-lys-arg; 21, bisdisaccharide-tri-tri-(dap-dap)-anhydro; 22, trisdisaccharide-tetra-tri-tri-(dap-dap)-lys-arg; 23, bisdisaccharide-tetra-tri-anhydro; 24, bisdisaccharide-tetra-tri-(dap-dap)-anhydro; 25, bisdisaccharide-tetra-tetra-anhydro; 26, trisdisaccharide-tetra-tetra-tri-anhydro; 27, trisdisaccharide-tetra-tetra-tri-(dap-dap)-anhydro; 28, Tris-disaccharide-tetra-tetra-tetra-anhydro; 29, bisdisaccharide-tri-tri-(dap-dap)-lys-arg-anhydro; 30, bisdisaccharide-tetra-tri-(dap-dap)-lys-arg-anhydro; and 31, trisdisaccharide-tetra-tetra-tri-(dap-dap)-lys-arg-anhydro.

The radioactivity of each fraction was measured by liquid scintillation, with Bray fluid as the scintillator (1).

Muropeptides have been grouped into the following families for this study: monomers (all of the disaccharide peptides [peak numbers 1–7, 12, and 17; Fig. 1]); dimers (all of the bisdisaccharide peptides [peak numbers 8–11, 13–16, 20, 21, 23–25, 29, and 30; Fig. 1]); trimers (all of the cross-linked tridisaccharide peptides [peak numbers 18, 19, 22, 26–28, and 31; Fig. 1]); diaminopimelyl-diaminopimelic acid muropeptides (all cross-linked muropeptides with a diaminopimelyl-diaminopimelic acid bridge in their molecule [peak numbers 9, 11, 16, 18, 20, 24, 27, 29–31; Fig. 1]); lipoprotein muropeptides (all muropeptides with a covalently bound molecule of lipoprotein [peak numbers 7, 16, 20, 22, 29–31; Fig. 1]); anhydromuropeptides: (all muropeptides with a (1-6)-anhydromuramyl residue in their molecule [peak numbers 12, 17, 21, and 23–31; Fig. 1]).

RESULTS

Composition of the peptidoglycan purified from cultures in exponential phase and 6 h after entering stationary phase. A 400-ml culture of *E. coli* W7 growing exponentially at 37°C for 6 generations was divided into two subcultures at an absorbance at 550 nm of 1. One was immediately used to isolate peptidoglycan; the second was incubated for 9 h more, until it had been in stationary phase for 6 h before peptidoglycan was isolated from it. Both samples were processed and analyzed by HPLC as indicated above. The relative amount of each muropeptide was determined by integration of the corresponding peaks on the UV absorption profiles of the chromatograms (Fig. 1).

Murein from stationary-phase cells (Table 1) was more cross-linked (41.8% of cross-linked muropeptides) than that from exponentially growing cells (33.3% of cross-linked

TABLE 1. Composition of the murein purified from the *E. coli* strains W7, KN126, and MC6 growing exponentially and after 6 h into stationary phase

Strains and (state of growth)	Composition in muropeptides[a] (molar fraction [×100])[b]					
	Monomers	Dimers	Trimers	Diaminopimelyl-diaminopimelic acid muropeptides	Lipoprotein muropeptides	Anhydro-muropeptides
W7 (Exponential)	67.6	30.3	2.9	5.8	7.7	3.0
W7 (Stationary)	57.0	37.1	4.7	11.3	11.1	5.8
KN126 (Exponential)	71.5	26.3	2.7	5.2	10.6	2.6
KN126 (Stationary)	60.0	31.7	7.7	13.7	14.4	6.8
MC6 (Exponential)	69.6	28.1	3.1	5.2	8.8	2.5
MC6 (Stationary)	58.3	33.9	7.9	14.8	13.0	6.7

[a] Muropeptides were grouped as indicated in the text.
[b] Calculated by integration of the UV (at 202 nm) absorption profiles of the chromatograms.

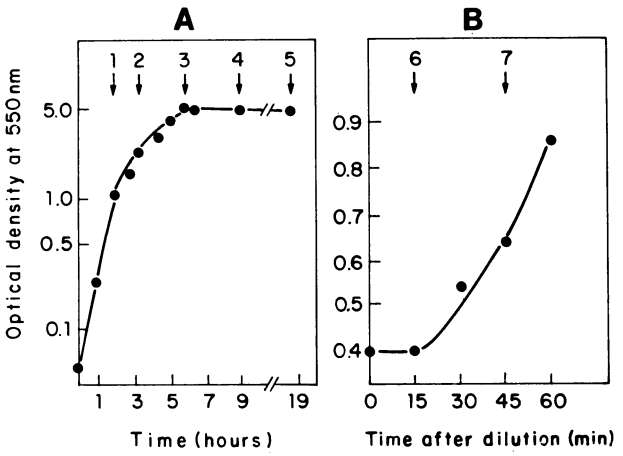

FIG. 2. Growth curve of the cultures of *E. coli* W7 used for the analysis of murein synthesized at various times during the transitions in the state of culture growth. Growth of the culture (in L broth; 37°C) was followed by measuring the optical density at 550 nm at regular time intervals. (A) Transition into stationary phase; (B) recovery of active growth after diluting the culture in prewarmed medium. At the times indicated by the arrows, samples containing 4 × 10^{11} cells were taken, and after a 15-min incubation in the presence of 0.3 μCi of *meso*-[^{3}H]diaminopimelic acid per ml (specific activity, 0.04 μCi/μg), cells were further processed to purify peptidoglycan (Tables 2, 3, and 4).

muropeptides), but the length of the glycan chains, determined as the inverse of the molar fraction of anhydromuropeptides, was longer in the peptidoglycan from exponentially growing cells (33 disaccharide units in exponential cells against 17 disaccharide units in stationary cells). In resting cells, the levels of cross-linked trimers and diaminopimelyl-diaminopimelic acid cross-linked muropeptides were much higher than in growing cells, with relative increments of 60 and 95%, respectively.

To determine whether the modifications detected in *E. coli* W7 were a peculiarity of this strain, the murein of the unrelated strains KN126 and MC6 was also analyzed (Table 1). Experimental conditions were identical to those indicated above. The two strains behaved like strain W7, indicating that the alteration of the murein at the end of active growth might be a fairly general characteristic of *E. coli* strains.

Time course analysis of the composition of murein during transitions in the state of growth of the cells. To study the timing of the alterations taking place on the peptidoglycan when the cell modifies its state of growth, the composition of the murein synthesized at various times during the transitions was analyzed by means of pulse-labeling experiments with *meso*-[^{3}H]diaminopimelic acid.

Samples of a convenient size (4 × 10^{11} total cells) were removed from a culture of *E. coli* W7 growing at 37°C at the following absorbances at 550 nm: 1.0 (exponentially growing cells), 2.0 and 5.0 (transition into stationary phase), 5.0 (3 h into stationary phase), 5.0 (12 h into stationary phase). After the dilution of culture, the following absorbances at 550 nm were made: 0.35 to allow for the resumption of growth, 0.4 (15 min after dilution), and 0.7 (45 min after dilution) (see Fig. 2). The samples were immediately transferred to prewarmed flasks containing *meso*-[^{3}H]diaminopimelic acid to obtain a final activity of 0.3 μCi/ml and 0.04 μCi/μg. After a 15-min incubation at 37°C, the samples were diluted to 1:1 in boiling 8% SDS, kept boiling for 45 min, and further processed as indicated to obtain the peptidoglycan.

The rate of incorporation of *meso*-[^{3}H]diaminopimelic acid into macromolecular murein in the various samples was estimated by taking portions from each sample after boiling in SDS. The samples were filtered through Millipore HAWP filters, washed with boiling water, dried, and measured by liquid scintillation (see Table 2).

With *meso*-[^{3}H]diaminopimelic acid pulse-labeling, it was possible to compare, at any given time, the mean composition of total murein obtained by UV monitoring of the chromatograms with that of newly made murein calculated by the distribution of radioactivity among the various muropeptides. Although this method allowed a parallel estimation of the relative changes in new and total murein, data from UV absorbance and radioactivity should not be strictly compared on a quantitative basis, because the molar extinction coefficients for each muropeptide had not yet been determined accurately. The evolution of the various groups of muropeptides in total and newly made murein are shown in Tables 3 and 4, respectively.

The results indicate that the alterations of the peptidoglycan during cessation of growth, as well as during its resumption, do not occur abruptly but gradually over considerable periods of time.

DISCUSSION

As shown by the data presented here, the transition of the cells from exponential growth into stationary phase is paralleled by a concomitant modification of the murein which becomes more cross-linked and richer in covalently bound lipoprotein as well as compounds of shorter glycan chains. The modification of the peptidoglycan occurs gradually, beginning as soon as the growth rate declines, although murein does not attain its final stable composition until the cells have been in stationary phase for ca. 12 h.

Hypercross-linking of murein in resting cells involves a remarkable rise in the level of trimeric cross-linked muropeptides (more than 50%) as well as in the amount of muropeptides that are cross-linked by direct diaminopimelyl-diaminopimelic acid peptide bridges. In fact, the increase in the mole fraction of the latter kind of muropeptides, from 5.8 to 10.6% (see Table 3 for a comparison of exponential cells and

TABLE 2. Incorporation of *meso*-[^{3}H]diaminopimelic acid by *E. coli* W7 in 4% boiling SDS-insoluble material at various times during transition into stationary phase and resumption of growth after dilution of the culture

State of growth of sample[a]	Optical density	Amt of *meso*-[^{3}H]diaminopimelic acid incorporated in (cpm) in 15 min by 10^8 cells
Exponential phase	1.0	1,942.200
Transition into stationary phase	2.0	381.000
Transition into stationary phase	5.0	57.000
Stationary phase after 3 h	5.0	16.030
Stationary phase after 12 h	5.0	7.930
Transition into exponential phase	0.4	249.750
Transition into exponential phase	0.8	1,106.330

[a] Samples correspond to those indicated in Fig. 1 and analyzed in Tables 3 and 4.

TABLE 3. Evolution of total murein from *E. coli* W7 during the transitions from exponential growth into stationary phase and during resumption of growth by dilution of the cultures

Sample state of growth[c]	Optical density (550 nm)	Composition in muropeptides[a] (molar fraction [×100])[b]					
		Monomers	Dimers	Trimers	Diaminopimelyl-diaminopimelic acid	Lipoprotein muropeptides	Anhydro-muropeptides
Exponential phase	1.0	67.6	30.3	2.9	5.8	7.7	3.0
Transition into stationary phase	2.0	67.6	29.7	3.2	6.2	9.6	2.8
Transition into stationary phase	5.0	62.0	33.3	5.1	8.9	11.8	4.3
Stationary phase after 3 h	5.0	59.6	35.9	4.8	10.6	11.0	5.1
Stationary phase after 12 h	5.0	54.8	40.4	4.8	12.8	11.7	6.6
Transition into exponential phase	0.4	58.8	36.8	5.2	11.5	10.8	5.4
Transition into exponential phase	0.8	67.1	30.4	3.0	6.5	7.8	4.0

[a] Muropeptides were grouped as indicated in the text.
[b] Calculated by integration of the UV (at 202 nm) absorption profiles of the chromatograms.
[c] Samples correspond to those indicated in Fig. 1 and Tables 2 and 4.

stationary cells after 3 h), accounts for ca. 60% of the increase of total cross-linked muropeptides which rose from 33.2 to 40.7% (see Table 3; results as described above).

The parallel increments in cross-linkage and in covalently bound lipoprotein in cells with arrested growth could be interpreted as modifications that reinforce the mechanical strength of the cell wall. However, the simultaneous shortening of the glycan chains casts some doubt on this idea, because there is no clear information, to our knowledge, on how the mean length of the peptidoglycan chains affects the strength of the murein network. From the data shown in Tables 3 and 4, the mean peptidoglycan chain in exponentially growing cells can be estimated to be 33 disaccharide units, cross-linked to neighboring chains by 11 peptide bridges and anchored to the outer membrane by 3 lipoprotein molecules. In the case of resting cells (12 h into stationary phase), the average is 15 disaccharide units, crosslinked by 7 peptide bridges and associated to the outer membrane by 1 to 2 lipoprotein molecules.

When growth stopped, the total amount of murein was compared with that newly made, and both types were found to be affected by the changes to a similar extent. The only remarkable discrepancy corresponded to the variation in the amount of anhydromuropeptides. These showed a relative increment of 38% in new murein and 70% in total murein (cf. Tables 3 and 4; exponential cells and stationary-phase cells after 3 h). The same kind of comparative analysis during the resumption of growth indicated that the murein does not acquire the composition characteristic of exponentially growing cells by the "dilution" of preexisting murein with new murein. Although new murein in cells restarting growth is very similar to that synthetized by cells under full exponential growth, the change in the composition of total murein was faster than could be expected if this assumption were correct. In fact, were it due to a dilution effect, the composition of murein after one doubling in cell mass should be the mean of the compositions of mureins from exponential cells and resting cells after 12 h. However, the experimental

TABLE 4. Evolution of newly synthetized murein from *E. coli* W7 during the transitions from exponential growth into stationary phase and during resumption of growth by dilution of the cultures

Sample state of growth[c]	Optical density (550 nm)	Composition in muropeptides[a] (molar fraction [×100])[b]					
		Monomers	Dimers	Trimers	Diaminopimelyl-diaminopimelic acid	Lipoprotein muropeptides	Anhydro-muropeptides
Exponential phase	1.0	74.7	24.6	1.5	3.5	3.7	2.1
Transition into stationary phase	2.0	72.6	26.2	1.8	4.5	4.0	2.4
Transition into stationary phase	5.0	72.4	26.2	2.4	5.2	5.1	2.4
Stationary phase after 3 h	5.0	70.7	28.2	2.3	6.0	6.1	2.9
Stationary phase after 12 h	5.0	69.4	28.2	4.1	8.6	6.9	6.7
Transition into exponential phase	0.4	76.0	23.1	1.7	4.4	2.6	2.5
Transition into exponential phase	0.8	75.1	24.1	1.5	2.8	3.7	1.1

[a] Muropeptides have been grouped as indicated in the text.
[b] Calculated from the distribution of *meso*-[^{3}H]diaminopimelic acid after fractionation of HPLC.
[c] Samples correspond to those indicated in Fig. 1 and Tables 2 and 3.

values found showed that the composition of murein after one doubling in cell mass was already similar to that of exponentially growing cells (see Table 3), with, again, the significant exception of the anhydromuropeptides. Their kinetics of change corresponds fairly closely with that expected from the dilution model.

The results discussed above suggest a working hypothesis in which the modifications taking place in murein during the transitions in the state of cell growth are coupled to the murein biosynthetic process itself. Affecting these changes are murein-modifying systems which are particularly active when resting cells commence growth.

Rather unexpected was the fact that even after a long period in stationary phase, cells continue synthesis of peptidoglycan. Furthermore, the murein synthesized at that time was very different from that synthesized at any period during the transitions in the state of growth of the cells, since it was particularly rich in diaminopimelyl-diaminopimelic acid bridges, muropeptides with anhydromuramyl residues, and cross-linked trimers. Interestingly, preliminary results from a study, now under way, on the nature of the incorporation of *meso*-[^{3}H]diaminopimelic acid into resting cells suggest that it might be due to a turnover process rather than to cumulative synthesis of the peptidoglycan.

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