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Transglycosylase and Endopeptidase Participate in the Degradation of Murein during Autolysis of *Escherichia coli*

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The cell wall degradation products released from *Escherichia coli* during autolysis triggered by cephaloridine or trichloroacetic acid were isolated and characterized. Murein was selectively lost from the disaccharide tetrapeptides and the bisdisaccharide tetrapeptide components. Two major autolytic products accounted for more than 85% of the released material. Compound 1 (60 to 80% of released material) was a disaccharide tetrapeptide monomer containing a 1,6-anhydromuramic acid residue. Compound 2 (15 to 30% of released material) was a mixture of a tritripeptide and a tritetrapeptide without hexosamines. Taken together the findings suggest that autolytic cell wall degradation in *E. coli* is selective and involves the activity of both the hydrolytic transglycosylase and an endopeptidase. Upon release, at least some of the wall components were also exposed to the activity of the *N*-acetylmuramic acid-L-alanine amidase.

Degradation of murein during autolysis of *Escherichia coli* was first described by Schwarz and Weidel (15). Murein degradation in cells exposed to cell wall inhibitors (5), chaotropic agents (7), or other treatments (5) is an enzymatic process catalyzed by murein hydrolases (autolysins). In contrast to other bacteria, *E. coli* contains several cell wall-degrading activities (7, 8): a degradative transglycosylase, an endopeptidase, and an *N*-acetylmuramyl L-alanine amidase, but it is unclear which of these multiple activities plays a role in autolysis. Mutants with alterations in the activity of the first two enzymes, but not the amidase, are tolerant to beta-lactam-induced autolysis (10, 11), suggesting that not all activities are required for autolysis. The purpose of the experiments to be described here was twofold: (i) to identify which of these hydrolases participate in the wall degradation triggered by inhibitors of cell wall synthesis (cephaloridine and penicillin) and by a chaotropic agent (trichloroacetic acid [TCA]) used in studies of autolysis-induction in *E. coli*, and (ii) to determine if autolysis involves selective degradation of certain murein components. We used two approaches in our studies. (i) The products of cell wall degradation were isolated from the surrounding medium and characterized by electrophoresis and amino acid analysis (techniques applicable to soluble peptidoglycan). (ii) After 40% of the murein was degraded, the residual (undegraded) murein was reisolated and analyzed by high-performance liquid chromatography (HPLC; a technique applicable to insoluble peptidoglycan) to determine which primary components were lost and contributed to the generation of the products seen in the first approach and whether the loss was selective. Results of previous studies have shown that pharmacologically important effects of beta-lactam antibiotics such as killing and lysis of bacteria are related to the uncontrolled activity of murein hydrolases, and the studies described here should help to elucidate the nature of such induced autolytic activity.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* χ1776 F⁻ *fhuA53 dapD8 minA1 supE42* Δ(*gal-uvrB*)*40* λ⁻ *minB2 rfb-2 gyrA25 oms-2 thyA57 metC65 oms-1* Δ(*bioH-asd*)*29 cycB2 cycA1 hsdR2* (1), *E. coli* GN-4 (*dap*) (9), and *E. coli* W7 (*dap lys*) (14) were used. *E. coli* χ1776 was cultured without aeration (a requirement of viability in this strain) at 32°C in Penassay broth (antibiotic medium number 3; Difco Laboratories, Detroit, Mich.) supplemented with 2,6-DL-*meso*-diaminopimelic acid (DAP; 20 μg/ml), biotin (0.2 μg/ml), and thymidine (30 μg/ml) (supplemented AM-3 medium). *E. coli* GN-4 was cultivated in NB medium supplemented with 0.1% *N*-acetylglucosamine at 32°C under aerobic conditions. *E. coli* W7 was grown in a synthetic salts medium (M9) supplemented with glycerol (4 mg/ml), DAP (5 μg/ml), and L-lysine (10 μg/ml) at 37°C with aeration, as described previously (5). Growth was measured by determining the absorbance (*A*) of suspensions with a Sequoia Turner spectrophotometer.

For radioactive labeling, [^{3}H]DAP (1 μCi/ml) or [^{3}H]*N*-acetylglucosamine (1 μg and 1 μCi/ml) was used. Murein of *E. coli* χ1776 was radioactively labeled by adding (DL-*meso*)-2,6-diamino-[U-^{3}H]pimelic acid (1.5 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill.) and L-lysine (1 mg/ml) to the medium ([^{3}H]DAP medium); 1.0 μCi of [^{3}H]DAP was added per ml of culture medium, giving a final DAP concentration of 3.126 μg/ml. Murein of *E. coli* GN-4 was labeled by adding [^{3}H]*N*-acetylglucosamine (1 μCi/ml; Amersham) and cold *N*-acetylglucosamine to give a final *N*-acetylglucosamine concentration of 116 μg/ml. For the labeling of cell walls of strain W7, [^{3}H]*N*-acetylglucosamine (1 μg and 1 μCi/ml) was added to the basic M9 medium.

Preparation of autolytic products from cephaloridine-triggered cells. Bacteria were cultivated in 100 ml of [^{3}H]DAP medium. At the mid-exponential phase of growth (A = 350, 5 × 10^8 CFU/ml), cells were centrifuged and transferred into 200 ml of isotope-free medium. After incubation for 50 min the cultures received 62.5 μg of cephaloridine or 60 μg of benzylpenicillin per ml and were incubated for another 10 min. After chilling in ice, cells were collected by centrifugation and washed with 0.1 M sodium phosphate buffer (pH 7.0) containing 10 mM $MgSO_4$. Parallel

cultures in isotope-free medium (2,000 ml) were also treated with cephaloridine by the same method and suspended in 2,000 ml of buffer. Both labeled and unlabeled cells were combined and incubated for 3 h at 30°C. The cells were removed by centrifugation, and the supernatant fluid received 30 g of charcoal. After mixing with a magnetic stirrer, the charcoal was recovered by centrifugation and washed twice with water. The absorbed products were then eluted with 300 ml of a 50% ethanol–1 M NH_4OH solution. A total of 92% of the radiolabel of the starting material was recovered from the charcoal. The eluates were dried in vacuo, suspended in 10 ml of water, and applied to a Bio-Gel P-6 column. In some cases, the supernatant of the autolysate was directly applied onto the Bio-Gel P-6 column.

Preparation of autolytic products from TCA-triggered cells. *E. coli* was cultivated in 100 ml of [^{3}H]DAP medium. At the exponential phase of growth (A = 300), cells were centrifuged, suspended in 200 ml of isotope-free medium, and incubated for another 60 min. Cells were collected again by centrifugation (8,000 × g, 5 min) and suspended in 10 ml of 0.01 M Tris-maleate buffer (pH 6.0) containing 0.01 M $MgSO_4$. An equal volume of 10% TCA was added to the cell suspension and allowed to react for 10 min on ice (7). The cells were washed three times by centrifugation in the buffer and suspended in 10 ml of the same buffer. Cells from 100 ml of isotope-free medium were also treated with TCA by the same method and suspended in 100 ml of the buffer. Both labeled and unlabeled cells were combined and incubated for 2 h. The reaction mixture was centrifuged, and the supernatant fluids were dried in vacuo. The residues were resuspended in 10 ml of water and then applied to a Bio-Gel P-6 column.

Preparation of lytic products of murein by murein transglycosylase. Sacculi were prepared essentially by a previously described method (11). *E. coli* χ1776 was cultivated in 100 ml of [^{14}C]DAP medium–200 ml of isotope-free medium at 32°C without aeration. At the exponential phase of growth (A = 300), cells were collected, washed with water, suspended in 10 ml water, and added slowly to 10 ml of a boiling solution of sodium dodecyl sulfate (1%; wt/vol) under vigorous stirring. The mixture was kept boiling for 10 min, after which the sacculi were collected by centrifugation (60 min, 36,000 × g), washed twice with 0.01 M Tris hydrochloride buffer (pH 7.5) containing 0.02 M NaCl, and suspended in 10 ml of 0.01 M Tris-maleate buffer (pH 6.2) containing 0.01 M Mg^{2+} and 1% Triton X-100.

Murein transglycosylase was prepared as described by Hakenbeck and Messer (6). Cells of *E. coli* χ1776 were collected from 300 ml of a culture in the logarithmic growth phase (A = 300), washed with ice-cold 0.01 M Tris hydrochloride buffer (pH 7.8), and suspended in 10 ml of the same buffer containing 5 mM EDTA and 2% Triton X-100. The cell suspension was incubated for 30 min at 30°C. After centrifugation at 12,000 × g for 30 min, the supernatant fluid was used as an enzyme source.

The reaction mixture containing 10 ml of substrate, 10 ml of enzyme, and 20 ml of 0.01 M Tris-maleate buffer (pH 6.2) with 0.01 M Mg^{2+} was incubated for 3 h at 32°C. After boiling for 5 min the supernatant fluid was collected by centrifugation (10 min at 12,000 × g) and applied to a Dowex 50 column (Cl^- type, 2.5 by 20 cm). The column was washed with water, and the absorbed material was eluted with 1 M NH_4OH. The eluted fluid was dried in vacuo, dissolved in 10 ml of water, and applied to a Bio-Gel P-6 column.

Gel filtration of autolytic products with the Bio-Gel P-6 column. Autolytic products in native or concentrated form (10 ml) were applied to a Bio-Gel P-6 column (2.5 by 80 cm), which was equilibrated and eluted with 0.05 M KCl (12). Fractions were collected and the radioactivity of each fraction was counted. The radioactive fractions were combined, concentrated, and desalted by Sephadex G-25 column chromatography (2.5 by 32.5 cm or 1.6 by 27 cm).

Paper chromatography and paper electrophoresis. Descending paper chromatograms were run on Whatman no. 1 or 3MM filter paper with solvent A (n-butanol–acetic acid–water; 4:1:5; vol/vol; upper phase) for 2 to 4 days, or with solvent B (isobutyric acid–1 M NH_4OH; 5:3; vol/vol) for 16 h. Paper electrophoresis was carried out on Whatman no. 1 or 3MM filter paper in 10% acetic acid (pH 2.2) at 60 V/cm for 60 to 90 min. Spots were visualized with ninhydrin, by autoradiography, or both.

Analytical procedures. Radioactivity in aqueous samples was measured in a ready-made scintillation cocktail (Biofluor; New England Nuclear Corp., Boston, Mass.). Radioactivity on paper was counted in a standard toluene-based scintillator. Amino acid analysis was done on a Durrum D-500 amino acid analyzer after hydrolysis with 6 N HCl in vacuo at 100°C for 16 h. Dinitrophenylation (DNP) studies were carried out as described by Mirelman and Sharon (12).

Analysis of residual insoluble peptidoglycan following autolysis. Reverse-phase HPLC was used to determine the composition of the peptidoglycan remaining in the sacculus following autolysis triggered by penicillin. Two 500-ml cultures of *E. coli* W7 (*dap lys*) were grown to the mid-logarithmic phase in M9 minimal salts medium supplemented with 5 μg of DAP and 10 μg of lysine per ml. One culture served as a control; the other was treated with 100 μg (20× MIC) of benzylpenicillin per ml for 2 h (a length of autolysis comparable to the time of autolysis in buffer used to generate the soluble products in the experiments described above). The other culture served as a control. Both cultures were chilled to 4°C and prepared for HPLC analysis by an adaptation of the method of Glauner and Schwarz (3) and Dougherty (2). Briefly, the samples were boiled in 5% sodium dodecyl sulfate, and the insoluble material was digested with 200 μg of alpha-amylase (Sigma Chemical Co., St. Louis, Mo.) for 1 h. It was found that digestion with 200 μg of pronase (Pronase-CB; Calbiochem-Behring, La Jolla, Calif.), preheated for 1 h at 60°C, had to be extended from 2 to 24 h to completely remove the lipoprotein attached to the peptidoglycan. The material was again extracted in boiling 5% sodium dodecyl sulfate, washed, lyophilized, and weighed. For HPLC analysis the samples were digested with *Streptomyces globisporus* muramidase (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) for 24 h and reduced with sodium borohydride (3). Cell wall material (15 μg) was applied to a reverse-phase HPLC column (Shandon ODS-Hypersil; 4.6 by 250 mm, 5-μm-diameter particles; Shandon Southern Instruments, Inc., Sewickey, Pa.) and eluted with a linear gradient of 50 mM sodium phosphate buffer (pH 4.33) to 50 mM phosphate buffer (pH 5.1) with 15% methanol over 120 min at a flow rate of 0.5 ml/min. Muropeptides were detected by monitoring UV absorbance of the eluted material at 205 nm.

For comparison of the cell wall composition of untreated and post-penicillin-treated cells, muropeptides were grouped into families as described by Pisabarro et al. (13): monomers (all of the disaccharide peptides [seven peaks at <52, 66, and 81 min]); dimers (all of the bisdisaccharide peptides [four peaks between 55 and 65 min; 4 peaks between 68 and 81, 89, and 95 min; three peaks between 100 and 109 min; three

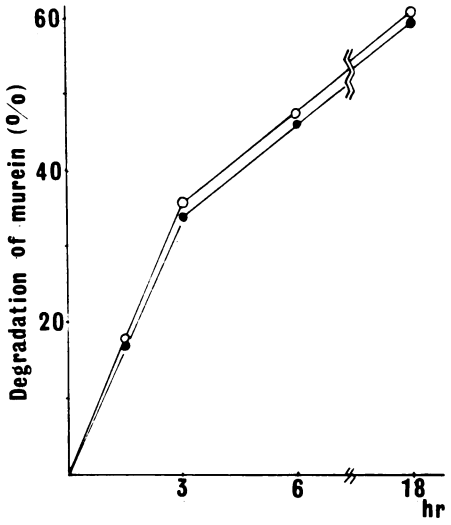

FIG. 1. Release of cell wall from cephaloridine-treated cells of *E. coli* χ1776. [^{3}H]DAP-labeled cells in the exponential growth phase were treated with 62.5 μg of cephaloridine per ml for 10 min at 32°C. The cells were then washed, suspended in 0.1 M sodium phosphate buffer (pH 7.0) containing 10 mM $MgSO_4$, and incubated at 32°C. Samples of the supernatant fluid were compared for the appearance of radiolabeled products of autolysis both as total counts per minute (○) and counts per minute soluble in 10% TCA (●).

peaks between 126 and 135 min]); trimers (all the cross-linked tridisaccharide peptides [82, 88, and 98 min; three peaks between 112 and 121 min, and 140 min]); muropeptides with diaminopimelyl-DAP cross-links (56, 64, 80, 89, 102, and 115 min; three peaks at >126 min); muropeptides linked to lipoprotein (50, 80, 89, and 98 min; three peaks at >126 min); muropeptides with 1,6-anhydromuramyl residues (65, 81, and 95 min; nine peaks at >100 min).

RESULTS

Release of cell wall fragments from cephaloridine-treated cells. In Fig. 1 is shown the release of cell wall fragments from cephaloridine-treated cells of *E. coli* χ1776. About 35% of murein was degraded in 3 h and 60% was degraded after overnight incubation. More than 95% of the released murein was TCA soluble at any time of incubation. In the case of *E. coli* GN-4, TCA-soluble components represented 60 to 80% of the total count. As *N*-acetylglucosamine is incorporated not only into muropeptide but also into lipopolysaccharide, some part of lipopolysaccharide might also be released together with the murein fragments.

Gel filtration patterns of cell wall fragments. In Fig. 2 is demonstrated the pattern of gel filtration with Bio-Gel P-6 of cell wall fragments released from *E. coli* χ1776. An identical pattern was obtained after treatment with benzylpenicillin (data not shown) and with cephaloridine (Fig. 2A). A total of 85 to 95% of the total radioactivity was recovered in four peaks. Peak II was the major product (about 75%), and peak III was the second largest (about 15 to 20%) of the fractions at any phase of autolysis. In Fig. 2B is shown the chromatogram of the autolytic products of TCA-treated cells. Most of the radioactivity was recovered in peaks II (about 50%) and III (about 45%). In Fig. 2C are shown the digestion products of the murein sacculus with murein transglycosylase. Peak II (about 85 to 90%) was the major product. It should be noted that peak II of each of the samples A, B, and C showed the same retention time on the Bio-Gel P-6 column. Most of the [^{3}H]*N*-acetylglucosamine-labeled material (>90%) from cephaloridine-treated cells of *E. coli* GN-4 eluted from the Bio-Gel P-6 column at the same position as peak II of the autolytic products from DAP-labeled cells (data not shown).

Characterization of cell wall fragments by paper chromatography and paper electrophoresis. Material from the major autolytic product (peak II of the various autolytic products) was concentrated and analyzed by paper chromatography and paper electrophoresis (Fig. 3). In all three systems the major products obtained from cephaloridine-treated (A) and TCA-treated (B) samples were identical and comigrated with the authentic major murein transglycosylase product (C) (Fig. 4) (8). In contrast, the major product was clearly separable from the egg white lysozyme digestion products of *E. coli* murein (Fig. 4) by paper chromatography in two solvent systems (but not by electrophoresis). The major product was not homogeneous (Fig. 3a). Prolonged (3 days) descending chromatography in solvent A (but not in solvent B and not by electrophoresis) separated the major autolytic product (A-II and B-II in Fig. 3), as well as the authentic transglycosylase product (C-II), into two components (II-1

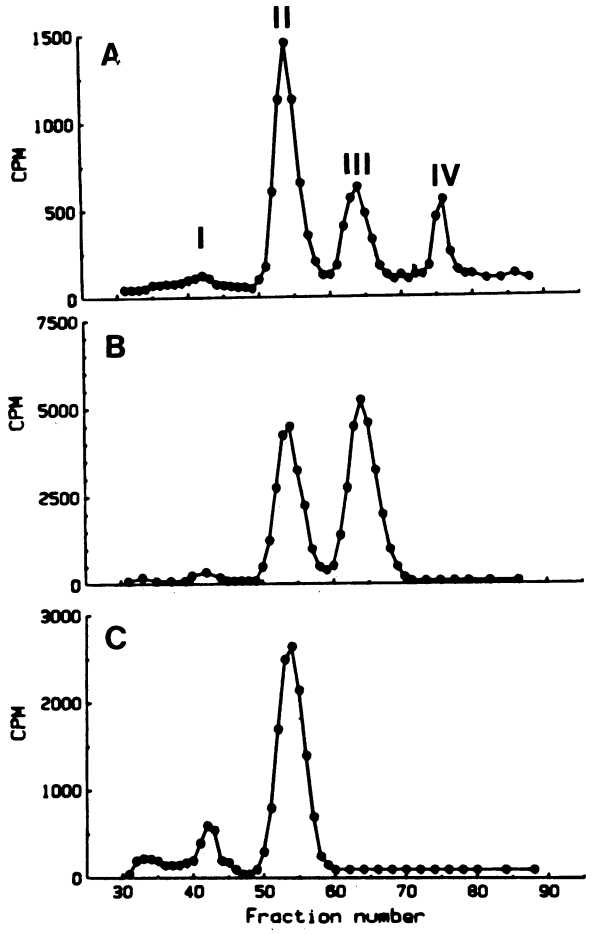

FIG. 2. Gel filtration of [^{3}H]DAP-labeled cell wall fragments of *E. coli* χ1776 on a Bio-Gel P-6 column. The supernatant fluid fractions from [^{3}H]DAP-labeled *E. coli* χ1776 cells treated as described below were applied directly to the column, as described in the text. (A) Autolytic products of cephaloridine-treated cells. (B) Autolytic products of TCA-treated cells. (C) Lytic products of murein by murein transglycosylase.

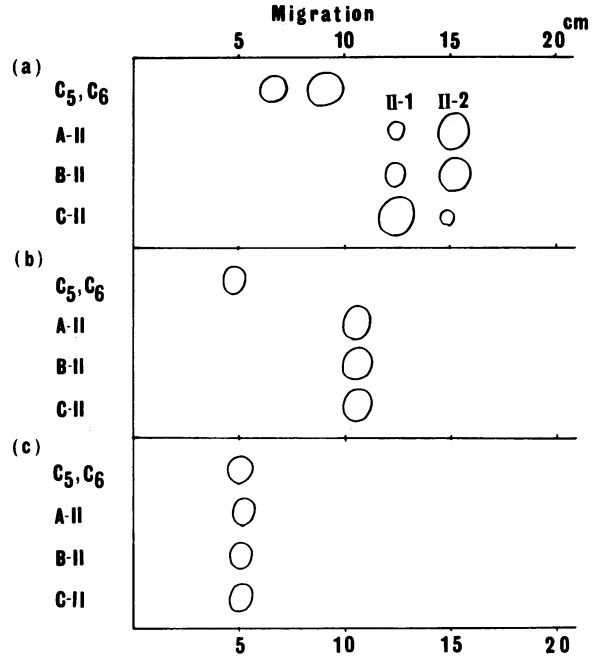

FIG. 3. Paper chromatography and paper electrophoresis of the major autolytic product (peak II from the Bio-Gel P-6 column shown in Fig. 2). (a) Descending paper chromatography on Whatman no. 1 filter paper in an upper phase of *n*-butanol–acetic acid–water (4:1:5) for 3 days. (b) Descending paper chromatography on Whatman no. 1 filter paper in isobutyric acid–1 M NH_4OH (5:3) for 16 h. (c) Paper electrophoresis in 10% acetic acid (pH 2.2) at 60 V/cm for 90 min. Peak II fractions of the Bio-Gel P-6 column were concentrated, desalted on a Sephadex G-25 column, and concentrated again; and paper chromatography and paper electrophoresis were carried out. A-II, Two products (II-1 and II-2) emerged from autolytic products of cephaloridine-treated cells; B-II, product from autolytic products of TCA-treated cells; C-II, product from lytic products of murein transglycosylase. The mixture of C_5 and C_6 bisdisaccharide tetrapeptides was prepared from the lytic products of *E. coli* murein sacculi with lysozyme.

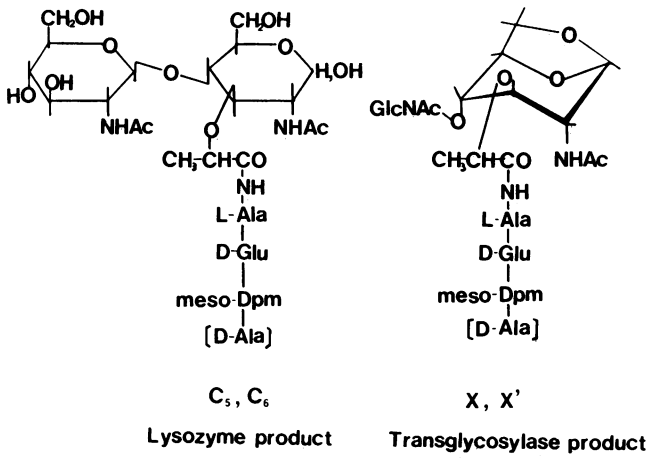

FIG. 4. Chemical structures of major murein degradation products of lysozyme and transglycosylase in *E. coli*. The 1,6-anhydro derivative that is characteristic of the transglycosylase product was the major degradation product during autolysis triggered by cephaloridine or TCA. Abbreviations: Ala, alanine; Glu, glutamic acid; meso-Dpm, DAP; GlcNAc, *N*-acetylglucosamine; NHAC, ammonium acetate. C_5, C_6, X, and X' are designations for these murein products commonly used in the literature.

and II-2) with different R_f values. These were isolated and analyzed separately.

Similar to the major product, the chromatographic and electrophoretic profiles of the minor autolytic product of cephaloridine- and TCA-treated cells (peak III of samples A and B) were also identical in the three systems (Fig. 5). The cephaloridine-treated minor product gave one spot with an R_f of 0.87 in solvent B and was relatively hydrophobic compared with other fragments. Paper chromatography in solvent A for 4 days again separated the minor product into two spots (III-1, III-2) which were analyzed separately.

Amino acid analysis of wall fragments. The two subcomponents of the major (II-1 and II-2) and the minor (III-1 and III-2) autolytic products were purified by preparative paper chromatography and paper electrophoresis. All peaks from the amino acid analysis are summarized in Table 1. Amino acids in the relative molar ratios characteristic of *E. coli* stem peptides were detected. Only the major autolytic subcomponent II-1, which was chromatographically identical to the murein transglycosylase product, contained *N*-acetylglucosamine and *N*-acetylmuramic acid; the other subcomponents apparently lost the hexosamine moieties.

DNP studies were used to detect free amino groups on DAP which would signify uncross-linked stem peptides. The epsilon amino group of DAP of both components of the major autolytic product was derivatized by DNP (II-1 and II-2) (uncross-linked); in contrast, the DAP of both components of the minor product (III-1) was partially free of DNP (cross-linked). Comparison of the size and total radioactivity in the spots of the major versus minor products on electro-

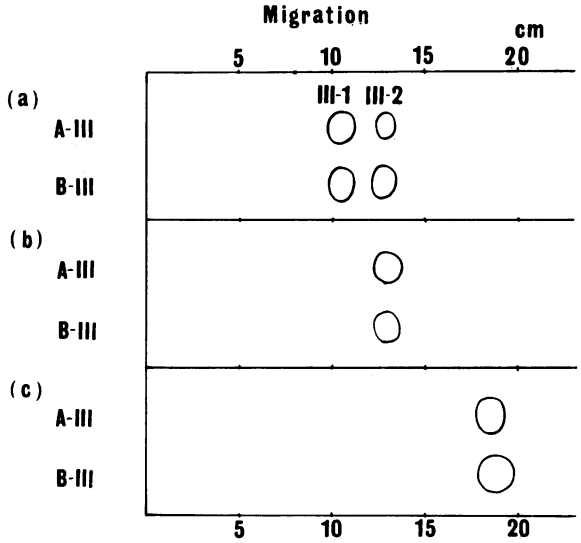

FIG. 5. Paper chromatography and paper electrophoresis of the minor autolytic product (peak III from the Bio-Gel P-6 column shown in Fig. 2). (a) Descending paper chromatography on Whatman no. 1 filter paper in an upper phase of *n*-butanol–acetic acid–water (4:1:5) for 4 days. (b) Descending paper chromatography on Whatman no. 1 filter paper in isobutyric acid–1 M NH_4OH (5:3) for 16 h. (c) Paper electrophoresis in 10% acetic acid (pH 2.2) at 60 V/cm for 75 min. Migration was toward the cathode. Samples were prepared from peak III fractions of the Bio-Gel P-6 column by the same procedure described in the legend to Fig. 3. A-III; Two products (III-1 and III-2) emerged from autolytic products of cephaloridine-treated cells; B-III, product from autolytic products of TCA-treated cells.

TABLE 1. Amino acid composition of the major cell wall degradation products

Constituent	Molar ratio of the following constituents[a]:			
	II-1[b]	II-2	III-1	III-2
Glucosamine	0.92	<0.05	<0.05	<0.05
Muramic acid	0.63	<0.05	<0.05	<0.05
DAP	0.92	0.99	0.84	0.90
Glutamic acid	1.00	1.00	1.00	1.00
Alanine	2.00	2.05	1.02	1.76
Dinitrophenylated DAP	1.00[c]	1.00	1.00	1.00
Free DAP	0.10	0.15	1.12	1.12

[a] Glutamic acid was taken as 1.00.
[b] Designations correspond to the doublet of spots obtained by electrophoresis of the major and minor peaks eluted from the Bio-gel P-6 column (Fig. 3 and 5A).
[c] The ratio of DAP resisting DNP (free DAP) is given as a value relative to the value for dinitrophenylated DAP, which is equal to 1.00.

phoresis (Fig. 3 A-II versus Fig. 5 A-III) indicated that the amount of the uncross-linked components (peak II) was much higher than that of the cross-linked components (peak III) among the cephaloridine-treated autolytic products.

Although peak IV was also purified by preparative paper chromatography in solvents A and B, no amino acids were detected in this sample. It seems likely that peak IV does not contain murein but may represent other (perhaps lipopolysaccharide) cell wall material containing some ^3H label from a metabolic product of DAP.

Composition of residual peptidoglycan following penicillin-induced autolysis. A total of 32% of the murein was solubilized during penicillin exposure, a value similar to the example shown in Fig. 1. In Table 2 is compared the composition of untreated *E. coli* W7 peptidoglycan with the structure of the residual *E. coli* W7 murein that remained nonhydrolyzed in the sacculus after penicillin-induced autolysis. The composition of the untreated cell walls varied somewhat from that of published data (13), presumably because of differences in growth media composition (minimal salts versus rich media). The residual peptidoglycan differed in composition from the starting material. Mean cross-linkage calculated from the relative amounts of monomer, dimer, and trimer families increased from 25.3 to 29.8%. Autolysis appeared to involve a selective loss of material from the monomer family (decrease from 51 to 44%), especially monomer tetrapeptides, and a sparing of trimers, which doubled in relative abundance. Although the overall amount of dimers in the cell wall was not greatly altered by autolysis, the nature of the dimer species within the family changed, as shown by the major loss of dimer tetrapeptide. Lipoprotein-rich regions doubled in amount, suggesting that they were spared autolytic degradation. A striking increase in anhydromuramyl peptides occurred, suggesting the appearance of new glycan chain ends after autolysis, a finding that is consistent with transglycosylase activity.

DISCUSSION

Analysis of murein degradation products released during autolysis was coupled with analysis of the mirror image residual murein remaining intact in the sacculus to determine which of the multiple murein hydrolase activities of *E. coli* is active in autolysis. The findings from these two approaches allow us to suggest that two enzyme activities are primarily responsible for autolytic wall degradation in *E. coli*: the transglycosylase (8) and an enzyme (endopeptidase) that is capable of splitting the transpeptide bonds between neighboring stem peptides in the murein.

Virtually all (>95%) of the labeled material released from the murein into the surrounding medium during autolysis consisted of low-molecular-weight components. The major murein degradation product, representing about 75 to 80% of [^3H]DAP-labeled material released after cephaloridine or penicillin treatment, was indistinguishable from the major authentic transglycosylase product (Fig. 4) by gel filtration size (Bio-Gel P-6), R_f values in two paper chromatographic systems, migration during electrophoresis, and chemical analysis. The material could be clearly distinguished (in two chromatographic solvents) from the major products (C_5 and C_6) of wall hydrolysis by egg white lysozyme. Chemical analysis confirmed a structure compatible with the transglycosylase product (glucosamine, muramic acid, glutamic acid, and DAP, in roughly equimolar proportions and 2 mol of alanine per mol of glutamic acid). The greater than 90% DNP derivatization of the epsilon amino group of DAP indicates that the species is uncross-linked. The most plausible structure for this component is that of a murein disaccharide tetrapeptide with a 1,6-anhydromuramic acid residue (see Fig. 4, transglycosylase product). Consistent with this proposition is the finding that during antibiotic-induced autolysis of *E. coli* with walls labeled in the glycan with radioactive *N*-acetylglucosamine, over 80% of the radioactive label released was reisolated as a single component, with the same gel filtration retention time as the major product released from [^3H]DAP-labeled bacteria.

In one solvent, the transglycosylase-like product could be resolved to two components with identical amino acid compositions, one with and the other without detectable hexosamines. Because the authentic transglycosylase product which served as the control behaved in the same way, we suggest that the loss of the disaccharide unit is due to chemical decomposition during the prolonged (3 days) exposure to the acidic solvent. The instability of the muramyl–L-alanine bond under alkaline conditions has been demonstrated (16). Alternatively, the hexosamines could have undergone secondary cleavage by the amidase; this would imply, however, that the purified transglycosylase also contains a previously unreported amidase.

A second, minor murein degradation product, representing 15 to 20% of the label released during cephaloridine-induced autolysis, was also characterized. It contained no

TABLE 2. Effect of autolysis on the composition of *E. coli* W7 murein, as determined by HPLC

Muropeptides	% total muropeptides by the following treatments[a]:	
	Control	Penicillin
Monomer family[b]	51.4	43.6
Dimer family	43.8	48.2
Trimer family	4.8	8.2
Mean cross-linkage[c]	25.3	29.8
Monomer tetrapeptide[d]	48.7	27.8
Dimer tetratetrapeptide[e]	37.6	24.3
Lipoprotein muropeptides	3.2	6.9
Total anhydromuropeptides	3.4	6.6

[a] Calculated by grouping muropeptides, as described in the text.
[b] (Area under the curve of all muropeptides in the family/total area under the curve of monomers, dimers, and trimers) × 100.
[c] [(0.5 × dimer) + (0.7 × trimer)/total area under the curve of monomer + dimer + trimer] × 100.
[d] Major monomer component with peak retention time of 35 min by HPLC.
[e] Major dimer component with peak retention time of 73 min by HPLC.

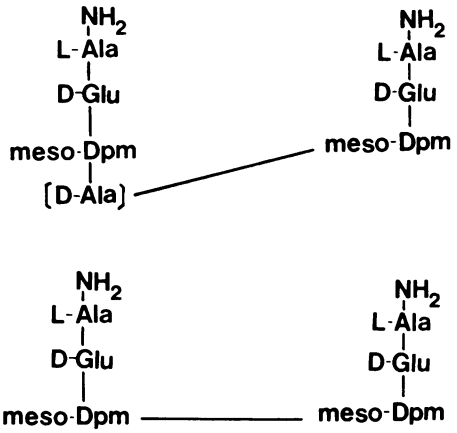

FIG. 6. Proposed chemical structures for the minor murein degradation products of *E. coli* autolysis. The tritripeptide and tritetrapeptides are most likely formed from the action of the transglycosylase, which liberates bisdisaccharide peptide units, followed by removal of the carbohydrate moieties by *N*-acetylmuramyl-L-alanine amidase. DAP-DAP cross-links have been described by Glauner and Schwarz (3). Abbreviations: meso-Dpm, DAP; Ala, alanine; Glu, glutamic acid.

detectable hexosamines, and both DAP and glutamic acid were present in molar ratios of about 1. Again, this minor component consisted of two subcomponents; one contained alanine in molar ratios (relative to glutamic acid) of 1.0, and the other contained alanine in molar ratios of 1.76. In both species approximately half of the DAP had a DNP-derivatized amino group, suggesting that this minor autolytic product represents a mixture of cross-linked and uncross-linked stem peptides. Tentative structures for the cross-linked species are shown in Fig. 6. These could be derived originally from bisdisaccharide-tetrapeptide dimers in the murein, which were released [presumably, by the same hydrolase(s) that released the major product], but which have been further degraded by additional hydrolases known to exist in *E. coli*, an amidase, an endopeptidase and a DL-carboxypeptidase. Importantly, no hexosamines were detected in any peak III (minor product) starting material. Thus chemical decomposition in acidic solvents (as suggested for peak II) does not explain stem peptides free of carbohydrate. Because the *E. coli* amidase does not seem to be able to attack intact sacculi (17) but can rapidly hydrolyse muramyl peptides, we assume that amidase action on the wall fragment(s) could occur only in a secondary manner to their release from the sacculus by the transglycosylase. The amidase activity may have a role in the metabolic recycling of cell wall fragments released during normal growth because of wall turnover (4).

The observations discussed so far suggest that the major hydrolase responsible for autolytic cell wall degradation induced by an antibiotic block of cell wall synthesis is the transglycosylase. Apparently, the major products of this enzyme under the conditions described were murein monomers. Nevertheless, the presence of a cross-linked DAP species among the minor autolytic products (Fig. 6) suggests that the transglycosylase probably also dissects murein dimers out of the wall during autolysis. The ratio of monomer (peak II) to dimer (peak IIIA) products released during autolysis was about 4 to 1, i.e., quite different from the relative abundance of these two murein components (about 1:1) in the intact wall of *E. coli* (3). This suggests that either the autolytic process is preferentially releasing preexisting monomers from the sacculi or that bisdisaccharide dimers are converted to monomers via breaking of the transpeptide cross-links by an endopeptidase, before or after release from the polymer network of the sacculus. To test these possibilities, we reisolated the undegraded portion of the *E. coli* sacculus from partial autolysates and undertook an analysis of their composition by the reverse-phase HPLC system developed by Glauner and Schwarz (3). These analyses fully confirmed and extended the observations made by the analysis of the released wall products. Results of the HPLC analyses showed that the autolytic process is selective, with the major loss occurring in the monomeric material, a finding that is consistent with the relative abundance of the monomers among the products that appear in the surrounding medium. However, an almost equal decrease (from 37.6 to 24.3%) in the dimer tetrapeptide fraction also occurred, an amount that is significantly greater than the 1:4 relative amount of cross-linked to uncross-linked species released into the medium. This evidence suggests that endopeptidase activity (conversion of dimers to monomers) is significant during autolysis. Another interesting change in the composition of the residual cell wall involved the substantial (twofold) increase in the relative proportion of wall components that contained anhydromuramyl residues. The increase in this fraction may represent chain ends created during incomplete (partial) activity of the transglycosylase.

Our results indicate that two major cell wall-hydrolyzing enzyme activities participate in the degradation of sacculi in response to inhibition of cell wall synthesis or chaotropic agents. The involvement of transglycosylase and endopeptidase in this autolytic process is consistent with the known cell wall-degrading capacities of these enzymes and also with the finding that antibiotic-tolerant mutants of *E. coli* (selected for poor lysis during antibiotic treatment) had lower specific activities of the transglycosylase and endopeptidase (10, 11). Secondary modification of released cell wall fragments by an amidase is consistent with the lack of ability of this enzyme to attack insoluble cell wall (17) and the lack of association between tolerance and loss of this enzyme activity (10, 11). It is conceivable that the amidase may function in the cell wall turnover described by Goodell and Schwarz (4) rather than in autolytic wall degradation per se, as triggered by beta-lactam antibiotics or chaotropic agents.

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LITERATURE CITED

1. **Curtiss, R., III.** 1978. Biological containment and cloning vector transmissibility. J. Infect. Dis. **137**:668–675.
2. **Dougherty, T. J.** 1985. Analysis of *Neisseria gonorrhoeae* peptidoglycan by reverse-phase, high-pressure liquid chromatography. J. Bacteriol. **163**:69–74.
3. **Glauner, B., and U. Schwarz.** 1983. The analysis of murein composition with high pressure liquid chromatography, p. 29–34, *In* R. Hakenbeck, J.-V. Holtje, and H. Labischinski (ed)., The target of penicillin, Walter de Gruyter, Berlin.
4. **Goodell, E. W., and U. Schwarz.** 1985. Release of cell wall peptides into culture medium by exponentially growing *Escherichia coli*. J. Bacteriol. **162**:391–397.
5. **Goodell, W., and A. Tomasz.** 1980. Alteration of *Escherichia*

coli murein during amino acid starvation. J. Bacteriol. **144:** 1009–1016.
6. **Hakenbeck, R., and W. Messer.** 1977. Activity of murein hydrolases in synchronized cultures of *Escherichia coli*. J. Bacteriol. **129:**1239–1244.
7. **Hartmann, R., B. S. Bock-Henning, and U. Schwarz.** 1974. Murein hydrolases in the envelope of *Escherichia coli*. Eur. J. Biochem. **41:**203–208.
8. **Holtje, J.-V., D. Mirelman, N. Sharon, and U. Schwarz.** 1975. Novel type of murein transglycosylase in *Escherichia coli*. J. Bacteriol. **124:**1067–1076.
9. **Imada, A., Y. Nozaki, F. Kawashima, and M. Yoneda.** 1977. Regulation of glucosamine utilization i n *Staphylococcus aureus* and *Escherichia coli*. J. Gen. Microbiol. **100:**329–337.
10. **Kitano, K., and A. Tomasz.** 1979. *Escherichia coli* mutants tolerant to beta-lactam antibiotics. J. Bacteriol. **140:**955–963.
11. **Kitano, K., and A. Tomasz.** 1980. Murein hydrolase defect in the beta lactam tolerant mutants of *Escherichia coli*. FEMS Microbiol. Lett. **7:**133–136.
12. **Mirelman, D., and N. Sharon.** 1967. Isolation and study of the chemical structure of low molecular weight glycopeptides from *Micrococcus lysodeikticus* cell walls. J. Biol. Chem. **242:**3414–3427.
13. **Pisabarro, A. G., M. A. de Pedro, and D. Vazquez.** 1985. Structural modifications in the peptidoglycan of *Escherichia coli* associated with changes in the state of growth of the culture. J. Bacteriol. **161:**238–242.
14. **Ryter, A., Y. Hirota, and U. Schwarz.** 1973. Process of cellular division in *Escherichia coli*: growth pattern of *E. coli* murein. J. Mol. Biol. **78:**185–195.
15. **Schwarz, U., and W. Weidel.** 1965. Zum wirkungsmechanismus von Penicillin. Z. Naturforsch. **206:**147–157.
16. **Tipper, D. J.** 1968. Alkali-catalyzed elimination of D-lactic acid from muramic acid and its derivatives and the determination of muramic acid. Biochemistry **7:**1441–1460.
17. **Van Heijenoort, J., C. Parquet, B. Flouret, and Y. van Heijenoort.** 1975. Envelope-bound N-acetylmuramic acid-L-alanine amidase. Eur. J. Biochem. **58:**611–619.