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Regulation of Murein Biosynthesis and Septum Formation in Filamentous Cells of *Escherichia coli* PAT 84

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Both the β-lactam antibiotic, cephalexin, and the deoxyribonucleic acid synthesis inhibitor, nalidixic acid, are known to inhibit cell division in *Escherichia coli* and induce the formation of filaments. The biosynthesis of murein was investigated in these filaments and compared with the murein synthesized by the normally dividing rods of *E. coli* PAT 84. Differences were found in the extent of peptide side-chain cross-linkage. Filamentous cells had higher extents of cross-linkages in their newly synthesized murein. Quantitative analyses of the D-alanine carboxypeptidase and transpeptidase reactions in the different cells revealed that the carboxypeptidase activity of the filamentous cells was partially inhibited. These results were similar to those previously found with filaments that were obtained after growth of the thermosensitive division mutant at its restrictive temperature. We conclude that the formation of new cell ends (septa) depends on the proper balance between the activities of the D-alanine carboxypeptidase that regulates the availability of precursor donors and the transpeptidase, which catalyzes cross-linking and attachment of newly synthesized murein.

Cell division in *Escherichia coli* has been shown to be inhibited by a great variety of chemical and physical methods as well as by several types of thermosensitive mutations (3, 9, 16). In most of these cases the result was that the rod-shaped bacteria continues to grow and forms nonseptated filaments.

Although it has been suggested that β-lactam antibiotics affect cell division, elongation and shape due to their interaction and binding to enzymes that are involved in the metabolism of the cell wall murein (1, 17), little evidence on the physiological function of the enzymes has been presented. In *E. coli* at least three enzymatic activities have been identified that are inhibited by penicillins: (i) the D-alanine carboxypeptidases that hydrolyze C-terminal D-Ala-D-Ala peptide bonds and which have been suggested to regulate the extent of peptide side-chain cross-linking in murein (10, 15); (ii) the endopeptidase (cross-link splitting enzyme) which is thought to be another manifestation of the D-alanine carboxypeptidase (2, 8); and (iii) the transpeptidases that catalyze the formation of cross-linkages (10, 18).

Using a novel in vitro system for studying the last stages of murein biosynthesis in *E. coli* we recently demonstrated a correlation between the extent of peptide cross-linking formed by the transpeptidation reaction and the D-alanine carboxypeptidase activity of the cell (14). We found that partial inhibition of the D-alanine carboxypeptidase activity by low concentrations of ampicillin caused an increase in the incorporation and cross-linkage of newly synthesized murein. In addition, we found that nonseptated filamentous cells of the thermosensitive division mutant *E. coli* PAT 84, when compared with the normally dividing rods, had lower levels of D-alanine carboxypeptidase activity and higher than normal degrees of peptide side-chain cross-linking in their newly synthesized murein (14). These findings suggested that the inability to form new dividing septa in cells of the thermosensitive division mutant was caused by an improper balance of activity between the peptide cross-linking and the D-alanine carboxypeptidase systems. To obtain further information on the possible role that these enzymatic activities may have in septum formation, we studied their activity as well as the synthesis of murein in two cases where antibiotics with a defined mode of action induce the formation of filaments (4, 5, 17). We now report that cells of *E. coli* that grew as filaments in the presence of either cephalexin or nalidixic acid had higher degrees of peptide cross-linkages and lower levels of D-alanine carboxypeptidase activity than the normally dividing cells of the same strain. These results lend

further support to our earlier observations (14) and, based on these, the mechanism and regulation of septum formation in *E. coli* are discussed.

MATERIALS AND METHODS

The organism used throughout these studies was the thermosensitive division mutant *E. coli* PAT 84 (DAP^-, Lys^-) (9). This mutant grows as normally dividing rod-like cells at 30°C and as polynucleated, nonseptated filaments at 42°C. The bacteria were grown with aeration to 10^8 cells/ml in the presence of *meso*-D,L-2,6 diaminopimelic acid (DAP) (4 μg/ml) and L-lysine (20 μg/ml) as described (14). Ether-treated cells were prepared from early log-phase cells (10^8 cells/ml) grown either at the permissive (30°C) or restrictive temperature (60 min, 42°C), according to the procedure of Vosberg and Hoffmann-Berling (20). Ether-treated cells were also prepared from filamentous cells of *E. coli* PAT 84 grown as above at the permissive temperature in the presence of cephalexin (Glaxo, England), 25 μg/ml for 60 min, or nalidixic acid (Sigma Co.), 10 μg/ml for 120 min.

Murein biosynthesis was studied in ether-treated cells as described (14). Assays for incorporation of murein routinely contained, in a final volume of 200 μl, uridine 5'-diphosphate (UDP) [^{14}C]*N*-acetylglucosamine (GlcNAc) (Radiochemical Centre, Amersham) (90 nmol, 0.9 cpm/pmol), UDP-*N*-acetylmuramyl (MurNAc)-L-Ala-D-isoGlu-*meso*-DAP-D-Ala-D-Ala (UDP-MurNAc-pentapeptide, 100 nmol) and approximately 0.8 mg of ether-treated bacteria. Reaction mixtures were incubated at either 30 or 42°C for 60 min, and the incorporation of radioactive precursors into insoluble cell wall material was determined after treatment with 4 ml of 5% CCl_3COOH (trichloroacetic acid) or with 1 ml of hot (100°C) 4% sodium dodecyl sulfate (SDS). The insoluble cell wall material remaining after these treatments was collected after filtration on membrane filters (0.45 μm; Millipore Corp.). Measurements of radioactivity were done in a scintillation counter, Packard-Tri-Carb 3003, with a toluene-based scintillation fluid at an efficiency of 74%.

The enzymatic release of the C-terminal D-alanine moiety of UDP-MurNAc-pentapeptide, which was used as a measure for the activities of D-alanine-carboxypeptidase and -transpeptidase, was assayed with UDP-MurNAc[^{14}C]D-Ala-pentapeptide (9 cpm/pmol, 160,000 cpm) as the labeled substrate. This was added to incubation mixtures with or without UDP-GlcNAc (100 nmol). After the incubation, reaction mixtures were treated with hot (100°C) 4% SDS (0.7 ml) for 30 min, and the amount of free [^{14}C]alanine was determined on an amino acid analyzer that was directly connected to a Packard flow analyzer scintillator (13).

Labeling of murein in intact cells with [^{3}H]DAP under different growth conditions. Cells of *E. coli* PAT 84 (200 ml) were grown with aeration at 30°C as described above to a density of 3×10^7 cells per ml. At this point (time zero), the cells were divided into four flasks. To one of the flasks nalidixic acid (10 μg/ml) was added and the cells were further incubated. After 60 min, cephalexin (25 μg/ml) was added to flask no. 2 and flask no. 3 was transferred to a 42°C bath. Bacteria in flask no. 4 were grown as the control at 30°C. After 60 min of incubation, the optical density of the cultures was monitored with a Klett-Summerson spectrophotometer (540-nm filter). Only a slight (10%) inhibition of growth was observed with nalidixic acid (10 μg/ml) after 2 h of incubation. At this stage 20 ml from each flask was removed and rapidly filtered (Sartorius membrane filter, 0.6 μm, Gottingen, Germany), and the cells on the filter were washed twice with 50 ml of growth medium lacking DAP and warmed either to 30 or 42°C depending on the temperature at which DAP incorporation was to take place. The washing medium for the cells grown in cephalexin or nalidixic acid also contained the respective antibiotic. The washed cells were suspended in 20 ml of medium lacking DAP, containing 500 μCi of [^{3}H]DAP (specific activity 13 Ci/mmol, Service de Molecules Marques [C.E.A.], France) as well as the respective concentrations of antibiotic. After incubation for 15 min at 30 or 42°C, respectively, the cells from each of the four flasks were filtered on membrane filters and rapidly washed as described above. The cells were then resuspended in 5 ml of medium and added to an equal volume of cold trichloroacetic acid (10%) and kept in ice for 30 min. The insoluble material was sedimented by centrifugation (30 min, 15,000 rpm) and the precipitate was suspended and washed three times with ice-cold water followed by sedimentation. The precipitate was then resuspended in 1.2 ml of ammonium acetate (0.01 M) and heated in a boiling-water bath for 15 min to destroy autolytic enzymes. After cooling, ribonuclease (100 μg/ml) and deoxyribonuclease (100 μg/ml) were added and the suspension was incubated overnight at 37°C. After this incubation, Pronase (100 μg/ml) was added and the suspension was incubated again for 6 h at 37°C, followed by heating in a boiling-water bath for 30 min. The digests were then dialyzed extensively against water and finally digested with lysozyme (100 μg/ml) in 0.05 M ammonium acetate, at 37°C overnight.

The degree of peptide cross-linkage, both in the murein synthesized by the intact cells and in ether-treated cells, was determined in lysozyme digests (14). Radioactive compounds were located by autoradiography, the ^{14}C-labeled fragments by Kodak X-ray film XP-54 and the ^{3}H-labeled materials with a Packard Radioactive Strip Scanner. The radioactive spots were cut and counted (14).

Other procedures. SDS-slab-gel electrophoresis was performed as described by Laemmli and Favre (11) using an acrylamide gradient from 10 to 20%. Cell membrane samples for electrophoresis were solubilized with Sarkosyl NL 97 as described (17). Protein was determined by the method of Lowry et al. (12). Radioactive amino acids and peptides were quantitatively determined as described (13).

RESULTS

Murein biosynthesis in nalidixic acid-induced filament. One of the antibiotics that has been shown to induce filament formation in *E.*

coli is nalidixic acid (4). This drug did not inhibit murein biosynthesis in ether-treated cells from normally dividing *E. coli* rods. No effect was observed with nalidixic acid (10 μg/ml) on the incorporation of labeled nucleotide sugar precursor into trichloroacetic acid- or SDS-insoluble murein. Furthermore, nalidixic acid (10 μg/ml) did not inhibit the release of C-terminal [^{14}C]alanine from UDP-MurNAc[^{14}C]D-Ala-pentapeptide nor the formation of peptide side-chain cross-linkages (Table 1).

The murein synthesized by the ether-treated filaments, which were prepared from *E. coli* cells grown for 120 min at 30°C in the presence of nalidixic acid (10 μg/ml), contained more peptide cross-links than the untreated, normally growing rods of the same organism (Table 1). Furthermore, the nalidixic acid-treated ether cells had a reduced level of D-alanine carboxypeptidase activity, and the ratio between the calculated transpeptidase and carboxypeptidase activities was different from that found in normal rod-like cells (Table 2). The total D-alanine carboxypeptidase activity of the nalidixic acid-treated cells, however, as measured after a brief sonication period (1 min at maximal amplitude; sonic oscillator, Measuring & Scientific Equipment, Ltd., London) was considerably higher than that observed for the nonsonicated ether-treated cells (Table 2).

Murein biosynthesis in nonseptated, cephalexin-induced filaments of *E. coli*. Two of the β-lactam antibiotics that most effectively induce the formation of nonseptated filamentous cells are cephalexin (5) and cefazolin. Inhibition of cell division seems to be the only effect of these antibiotics; the increase in cell mass was normal and the cells did not lyse even after several hours of incubation over a wide range of concentrations (cephalexin, 5 to 50 μg/ml [5]; cefazolin, 0.5 to 5.0 μg/ml). The main effect of these antibiotics on murein biosynthesis as determined from experiments with ether-treated cells of normally dividing *E. coli* PAT 84 rods was on the D-alanine carboxypeptidase activity which, as shown in Fig. 1, was inhibited approximately 80% by cefazolin (1 μg/ml) and cephalexin (5 μg/ml). At these concentrations almost no effect was observed on the incorporation and attachment of newly synthesized murein to the preexisting cell wall (sacculus) (Fig. 1) or on the formation of peptide side-chain cross-linkages by the transpeptidation reaction (Table 1).

The murein synthesized by ether-treated filaments prepared from *E. coli* cells grown for 60 min at 30°C in the presence of cephalexin (25 μg/ml) had a higher degree of peptide side-chain cross-linkage than the untreated rod-like cells which served as a control (Table 1). Furthermore, in these filaments the D-alanine carboxypeptidase activity was considerably re-

TABLE 1. *Peptide side-chain cross-linkages in murein of normal rods and filaments*

Growth conditions of *E. coli* PAT 84	Cell shape	Peptide side-chain cross-linkage ratios[a]		
		Ether-treated cells incubated[b]		Intact bacteria[c]
		Without antibiotic	In the presence of antibiotic (μg/ml)	
30°C	Normal rods	1.01	Ampicillin (0.5), 1.19	0.67
30°C, + cephalexin (25 μg/ml), 60 min	Filaments	1.23	Cephalexin (25), 1.16	0.86
30°C, + nalidixic acid (10 μg/ml), 120 min	Filaments	1.45	Nalidixic acid (10), 1.41	0.87
42°C, 60 min	Filaments	1.39	Ampicillin (0.5), 1.42	0.90

[a] Ratios of cross-linked (C_3 + C_4) to uncross-linked (C_5 + C_6) murein fragments obtained after chromatography of lysozyme digests. Labeled, newly synthesized murein was incubated with lysozyme and the digests were separated on paper chromatograms as described in the text. The amount of cross-linked and uncross-linked fragments in the digests was computed from the amount of radioactivity in each of the respective muropeptides. Differences of less than 15% were obtained in two independent experiments.

[b] Ether-treated bacteria were prepared as described in the text. Incubation mixtures, in a final volume of 200 μl, contained tris(hydroxymethyl)aminomethane buffer (50 mM, pH 8.3), NH_4Cl (50 mM), $MgCl_2$ (20 mM), adenosine 5′-triphosphate (10 mM), 2-mercaptoethanol (0.5 mM), UDP-MurNAc pentapeptide (100 nmol), UDP[^{14}C]GlcNAc (specific activity, 40 cpm/pmol, 180,000 cpm) and ether-treated bacteria (~0.8 mg of protein). Incubations were done at 30°C for 60 min (cells grown at 42°C were incubated at 42°C) in the absence or presence of antibiotic as indicated. After the reaction, 1 ml of Cl_3COOH (10%) was added. Insoluble material was filtered and washed on membrane filters (0.45 μm, Millipore Corp.). The filters were suspended in vials in 1.0 ml of ammonium acetate (0.05 M, pH 7.0) and heated in a boiling-water bath for 15 min to destroy lytic enzymes and then digested with lysozyme as described.

[c] Pulse labeling of murein in intact cells was with [^{3}H]DAP as described in the text. Extent of peptide cross-linkage was determined as for the murein synthesized with ether-treated bacteria.

TABLE 2. *Enzymatic release of C-terminal [^{14}C]alanine from UDP-MurNAc[^{14}C]D-Ala-pentapeptide by ether-treated rod-like or filamentous cells*[a]

Growth conditions of *E. coli* PAT 84	Cell shape	Transpeptidase activity[b]	DD-carboxypeptidase activity[c]		Transpeptidase/ carboxypeptidase ratio
			Ether-treated[c] cells	Sonically treated cells[d]	
30°C	Normal rods	260	380	910	0.7
30°C, + cephalexin (25 μg/ml), 60 min	Filaments	320	205	290	1.5
30°C, + nalidixic acid (10 μg/ml), 120 min	Filaments	240	210	690	1.15
42°C, 60 min	Filaments	290	210	780	1.4

[a] Ether-treated cells were prepared as described in the text. Incubation mixtures in a final volume of 200 μl contained ether-treated bacteria (~0.8 mg of protein). Tris(hydroxymethyl)aminomethane buffer (50 mM, pH 8.3), NH_4Cl (50 mM), $MgCl_2$ (20 mM), adenosine 5'-triphosphate (10 mM), 2-mercaptoethanol (0.5 mM), UDP-MurNAc[^{14}C]D-Ala-pentapeptide (specific activity 9 cpm/pmol, 160,000 cpm), and UDP-GlcNAc (100 nmol) were added as indicated (see footnotes b, c). Reaction mixtures were incubated for 60 min at 30°C. For cells grown at 42°C, the incubation mixtures were also done at 42°C. After the reaction, 1 ml of SDS (4%) was added, and the solution was heated (100°C, 15 min) and then analyzed for free [^{14}C]alanine as described (13). Values shown are in picomoles of [^{14}C]alanine released per milligram of cell protein.

[b] Transpeptidase activity was calculated from the difference in amounts of [^{14}C]alanine released by reactions done in the presence or absence of UDP-GlcNAc and 0.5 μg of ampicillin per ml.

[c] The ampicillin-sensitive D-alanine-carboxypeptidase activity was determined from the difference in amounts of [^{14}C]alanine released by reactions carried out with UDP-GlcNAc in the absence and presence of ampicillin (0.5 μg/ml).

[d] Same ether-treated cell preparation was sonically treated before incubation for 1 min at maximal amplitude in a Measuring & Scientific Equipment sonic oscillator. The release of [^{14}C]alanine by these preparations was inhibited (>95%) by ampicillin (0.5 μg/ml).

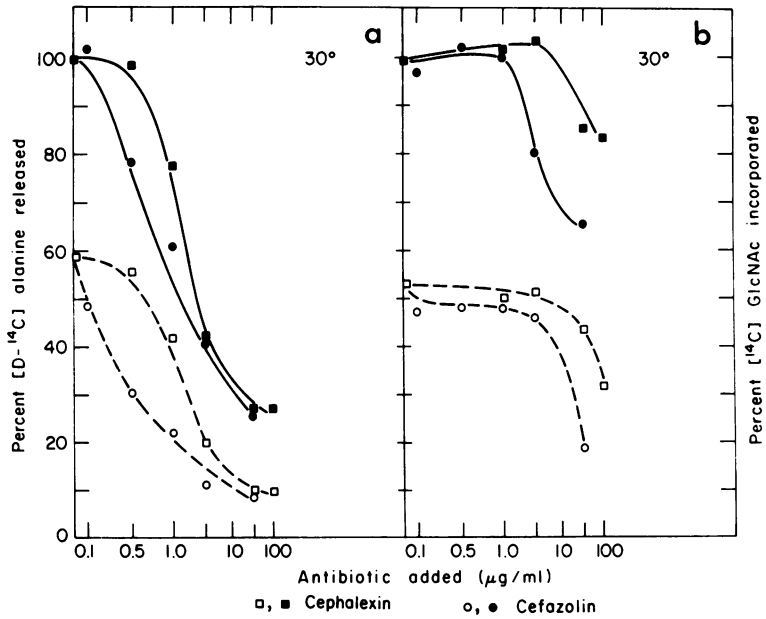


FIG. 1. *(a) The inhibitory effect of increasing concentrations of cephalexin* (□) *or cefazolin* (○) *on the release of C-terminal [^{14}C]alanine from UDP-MurNAc[^{14}C]D-Ala-pentapeptide. Ether-treated cells from E. coli PAT 84 grown as normally dividing rods at 30°C were incubated with* (■, ●) *or without* (□, ○) *UDP-GlcNAc as described in the text. (b) The inhibitory effect of cephalexin* (□) *and cefazolin* (○) *on the incorporation of UDP[^{14}C]GlcNAc by ether-treated cells, as above. Radioactivity was measured in precipitates obtained with trichloroacetic acid* (——) *and in sacculi after boiling of the samples with SDS* (-----).

duced and the ratio between the calculated transpeptidase and carboxypeptidase activity was almost twice that in the normal rod-like cells (Table 2). In addition, in the cephalexin-induced filaments, approximately 75% of the total murein that was synthesized from the labeled precursors became covalently bound to the preexisting cell wall sacculus, as compared with about 55 to 60% in the normally dividing rods (Fig. 2) (14). This was concluded from the small difference observed between the total murein strands synthesized and determined as CCl_3COOH-insoluble material and the murein that was determined after heating the cells in SDS, which removed the material that was not covalently attached to the preexisting sacculus (14).

Extent of peptide side-chain cross-linking in intact cells grown under different conditions. Chromatographic analysis of lysozyme digests of the high-molecular-weight [^{3}H]DAP-labeled murein synthesized by intact cells of the four different types showed that the three filamentous cells (induced by cephalexin, nalidixic acid, or restrictive temperature) were more cross-linked (by approximately 30%) than the rod-like cells (Table 1). A similar level of increase in the extent of cross-linkage of the newly synthesized murein was also obtained with the corresponding ether-treated cell preparations.

Slab-gel electrophoresis of cell membrane proteins. A preliminary comparison between the cell membrane proteins of the rod-like and the filamentous cells of the thermosensitive mutant *E. coli* PAT 84 on slab-gel electrophoresis showed that they differ in a number of protein bands (Fig. 3). No difference was observed, however, in the protein bands of the cephalexin-induced filaments.

DISCUSSION

The present investigation shows that in three forms of filamentous cells of *E. coli* PAT 84 the newly synthesized murein had a higher level of peptide side-chain cross-linkage as compared with that found in the normally dividing rod-like cells of the same strain (Table 1). The stimulation of cross-linkage in newly synthesized murein of filamentous forms was observed both in experiments done with ether-treated cells as well as with intact cells. Furthermore, we have found that in these filaments there was also a depressed level of D-alanine carboxypeptidase activity and that cephalexin almost completely inhibited the D-alanine carboxypeptidase without inhibiting peptide cross-linkage formation (Fig. 1).

Nalidixic acid, though without any direct effect on murein biosynthesis (Tables 1 and 2), leads nevertheless to a partial inactivation of D-alanine carboxypeptidase activity in the cell.

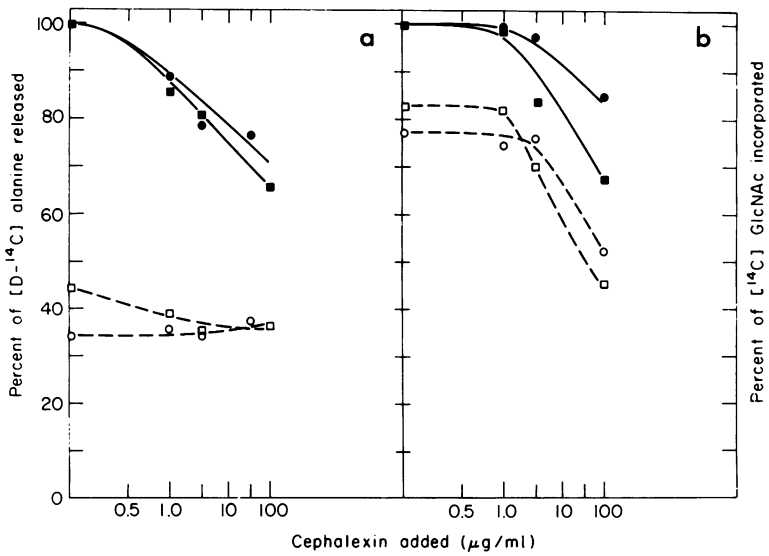


FIG. 2. *(a) The effect of cephalexin on the release of C-terminal [^{14}C]alanine from UDP-MurNAc[^{14}C]D-Ala-pentapeptide by ether-treated filaments obtained from E. coli PAT 84 grown at 42°C* (○, ●) *for 60 min or after growing them in the presence of cephalexin* (□, ■) *(25 μg/ml, 60 min). Incubations were done with* (■, ●) *or without* (□, ○) *UDP-GlcNAc as described in the text. (b) The effect of cephalexin on the incorporation of UDP[^{14}C]GlcNAc by the same ether-treated cells. Radioactivity was measured in precipitates obtained with trichloroacetic acid* (——) *and in sacculi after boiling of the samples with SDS* (-----).

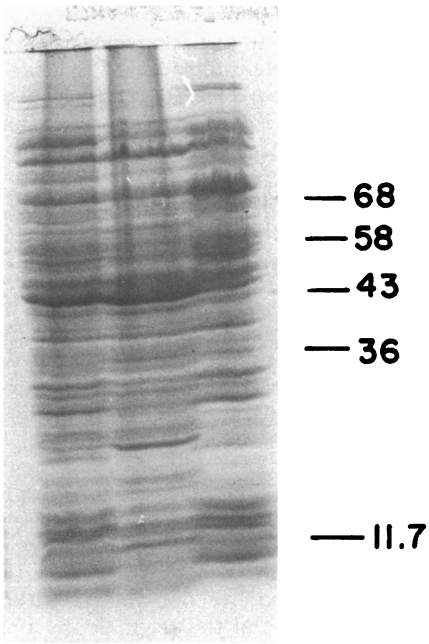


FIG. 3. *Membrane proteins of E. coli PAT 84 cells. Membrane preparations were obtained by differential centrifugation after extraction with 1% Sarkosyl from cells grown (a) in the presence of cephalexin (25 μg/ml, 60 min); (b) at the restrictive temperature (42°C, 60 min); (c) at the permissive temperature (30°C). SDS-acrylamide-gradient (10 to 20%) slab-gel electrophoresis was performed as described (11). Molecular weights (in 1,000's) are shown on the right of the figure.*

This effect was again correlated with an increase in the extent of peptide cross-linking and the inability of the cells to divide in the presence of the antibiotic.

The effects of a block of septum formation on murein biosynthesis are strikingly similar in all three cases studied. In filaments, induced by completely different interferences with the septum formation, the ability to divide seems to depend on the proper function and relative activity of the D-alanine carboxypeptidase(s) as compared with the transpeptidase.

A major difficulty in the interpretation of our findings with regard to the specific role of the D-alanine carboxypeptidase in cell division lies in the fact that the enzymes under discussion show at least two different specificities: (i) they can operate as a D-alanine carboxypeptidase or (ii) as an endopeptidase (peptide cross-link splitting enzyme [2, 8, 15, 19]). If in the cell the enzyme acts predominantly as a D-alanine carboxypeptidase, its physiological function could be to remove C-terminal D-alanine residues from part of the newly synthesized murein strands, converting them into inert murein acceptors which begin to accumulate at the site of synthesis. Another portion of the newly synthesized murein strands that retain their C-terminal D-alanine residues would be the donors for the transpeptidation reaction, and their subsequent attachment to the accumulated acceptors would lead to the formation of newly synthesized septum (Fig. 4). Thus, under growth conditions where a partial inhibition of D-alanine carboxypeptidase occurs (cephalexin, nalidixic acid, or restrictive growth temperature [14]), there is a diminished accumulation of acceptor and a greater proportion of the newly synthesized murein strands remain with their C-terminal D-alanine as intact donors. These strands may continue to be attached by the transpeptidation reaction to acceptors that are present on the preexisting lateral wall, causing it to elongate without synthesizing the septum region. On the other hand, if the enzyme acts mainly as an endopeptidase, it may function under topological control at the site of cell division to loosen up the structure of the preexisting murein. The hydrolysis of peptide cross-linkages could initiate the necessary morphological conversion of the wall cylinder into the hemispherical cell ends, and the liberated peptide chains could then serve as acceptors for incoming murein donor strands. Partial inhibition of these processes could interfere with the cell's ability to divide and cause the formation of filaments.

The mechanism by which the D-alanine carboxypeptidase is activated during septum formation of a normal *E. coli* cell or inhibited in the nalidixic acid- or thermosensitive-induced filaments is far from being understood.

Nalidixic acid, which inhibits deoxyribonucleic acid synthesis (4), has recently been shown to stimulate the synthesis of a protein X and also to cause the inhibition of an envelope protein D in *E. coli* (6, 7). Although it has been suggested that this protein D may provide a metabolic link between murein synthesis, protein synthesis, deoxyribonucleic acid initiation, and cell division, no evidence on its physiological function has been presented (6, 7). Growth of the cells in nalidixic acid must somehow cause an alteration in the cell membrane protein components, and one of the consequences of this may be that it prevents the activation of the D-alanine carboxypeptidase at the site of murein synthesis. The alteration in the composition of the membrane proteins of cells grown at the restrictive temperature (42°C) may have

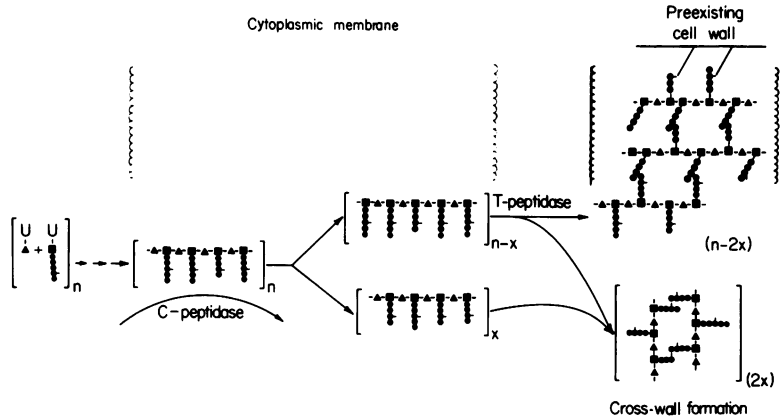
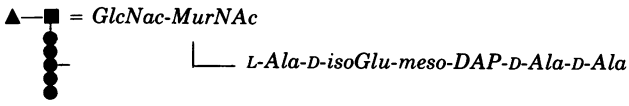


FIG. 4. *Schematic representation of the regulatory role of the D-alanine carboxypeptidase (C-peptidase) activity in the incorporation and attachment of newly synthesized strands by transpeptidation (T-peptidase) to the preexisting murein in E. coli cell walls. Murein strands (n) from which most of their C-terminal D-alanine moieties have been removed by a D-alanine carboxypeptidase activity are converted into acceptors (x) awaiting cross-linkage to donors (n-x). Formation of the crosswall is then achieved by transpeptidation of newly synthesized donor and acceptor strands. The acceptor strands (x) may begin accumulating before cell division.*



an effect similar to that observed with nalidixic acid (6, 7). This alteration appears to be the cause that leads to the partial inhibition of D-alanine carboxypeptidase activity in these filamentous cells.

More work is undoubtedly needed to unravel the mode of action of these enzymes and to understand the regulation of their activity in the intact cell.

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