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Effect of D-Amino Acids on Structure and Synthesis of Peptidoglycan in *Escherichia coli*

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Growth of *Escherichia coli* in the presence of certain D-amino acids, such as D-methionine, results in the incorporation of the D-amino acid into macromolecular peptidoglycan and can be lethal at high concentrations. Previous studies suggested that incorporation was independent of the normal biosynthetic pathway. An enzymatic reaction between the D-amino acid and macromolecular peptidoglycan was proposed as the mechanism of incorporation. The application of more advanced analytical techniques, notably high-pressure liquid chromatography, revealed that the presence of a D-amino acid susceptible to incorporation induced a multiplicity of alterations in peptidoglycan metabolism. Results derived basically from the study of samples treated with D-Met, D-Trp, and D-Phe indicated that the incorporation of a D-amino acid results in the accumulation of two major new muropeptides whose general structures most likely are GlucNAc-MurNAc-L-Ala-D-Glu-*m*-diaminopimelic acid-D-aa and GlucNAc-MurNAc-L-Ala-D-Glu-*m*-diaminopimelic acid-D-Ala–GlucNAc-MurNAc-L-Ala-D-Glu-*m*-diaminopimelic acid-D-aa, where D-aa represents a residue of the added D-amino acid. Resting cells are proficient in the incorporation of D-amino acids and can reach peptidoglycan modification levels comparable to those in growing cells. Under our conditions, D-amino acids had no apparent effect on growth or morphology but caused a severe inhibition of peptidoglycan synthesis and cross-linking, possibly leading to a reduction in the amount of peptidoglycan per cell. The properties of the reaction support the involvement of a penicillin-insensitive LD-transpeptidase enzyme in the synthesis of modified muropeptides and a possible inhibitory action of D-amino acids on high-molecular-weight penicillin-binding proteins.

Biosynthesis of the peptidoglycan sacculus, the stress-bearing structure of the cell wall, is one of the most complex metabolic processes in the bacterial cell, in particular from the physiological point of view. Cell growth is strictly dependent on the enlargement of the sacculus, and cell division is concomitant with the formation of a transverse wall, the septum, in the sacculus (11, 21). Therefore, a strict coordination among growth rate, cell division, and peptidoglycan synthesis is required to ensure viability. Furthermore, as the sacculus supports the turgor pressure of the cell, it must grow without the formation of discontinuities. Otherwise, cell lysis will follow, as often happens when peptidoglycan synthesis is disturbed (12, 13, 17, 27). As a further complication of this scheme, the sacculus itself is not a static structure but rather is a macromolecule subject to highly dynamic metabolic activity comprising maturation, turnover, and growth-phase-dependent structural changes (4, 5, 8–10, 22).

A breakthrough in the investigation of peptidoglycan structure was the introduction by Glauner et al. of high-pressure liquid chromatography (HPLC)-based analytical methods for the determination of the muropeptide composition (6, 7). These techniques permitted routine analyses of unprecedented accuracy and resolution. More than 30 types of muropeptide were demonstrated as constitutive elements of *Escherichia coli* peptidoglycan. The structures of many of them were either known or understandable on the basis of identified enzymatic reactions. However, a new family of relatively abundant (3 to 5%) cross-linked muropeptides was discovered. The distinctive feature of these muropeptides is that cross-linking occurs by a direct *meso*-diaminopimelyl-*meso*-diaminopimelic acid peptide bridge (dap-dap bridge) of LD configuration instead of by the classical D-alanyl-*meso*-diaminopimelic acid bridge (Ala-dap bridge) of DD configuration. At present, the genesis and physiological role of this family of muropeptides are unknown. Nevertheless, synthesis via penicillin-binding proteins (PBPs) seems improbable, suggesting the existence in the cell envelope of specific enzymes to catalyze the formation and cleavage of LD-dap-dap bridges (5–7, 12, 22).

A large number of enzymes specifically involved in peptidoglycan metabolism have been identified (11, 12). However, the enzymes involved in such important processes as the binding of lipoprotein and the formation of the dap-dap bridges mentioned above remain unknown. The search for enzymes involved in the metabolism of macromolecular peptidoglycan often has been hampered by a lack of suitable assays. However, in some instances indirect methods based on the exploitation of side reactions have proved very valuable. As a matter of fact, the ability of the PBPs to covalently bind β-lactam antibiotics enormously facilitated their characterization by means of relatively simple binding assays with radioactive β-lactams (24, 25). Therefore, it seems reasonable that the investigation of side reactions related to peptidoglycan synthesis could help in the search for the missing enzymes and provide the basis for suitable assays. Furthermore, the study of these reactions may have a practical return in helping to identify new inhibitors of relevant enzymes with a potential application as antibacterial drugs.

With this idea in mind, our attention was drawn by the observation that the addition of some D-amino acids (D-aa) other than the regular constituents of the cell wall to cultures of *E. coli* results in the incorporation of the D-aa into macromolecular peptidoglycan (3, 15, 27, 28). Although

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some D-aa can be lethal, wild-type *E. coli* strains are rather resistant and damage becomes evident only at relatively high concentrations. Nevertheless, D-aa are likely to induce physiologically relevant alterations in peptidoglycan structure or synthesis at sublethal concentrations. The synergistic nature of some mixtures of β-lactams and D-aa and the hypersensitive phenotype of mutants defective for PBP 1B support this assumption (3, 27). Incorporation apparently occurs by means of a penicillin-insensitive reaction involving the α-NH_2 group of the D-aa. It has been proposed that the reaction could be catalyzed by a hitherto-unknown lipoprotein-binding enzyme (28).

The results of our investigations indicate that peptidoglycan synthesis and structure are both severely altered by the presence of D-aa in the growth medium and suggest that the incorporation of these compounds into macromolecular peptidoglycan is mediated by an enzyme endowed with LD-transpeptidase activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* MC6 RP3 (K-12 F^- *proA leuA thr dra drm lysA dapA thi*) (30) was used throughout this work. Cultures were routinely grown either in LB (18) or in minimal citrate (MC) (29) medium at 37°C under vigorous aeration. Media were systematically supplemented with 8 μg of 2,6-*meso*-diaminopimelic acid (*m*-dap) and 40 μg of L-lysine per ml. In addition, MC medium was supplemented with the required amino acids at 40 μg/ml, 200 μg of Casamino Acids per ml, and 0.2% (wt/vol) glucose as a carbon and energy source. Growth was monitored by measuring the optical density at 550 nm (OD_{550}) of the cultures.

Analysis of peptidoglycan by HPLC. Peptidoglycan was purified and analyzed by HPLC with a Hypersil RP18 column (3-μm particle size; 250 by 4 mm) (Teknochroma, Barcelona, Spain) as described by Glauner et al. (5, 7). Muropeptides were identified by their retention times (Rt) after periodic calibration of the HPLC equipment with a mixture of known, purified muropeptides. Muropeptides were grouped into structurally related families as described previously (22). Specific detection of tryptophan- and phenylalanine-containing muropeptides was performed with two in-series UV detectors set at 204 nm to detect muropeptides and at either 280 or 260 nm to detect Trp or Phe, respectively. Quantitation was performed by automatic integration of the absorption peak areas. For analysis of radioactively labeled peptidoglycan, a radioactivity detector (Laboratorium Prof. Dr. Berthold, Wildbad, Germany) was connected in series with the UV detector. Radioactivity was measured with Pico-Fluor 40 (Packard Instruments, Downers Grove, Ill.) as the scintillator.

Incorporation of ^{3}H-dap into SDS-insoluble material. Aliquots (1 ml) of cultures radioactively labeled with *meso*-[3,4,5-^{3}H]diaminopimelic acid (^{3}H-dap) were mixed 1:1 with 10% (wt/vol) boiling sodium dodecyl sulfate (SDS), and the mixtures were incubated for 45 min in a boiling water bath. Insoluble material was collected onto Millipore HAWP membrane filter disks (Millipore España, Madrid, Spain) presoaked in 10 μg of nonlabeled *m*-dap per ml and extensively washed with hot water (60°C, 20 ml, four times). The filters were placed in vials containing 2 ml of Pico-Fluor 40 and soaked for 12 h, and then radioactivity was measured by liquid scintillation in a model LS3801 (Beckman Instruments, Fullerton, Calif.) liquid scintillation counter.

Amino acid analysis of purified muropeptides. Muropeptides of interest were purified by HPLC and lyophilized. Desalting was performed by HPLC. Lyophilized material was dissolved in water and injected into the Hypersil RP18 HPLC column, equilibrated with 0.05 M sodium phosphate buffer (pH 4.31), at a flow rate of 0.9 ml/min. The column was eluted first with distilled water (10 min) and then with a steep gradient (10 min) of 0 to 30% (vol/vol) methanol. Muropeptides were eluted at the end of the gradient essentially salt free. Once desalted, the muropeptides were subjected to acid hydrolysis in 6 M HCl–0.2% (vol/vol) β-mercaptoethanol at 105°C for 18 h, vacuum dried, and dissolved in distilled water. Samples were then subjected to precolumn derivatization essentially as described previously (19) by being mixed 1:1 with a 50-μg/ml solution of *ortho*-phthaldialdehyde in 150 mM β-mercaptoethanol–0.5 M potassium borate buffer (pH 10). After 5 min at room temperature, the samples were injected into the Hypersil RP18 HPLC column, equilibrated in methanol–tetrahydrofuran–(50 mM Na_2PO_4H–50 mM Na acetate) (pH 7.5) (2:2:96), at a flow rate of 0.9 ml/min. Elution was performed with the following gradient: 8 min of isocratic elution in starting buffer; change to 30% of 65% (vol/vol) methanol in water (buffer B) in 3 min; change to 50% of buffer B in 22 min; change to 100% of buffer B in 22 min; 15 min of isocratic elution in buffer B. The eluate was monitored by measuring the A_{365}; 365 nm was the excitation wavelength for the derivatized amino acids.

Dinitrophenylation of purified muropeptides. Muropeptides purified as described above were subjected to dinitrophenylation as described previously (20). Dried compounds (5 to 50 nmol) were dissolved in 50 μl of water, and the solution was mixed with 15 μl of 10% (vol/vol) triethanolamine in ethanol and 25 μl of 18 mg of 1-fluoro-2,4-dinitrobenzene per ml in ethanol. Samples were incubated for 45 min at 60°C in the dark and vacuum dried at room temperature. The dried matter was dissolved in 200 μl of 6 N HCl. Excess reagent was removed by repeated washings with diethyl ether, and the samples were hydrolyzed overnight in sealed ampoules at 105°C. Hydrolyzed samples were further processed for amino acid analysis as described above.

RESULTS

Accumulation of modified muropeptides in the peptidoglycan of cells grown in the presence of D-aa. To investigate how the presence of D-aa in the growth medium affected peptidoglycan metabolism, we selected D-Met as the representative compound because it has been used in a number of previous studies of *E. coli* and other bacteria (3, 15, 16, 27, 28). D-Trp and D-Phe were also used because of their characteristic UV absorption maxima at 280 and 260 nm, respectively, which facilitate identification.

At high concentrations (>45 mM), D-Met, D-Phe, and D-Trp are lethal for *E. coli* (3). However, under our experimental conditions, cells were capable of unlimited growth (>16 mass doublings) without detectable alterations in growth rate or morphology, as assessed by phase-contrast microscopy. To obtain an initial estimate of the alterations induced in the sacculus by D-aa, we grew cells in LB medium supplemented with the D and L isomers of Met, Trp, and Phe each at 20 mM and also grew a control culture. After 4 h of incubation, peptidoglycan was purified and further processed to determine the muropeptide composition by HPLC. The L isomers had no effect on the structure of peptidoglycan, as expected. However, the induction of severe alterations by the D isomers was evidenced by the appearance of new absorption peaks on the chromatograms (Fig. 1). Al-

though the Rt for the new components differed widely, depending on the D-aa added, the general patterns were remarkably similar; two major peaks accounted for more than 90% of the new material, and there were a small number (three to five) of minor components. These similarities and the strong UV absorption maxima at 280 and 260 nm of the new components in the D-Trp- and D-Phe-treated samples, which are negligible for normal muropeptides, suggested the presence of at least one residue of the foreign D-aa in the new components. Hereafter these new compounds are identified by the standard three-letter abbreviation of the D-aa followed by a number indicating the order of elution in HPLC. For instance, Met-1 and Met-2 refer to peaks with Rt of 54.3 and 78.5 min, respectively, in Fig. 1B.

To gain insight into the specificity of the reaction, we subjected peptidoglycan samples from cultures supplemented with different amino acids and related compounds to HPLC analysis as described above (Table 1). The reaction seemed to be rather nonspecific. Most D-aa assayed were incorporated, producing the patterns described above. The similarities of the patterns support the idea of a common mechanism of incorporation, leading to an accumulation of equivalent modified muropeptides differing only in the nature of the foreign amino acid.

Interestingly, the nonincorporated compounds among those tested fell into three well-defined groups: amino acids without an α-primary amino group (D-Pro, β-D-aminobutyric acid, and β-Ala), diamino acids (D-Lys and *m*-dap), and achiral amino acids (α-aminoisobutyric acid and β-Ala).

Commercially available derivatives of D-Trp with modified amino and carboxyl groups (*N*-acetyl-D-Trp and D-Trp-methyl ester, respectively) were also assayed to study how a blockade of the functional groups would interfere with the incorporation reaction. As shown in Table 1, D-Trp-methyl ester was clearly incorporated, whereas the *N*-acetyl derivative was not. These results indicated that a free α-D-amino group is required for the reaction but that a free carboxyl group is dispensable.

In a previous report, it was suggested that the incorporation of D-aa could be a side reaction catalyzed by the enzyme binding lipoprotein to peptidoglycan (28). In this reaction, a peptide bond is formed between the L center of a residue of *m*-dap and the ε-NH_2 group of the C-terminal L-Lys of the lipoprotein. As lysine enantiomers were not incorporated, the acylated derivatives *N*-α-acetyl-L-Lys and *N*-α-acetyl-L-Lys-methyl ester were also assayed to determine whether the conversion of the α-NH_2 group into an amide, as in lipoprotein, would favor incorporation. Neither of these compounds was incorporated (Table 1). Furthermore, the quantitation of lipoprotein-bound muropeptides indicated that the acylated derivatives did not compete for the binding reaction. The proportions of lipoprotein-bound muropeptides in peptidoglycan from control and *N*-α-acetyl-L-Lys- and *N*-α-acetyl-L-Lys-methyl ester-treated cells were 14.3, 12.5, and 13.2 mol%, respectively.

To find out whether the new A_{204} peaks represent modified muropeptides, we performed experiments similar to those described above but grew the cells in the presence of a D-aa and ^{3}H-dap for 2 h. Labeled peptidoglycan was analyzed by HPLC, monitoring the A_{204} and radioactivity of the eluate. As shown in Fig. 2, the new compounds did contain a substantial proportion of ^{3}H-dap as a constitutive element. Furthermore, the A_{204}/radioactivity ratios for normal and D-Met-modified material were similar, as indicated by the values calculated for the most abundant compounds, GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap-D-Ala (disaccharide tetrapeptide) and Met-1 (274 and 288 A_{204} integration units/cpm, respectively), while the value for D-Trp-modified material was higher (320 A_{204} integration units/cpm for Trp-1).

Interestingly, the A_{280}/radioactivity ratio for Trp-1 (42 A_{280} integration units/cpm) was about twice that for Trp-2 (17.5 A_{280} integration units/cpm). If we assume that the A_{280} was exclusively due to Trp, then the difference in the ratios would fit well with that expected for monomeric (one ^{3}H-dap residue) and dimeric (two ^{3}H-dap residues) muropeptides with equal numbers of Trp residues per molecule.

For more precise structural information, modified muropeptides were subjected to amino acid analysis following purification and desalting by HPLC. Because of its chemical stability and massive incorporation, D-Phe proved to be the most appropriate compound for this purpose. The two natural major muropeptides of *E. coli*, disaccharide tetrapeptide and GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap-D-Ala–GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap-D-Ala (*bis*-disaccharide tetrapeptide), were used as standards. The results are shown in Table 2. The presence of Phe on both compounds, expected from the A_{260} of Phe-1 and Phe-2 (Fig. 1E), was confirmed. Furthermore, the relative proportions of the different amino acids fit well with Ala-Glu-dap-Phe ratios of 1:1:1:1 and 3:2:2:1, suggesting monomeric and dimeric natures for Phe-1 and Phe-2, respectively. The presence of Met in Met-1 and Met-2 could be confirmed, but no good data could be obtained by this method for quantification (data not shown).

To further assess the monomeric and dimeric natures of Phe-1 and Phe-2, we determined the fraction of dap amenable to dinitrophenylation in these compounds. Whereas all dap should react in monomeric material, only one-half of it should do so in dimeric material, because the dap residue in the acceptor moiety of the muropeptide has no free amino group. Purified Phe-1 and Phe-2, as well as regular disaccharide tetrapeptide and *bis*-disaccharide tetrapeptide, were reacted with dinitrophenyl benzene, hydrolyzed in 6 M HCl, and subjected to amino acid analysis. The results in Table 2 clearly support our assumption. While almost all the dap in Phe-1 and disaccharide tetrapeptide was susceptible to dinitrophenylation, only about one-half of it was modified in Phe-2 and *bis*-disaccharide tetrapeptide.

In view of these results and keeping in mind the general structure of muropeptides, it seems reasonable to assume that the major muropeptides accumulating in the presence of D-aa are the equivalents to disaccharide tetrapeptide and *bis*-disaccharide tetrapeptide with a residue of the added D-aa instead of the D-ala residue normally present at the terminal position.

Kinetics of incorporation of D-aa into the peptidoglycan of exponentially growing cells of *E. coli*. To obtain a more detailed estimate of the magnitude and rate at which D-Met and D-Trp were incorporated into peptidoglycan, we studied the variation in the proportions of modified muropeptides in time course and dose-response experiments.

Figures 3A and B show the time course for the incorporation of D-Met and D-Trp as measured by monitoring the variation with time in the proportions of modified muropeptides in the peptidoglycan from cells growing in the presence of 20 mM D-Met or 15 mM D-Trp. Incorporation was rather fast for the first 30 min, and then declined progressively in both cases, probably because of saturation of potentially reactive sites in the peptidoglycan.

In dose-response experiments, a number of parallel cultures (100 ml) growing exponentially in LB medium were

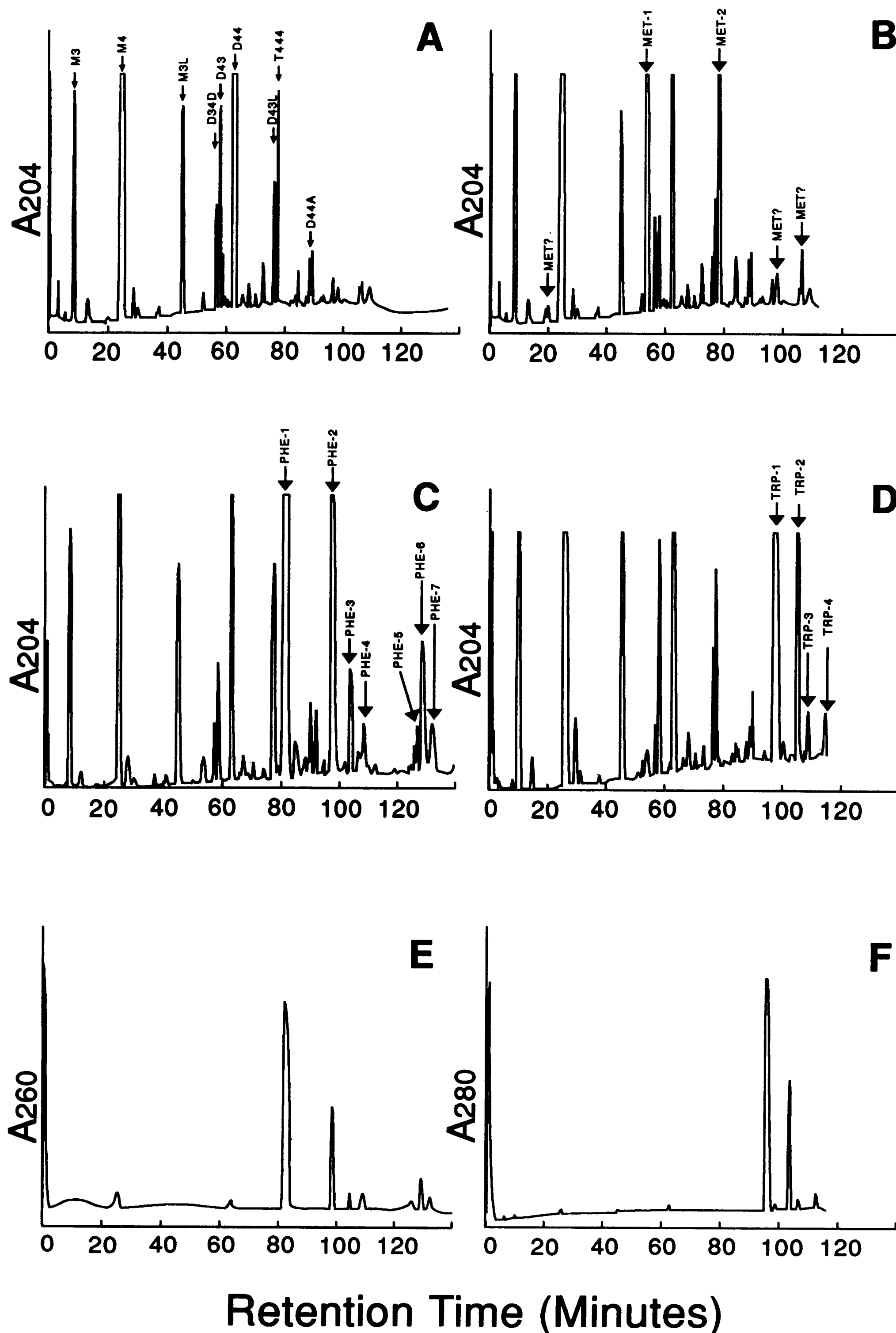

FIG. 1. Chromatographic analysis of peptidoglycan purified from cells grown in the presence of D-aa. Peptidoglycan was purified from cultures (250 ml) grown for 2 h in the presence of 20 mM each D-Met, D-Phe, and D-Trp and from an untreated, control culture. Samples were subjected to HPLC analysis as described in Materials and Methods. Shown are the UV absorption profiles of the eluates. (A to D) A_{204} records of the control (A) and D-Met (B)-, D-Phe (C)-, and D-Trp (D)-treated samples. (E) A_{260} record of the D-Phe-treated sample. (F) A_{280} record of the D-Trp-treated sample. In panel A, the positions of the more conspicuous muropeptides are indicated: M3, GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap; M4, GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap-D-Ala; M3L, GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap-(ε)-L-Lys-L-Arg (lipoprotein-bound M3); D34D, GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap–GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap-D-Ala (dap-dap bridge-cross-linked dimer); D43, GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap-D-Ala–GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap; D44, GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap-D-Ala–GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap-D-Ala; D43L, GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap-D-Ala–GlucNAc-MurNAc-

TABLE 1. Incorporation of amino acids and related compounds into the peptidoglycan of growing cells

Compound[a]	Incorporation	Characteristic of major modified muropeptide[b]: 1			2		
		Rt	R_{t-t}	%	Rt	R_{t-t}	%
D-Met	+	50.3	0.86	21.1	74.5	1.33	10.6
D-Trp	+	98.6	1.76	18.9	106.0	1.89	7.1
D-Trp-methyl ester	+	95.2	1.71	5.3	105.0	1.87	2.5
N-Acetyl-D-Trp*	–						
D-Phe	+	79.5	1.42	29.4	96.0	1.71	14.9
D-Val	+	51.2	0.91	28.7	71.2	1.27	14.5
D-Asp	+	7.0	0.12	2.5	16.2	0.28	1.5
D-Lys	–						
N-α-Acetyl-L-Lys	–						
N-α-Acetyl-L-Lys-methyl ester	–						
D-Norleucine	+	77.6	1.38	8.5	98.1	1.75	5.0
D-Pro*	–						
α-Aminoisobutyric acid	–						
D-α-Aminobutyric acid	+	32.2	0.57	24.2	65.7	1.17	12.9
DL-β-Aminobutyric acid*	–						
β-Ala	–						
LD-*m*-dap*	–						
D-α-Aminopimelic acid	+	45.0	0.80	9.2	72.5	1.30	3.4

[a] The L isomers of all the compounds listed, except for those marked with an asterisk, were checked under identical conditions. None of them was incorporated.

[b] Data are for the two most abundant new muropeptides accumulated after 4 h of growth in the presence of the indicated compounds each at a concentration of 20 mM. Rt and R_{t-t} (retention time of the compound relative to the retention time of *bis*-disaccharide tetrapeptide) are reported in minutes. Percent represents the relative abundance of the modified muropeptide. The values indicate the fraction of the total integrated area that corresponds to each of the modified muropeptides. The values have been corrected for the differences in the extinction coefficients at 204 nm of the added compounds relative to alanine. It was assumed that the modified muropeptides contain a residue of the added compounds instead of the terminal residue of D-alanine.

incubated for 5 min in different concentrations of D-Met or D-Trp. Incorporation was stopped by rapid chilling of the cultures in a water-ice-salt bath; the time required to reach 5°C was 1.5 min. Cells were centrifuged (10,000 × *g*, 15 min), resuspended in 8 ml of fresh LB medium, mixed 1:3 with boiling 10% SDS, and further processed for HPLC analysis of peptidoglycan. The results of the experiment shown in Fig. 3C and D indicated that incorporation followed saturation kinetics for both D-aa. The apparent half-saturating concentrations were 3 mM for both Met-1 and Met-2 and 6.5 and 9.5 mM for Trp-1 and Trp-2, respectively. The proportions of total modified muropeptides were below 10% in all instances; therefore, the results should not be significantly influenced by depletion of the insoluble substrate.

The results of an experiment designed to study the relationship between the incorporation of D-aa (D-Met) and the growth rate indicated that incorporation was essentially independent of growth rate. The proportions of D-Met-containing muropeptides in peptidoglycan from cells grown in MC medium at 30°C and in LB medium at 37°C (96- and 40-min mass doubling times, respectively) and incubated in 20 mM D-Met for 40 min were very similar (19.3 and 23.2% for the MC and LB cultures, respectively), despite the different increments in cell masses (40 and 100%, respectively).

Incorporation of D-aa into the peptidoglycan of resting cells and effect of ampicillin. To find out whether the incorporation of D-aa was restricted to actively growing cells and to what extent it would be influenced by growth-limiting conditions, we performed an incorporation experiment with stationary-phase cells of *E. coli* grown in LB and MC media. Cultures were grown to the stationary phase; 1 h after the OD_{550} reached constant values (2.3 and 1.0 for LB and MC cultures, respectively), each culture was divided into two equal subcultures, and D-Met (20 mM) was added to one member of each set. After 12 h of incubation, the cells were harvested and further processed for peptidoglycan analysis. Resting cells efficiently incorporated D-Met; 35 and 17% of total muropeptides were accounted for by Met-1 plus Met-2 in LB and MC cultures, respectively. The clear influence of the media on the magnitude of the incorporation is certainly in contrast to the observations made above for growing cells, suggesting important differences in the properties of the reaction in growing and resting cells.

The ability of resting cells to incorporate D-aa into peptidoglycan opened an easy and straightforward way to test the possible role of PBPs in the process. As resting cells are tolerant of β-lactams (14, 26), a stationary-phase culture of *E. coli* grown in LB medium was divided into two equal parts 1 h after the OD_{550} reached a constant value. Ampicillin (1 mg/ml) was added to one subculture to inhibit the activity of PBPs, and 30 min later D-Met (20 mM) was added to both subcultures. After 12 h of incubation, the cells were treated as described above to analyze the composition of peptidoglycan. The proportions of modified muropeptides in both cultures (17 and 23% for the ampicillin-treated and control cultures, respectively) indicated that the incorporation of D-Met was essentially independent of the activity of PBPs, as previously suggested (28).

Effect of D-aa on the structure of peptidoglycan. Up to this point, we have considered the accumulation of new muropeptides the only relevant alteration of peptidoglycan structure induced by the presence of D-aa. However, alterations in the proportions of normal muropeptides were also likely. Table 3 shows the muropeptide composition of peptidoglycan from cells growing exponentially in the presence of 20 mM D-Met for different periods of time. With one exception, the glycan-chain-terminating anhydro-muropeptides, the proportions of all other groups of muropeptides were significantly affected. The presence of D-Met brought about a drastic reduction in cross-linked muropeptides, particularly severe for muropeptides cross-linked by dap-dap bridges, whose proportion dropped to 45% of the initial value. The proportion of lipoprotein-bound muropeptides decreased moderately, to 73% of the control value. The modification took place quickly, leading to a new, apparently steady muropeptide composition in about 60 min.

The data discussed above merely reflect the change in the mean composition of total peptidoglycan, giving no clues to discern whether modifications occurred at the stage of synthesis or at a later stage. To study this aspect, we performed an experiment in which ^{3}H-dap (0.1 mCi/mg; 6

L-Ala-D-Glu-*m*-dap-(ε)-L-Lys-L-Arg (lipoprotein-bound D43); T444, GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap-D-Ala–GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap-D-Ala–GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap-D-Ala; D44A, GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap-D-Ala–GlucNAc-(1,6-anhydro)-MurNAc-L-Ala-D-Glu-*m*-dap-D-Ala. Arrows in panels B, C, and D indicate the positions of the new absorption peaks.

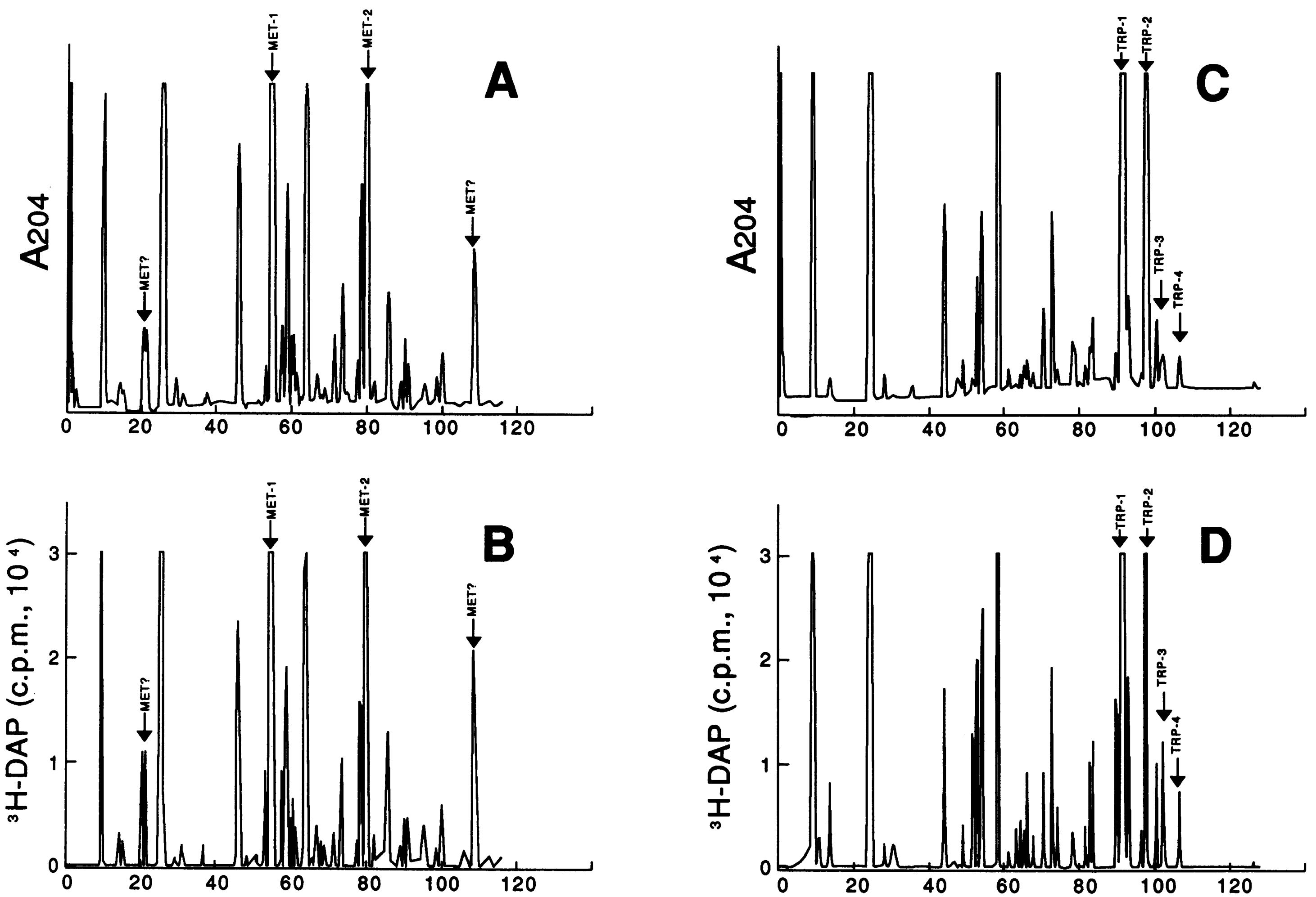

FIG. 2. Chromatographic analysis of radioactively labeled peptidoglycan purified from cells grown in the presence of D-Met and D-Trp. Peptidoglycan was purified from cultures (100 ml) grown for 2 h in the presence of 20 mM D-Met or D-Trp and ^{3}H-dap (0.05 mCi/mg; 6 μg/ml) and analyzed by HPLC, monitoring the A_{204} and radioactivity of the eluate. (A) A_{204} record of the D-Met-treated sample. (B) Radioactivity record of the D-Met-treated sample. (C) A_{204} record of the D-Trp-treated sample. (D) Radioactivity record of the D-Trp-treated sample. The positions of peaks corresponding to modified muropeptides are indicated by arrows.

TABLE 2. Amino acid compositions of native (−) and dinitrophenylated (+) modified muropeptides from cells grown in the presence of D-phenylalanine

Muropeptide	DNP	Nmol of:				Molar ratio relative to Glu			
		Ala	Glu	dap	Phe	Ala	Glu	dap	Phe
Disaccharide tetrapeptide	−	0.59	0.32	0.34		1.84	1	1.1	
Disaccharide tetrapeptide	+	0.35	0.17	0		2.05	1	0	
bis-Disaccharide tetrapeptide	−	2.72	1.6	1.55		1.7	1	0.95	
bis-Disaccharide tetrapeptide	+	2.78	1.56	0.71		1.8	1	0.46	
Phe-1	−	0.67	0.81	0.63	0.82	0.87	1	0.81	1.01
Phe-1	+	0.32	0.37	0.03	0.37	0.86	1	0.08	1.16
Phe-2	−	0.39	0.29	0.27	0.18	1.34	1	0.93	0.62
Phe-2	+	0.31	0.20	0.09	0.09	1.55	1	0.45	0.45

μg/ml) was added to exponentially growing cultures (50 ml) 2 and 30 min after the addition of D-Met (20 mM) and to a control culture. Cultures were incubated for 5 min, and incorporation was stopped by pouring the cultures into 2-liter flasks precooled in a water-ice-salt bath. Cells were sedimented by centrifugation (10,000 × *g*, 10 min, 4°C), resuspended in 10 ml of water, slowly mixed with 20 ml of boiling 10% SDS, and further processed for peptidoglycan analysis. Unfortunately, because of the unavailability of high-specific-activity ^{3}H-dap, we could only determine the amounts of the major muropeptides. Despite this difficulty, the results indicated a strong effect of D-Met on the structure of newly made peptidoglycan. Whereas 35% of the total radioactivity was present in cross-linked muropeptides in the control sample, in the D-Met-treated samples this proportion was reduced to 24% in both preincubated and nonpreincubated samples. The magnitude of the reduction (34%) was similar to that found in total peptidoglycan after a long incubation time (32%) (Table 3). The similarity of the results observed for both D-Met-treated cultures suggested that the disturbance of biosynthesis was more likely due to the presence of D-Met itself rather than to an accumulation of modified muropeptides in the peptidoglycan, which was drastically different in the cultures (5.6 and 28.7 mol% for 2 and 30 min of preincubation, respectively). Incidentally, no radioactivity was detected at the position of modified muropeptides, even though the molar fraction of Met-1 at the end of the labeling period (5.4 mol%) was about one-fifth that of disaccharide tetrapeptide (28.2 mol%) in the culture preincubated with D-Met for 2 min.

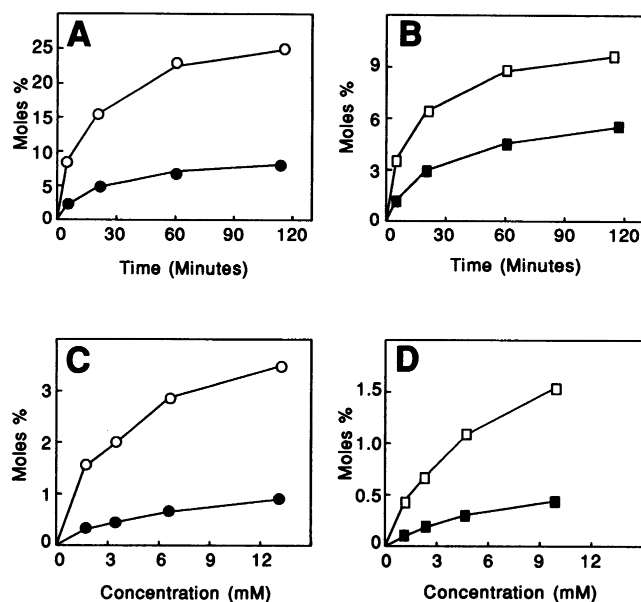

FIG. 3. Incorporation of D-Met and D-Trp into the peptidoglycan of exponentially growing cells. Incorporation of the indicated D-aa was studied in time course (A and B) and dose-response (C and D) experiments. (A and B) Peptidoglycan was purified from cultures (100 ml) growing in the presence of 20 mM D-Met (A) and D-Trp (B) for increasing periods of time and analyzed by HPLC. (C and D) Peptidoglycan was purified from cultures (100 ml) growing for 5 min in the presence of D-Met (C) and D-Trp (D) at different concentrations and analyzed by HPLC. The relative abundance of the individual muropeptides was estimated by automatic integration of the corresponding A_{204} peaks and is expressed as a molar fraction. Values have been corrected as indicated in Table 1, footnote *b*. Symbols: ○, Met-1; ●, Met-2; □, Trp-1; ■, Trp-2.

Peptidoglycan is known to suffer a global modification concomitantly with the transition of the culture into the stationary phase. The most conspicuous change is the increase in the proportions of cross-linked muropeptides (particularly of the dap-dap bridge kind), lipoprotein-bound muropeptides, and anhydro-muropeptides (22). To test whether D-aa would affect this process, we harvested cells growing in LB medium with and without 20 mM D-Met in the late exponential phase after eight generations of exponential growth and after 2 h in the stationary phase (9 h of total incubation time) and processed them for HPLC analysis. The muropeptide compositions of the different samples are shown in Table 3. Although the muropeptide compositions were greatly influenced by the presence of D-Met in growing and resting cells, peptidoglycan suffered similar modifications in the presence and absence of this compound, with the exception of the variation in the molar fraction of dap-dap bridge-cross-linked muropeptides, which remained nearly constant in the D-Met-treated cultures. The amount of peptidoglycan-bound lipoprotein increased in both cultures concomitantly with the transition into the stationary phase; however, the relative magnitude of the change was severely reduced by the presence of D-Met (Table 3).

Effect of D-aa on the synthesis of peptidoglycan in actively growing and resting cells of *E. coli*. Apart from the effects on the muropeptide composition of peptidoglycan, D-aa are likely to affect the biosynthesis of this cellular component either as a consequence of the accumulation of modified muropeptides or by directly interfering with biosynthetic enzymes. Therefore, the effect of D-Met and D-Trp on the incorporation of the specific precursor ^{3}H-dap into macromolecular peptidoglycan was studied.

TABLE 3. Effect of D-Met on the muropeptide composition of peptidoglycan in exponentially growing cells and during the transition into the stationary phase

Expt[a]	Sample	Phase of growth	Relative abundance (mol%) of[b]:						
			Monomers	Dimers	Trimers	dap-dap[c]	lpp mur[d]	anh mur[e]	D-Met mur[f]
1	Control	Exponential	70.7	26.5	2.8	2.8	9.3	2.4	0.0
	D-Met (5 min)	Exponential	73.3	24.3	2.3	2.4	8.2	2.7	11.1
	D-Met (20 min)	Exponential	77.7	20.7	1.6	1.3	6.9	2.5	21.0
	D-Met (60 min)	Exponential	79.8	18.5	1.7	1.5	6.2	2.5	30.5
	D-Met (120 min)	Exponential	80.3	18.1	1.6	1.3	6.5	2.6	33.6
2	Control	Late exponential	70.7	26.5	2.8	3.6	11.4	2.4	0.0
	D-Met (6 h)	Late exponential	78.1	20.5	1.5	2.1	6.9	3.2	34.0
	Control	Stationary	63.5	29.1	7.4	7.5	14.3	4.1	0.0
	D-Met (9 h)	Stationary	72.0	24.8	3.2	2.7	7.8	5.6	44.8

[a] Experiment 1 tested the evolution of peptidoglycan in exponentially growing cells, and experiment 2 tested that during the transition into the stationary phase.
[b] Muropeptides were quantified and grouped in structurally related families as described previously (22). Dimers and trimers were cross-linked.
[c] Muropeptides cross-linked by dap-dap bridges.
[d] Lipoprotein-bound muropeptides.
[e] Muropeptides with a residue of the glycan-chain-terminating sugar (1,6-anhydro)-MurNAc.
[f] D-Met-containing muropeptides (Met-1 plus Met-2).

To do so, we divided a culture growing exponentially in LB medium at 37°C into five equal subcultures. One was kept growing in plain medium, and the rest were treated with the D and L forms of Met (20 mM) and Trp (20 mM). After four generations of growth in the presence of the amino acids, ^{3}H-dap (0.1 mCi/mg; 6 μg/ml) was added to the cultures. One hour later, triplicate samples were removed and further processed to measure the radioactivity incorporated into peptidoglycan. The incorporation of ^{3}H-dap was similar in the control and L-form-treated cultures (10,368 cpm/ml for the control and 14,624 and 11,020 cpm/ml for L-Met and L-Trp, respectively). However, D-Met and D-Trp had a strong inhibitory effect, reducing incorporation to 26% (2,680 cpm/ml) and 34% (3,832 cpm/ml) of the control value, respectively. This inhibitory effect could have been due to the accumulation of modified muropeptides in peptidoglycan (>30% under the conditions used) and/or to a direct effect of the D-aa on the biosynthesis of new material. In an attempt to decide on this alternative, we studied the effect of D-aa on the incorporation of ^{3}H-dap in cells that had not been growing in the presence of the D-aa and whose peptidoglycan was therefore normal. Aliquots from an exponentially growing culture were transferred to flasks containing ^{3}H-dap (0.1 mCi/mg; 6 μg/ml) and either the D or the L form of Met (20 mM final concentration) or Trp (20 mM final concentration) and to a control flask. Cultures were incubated for 30 min. Afterwards, samples were withdrawn and treated as described above to quantify the incorporation of radioactivity into peptidoglycan. The results were similar to those reported above. Radioactivity incorporated by the control and L-form-treated samples was about the same (11,700, 9,340, and 9,500 cpm/ml for control and L-Met- and L-Trp-treated cultures, respectively), whereas D-Met and D-Trp reduced the incorporation to 25% (3,020 cpm/ml) and 34% (3,950 cpm/ml) of the control value, respectively. These results suggest an inhibitory action of the D-aa on biosynthetic enzymes which, nevertheless, permits apparently normal growth of the cells.

Previous work showed that resting cells of *E. coli* synthesize peptidoglycan at a reduced although significant rate (1). The physiological meaning of this residual synthesis is still obscure. A likely possibility is that peptidoglycan turnover or repair processes are active in resting cells for a given period of time. To investigate the sensitivity of this biosynthetic process to D-aa, we grew parallel cultures at 37°C in LB medium plus either D-Met or L-Met (20 mM) and D-Trp or L-Trp (20 mM) to the stationary phase. Two hours after the OD_{550} reached a constant value, ^{3}H-dap (0.1 mCi/mg; 6 μg/ml) was added to the cultures. After 90 min of incubation, samples were withdrawn and processed to measure the incorporation of ^{3}H-dap into insoluble peptidoglycan. D-Met and D-Trp had a clear inhibitory effect, although it was not as severe as in exponentially growing cells. In fact, while the control and L-form-treated cultures incorporated equal amounts of radioactivity (2,750, 2,600, and 2,640 cpm/ml for the control and L-Met- and L-Trp-treated cultures, respectively), in the presence of D-Met and D-Trp the incorporation was reduced to 51% (1,425 cpm/ml) and 72% (1,980 cpm/ml), respectively.

DISCUSSION

The growth of *E. coli* cells in medium supplemented with certain D-aa results in the incorporation of the D-aa into macromolecular peptidoglycan (15, 28). Analysis by HPLC of peptidoglycan from cells grown in the presence of a number of D-aa clearly confirmed this observation. The incorporation of D-aa resulted in the accumulation of new muropeptides in the sacculus, producing a characteristic HPLC elution pattern. In all instances, two major components (>90%) and a small number (three to five) of minor components were identified. The Rt of the modified muropeptides roughly correlated with the hydrophobicity of the particular D-aa added to the growth medium, as would be expected for a series of analogous compounds incorporating a fixed number of residues of the foreign amino acid. The accumulation of the modified muropeptides was concentration and time dependent but apparently independent of the growth rate. Modified muropeptides can account for as much as 45% of total muropeptides at concentrations of D-aa compatible with normal growth and morphology.

Spectrometric data and chemical analysis of the major modified muropeptides accumulated upon growth of the cells in the presence of D-Met, D-Trp, and primarily D-Phe positively identified the foreign D-aa as components of their molecules. Our results support as the most likely structures

for these two muropeptides those of the disaccharide tetrapeptide GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap-D-aa and the cross-linked dimer GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap-D-Ala–GlucNAc-MurNAc-L-Ala-D-Glu-*m*-dap-D-aa, for the early- and late-eluting components, respectively. Nevertheless, work oriented to the definite elucidation of the structures of both the major and the minor components accumulated in the presence of a large number of compounds by means of more sophisticated analytical techniques, like mass spectrometry, is in progress.

Resting cells were proficient in the incorporation of D-aa, reaching a level of peptidoglycan modification comparable to that in growing cells. The reaction therefore was not circumscribed to a particular phase of growth. This fact was particularly fortunate, because it permitted us to study how β-lactams would affect the reaction under conditions preventing bacteriolysis. Our experiments confirmed previous observations indicating that the incorporation of D-Met was essentially insensitive to β-lactams (1 mg of ampicillin per ml). The relatively lower level of modification in β-lactam-treated cultures was probably due to premature cell death or metabolic decay, as suggested by the extreme fragility of stationary-phase cells incubated simultaneously with ampicillin and a D-aa. These cells were lysed to an appreciable extent upon low-speed centrifugation and were much easier to solubilize with detergents than untreated cells. The insensitivity of the incorporation of D-Met to β-lactams excludes the involvement of the PBPs in the reaction and therefore of the normal biosynthetic pathway. Assuming that the structures proposed for the major modified muropeptides were correct, their synthesis could be performed by a periplasmic enzyme catalyzing the transfer of the *m*-dap-D-Ala LD-peptide bond of a tetrapeptidic side chain in the peptidoglycan to the α-D-NH_2 group of a molecule of D-aa in solution. Such a reaction would formally be a transpeptidation reaction conceptually identical to the "transpeptidase model reaction" catalyzed by certain DD-transpeptidases in vitro. These enzymes are able to mediate the exchange of the terminal D-Ala of UDP-muramyl-pentapeptide by a molecule of glycine (30). The most interesting and obvious difference is that in our case, the stereochemistry and stereoselectivity of the reaction would define the enzyme as an LD-transpeptidase. To our knowledge, enzymes with this activity have not been detected before in the cell envelope of *E. coli*. The LD character of this activity would explain its insensitivity to β-lactams, powerful inhibitors of DD activities but without effect on other LD activities, such as that of the LD-carboxipeptidase of *E. coli* (22).

A peculiar aspect of the incorporation of D-aa into peptidoglycan is the poor substrate specificity of the reaction with respect to the D-aa. Although not all D-aa are accepted, compounds with as chemically different side groups as D-Met, D-Trp, D-Phe, D-Val, or D-aminopimelic acid are accepted. Experiments with derivatives of D-Trp indicated that a free amino group, but not a carboxyl group, is required for the reaction, as expected for the mechanism postulated above. Despite the apparent lack of substrate specificity of the incorporation of D-aa, some of them, e.g., D-Pro, D-Lys, *m*-dap, and β-D-amino acids, were not incorporated into the sacculus. The requirement of the reaction for a free amino group could explain the case of D-Pro easily. D-Lys and *m*-dap have in common a second NH_2 group distal to the D center. In both cases, the equivalent α-mono-amino acids (D-norleucine and D-aminopimelic acid) were incorporated. This result suggests that it is the presence of a second NH_2 group distal to the α-D-NH_2 group that impedes the reaction. It is worth noting that dicarboxylic amino acids (D-Asp and D-aminopimelic acid) were indeed incorporated, suggesting that it is not the presence of a charged group per se that hampers the reaction but rather the charge or a steric constraint.

The stereospecificity of the incorporation of D-aa seems to be strict enough to exclude achiral amino acids, as indicated by the results obtained with α-amino-isobutyric acid, which was not incorporated, while the structurally related D-Val was a good substrate for the reaction. Glycine could not be tested because it is normally incorporated into peptidoglycan through the normal biosynthetic pathway (6).

In this context it is worth noting the absence of radioactivity associated with Met-1 after a short time of labeling of peptidoglycan with ^{3}H-dap in the presence of D-Met. Although the sensitivity of the experiment was low for the reasons stated above, if D-Met were uniformly incorporated into peptidoglycan, then radioactivity should have been detectable in Met-1, which was one-fifth as abundant as disaccharide tetrapeptide. This observation suggests that the incorporation of D-Met might occur preferentially on "old" peptidoglycan.

The presence of D-Met or D-Trp and likely of other incorporable D-aa not only results in the accumulation of modified muropeptides but also has drastic effects on the synthesis and structure of peptidoglycan.

D-Met and D-Trp were inhibitors of peptidoglycan synthesis in growing cells. In fact, D-Met could inhibit the incorporation of ^{3}H-dap into peptidoglycan by as much as 60 to 70% at a concentration (20 mM) permitting normal growth. The inhibitory effect of D-Met was apparently independent of the degree of modification of peptidoglycan, suggesting a direct effect of D-Met on the biosynthetic process.

The fact that peptidoglycan synthesis could be severely inhibited by D-aa while a normal growth rate and morphology were maintained indefinitely was surprising. Such a situation should have led to a progressive reduction in the amount of peptidoglycan per cell. Assuming that all parameters remained constant in treated cells, a final steady condition would be reached when the amount of peptidoglycan per cell was reduced in the same proportion as the rate of synthesis. At this moment, the cell could again be able to double its absolute amount of peptidoglycan in a cell cycle and would therefore reach a new steady condition. According to these arguments, *E. coli* cells would be able to grow and divide normally with as little as 40% of their normal peptidoglycan content. The ability of *E. coli* cells to grow and divide properly with approximately one-half the normal amount of peptidoglycan has been postulated before on different grounds (23). The good concordance between previous and present data further supports this idea.

Another relevant alteration in peptidoglycan structure caused by D-aa was a remarkable reduction in cross-linkage. Although the effect on the LD dap-dap bridge-cross-linked muropeptides was particularly severe (ca. 50%), the proportion of normal DD Ala-dap bridge-cross-linked muropeptides was also considerably reduced (ca. 25%) (Table 3). An analysis of the effect of D-Met on the structure of newly made murein suggested an inhibitory action of D-Met on the synthesis of normal *bis*-disaccharide tetrapeptide. This fact, together with the concomitant reduction in the rate of peptidoglycan synthesis, suggests a direct inhibitory action of D-Met on one or more of the high-molecular-weight PBPs. These enzymes (PBPs 1 to 3), endowed with transglycosylase and DD-transpeptidase activities, catalyze the synthesis and cross-linking of high-molecular weight peptidoglycan

(11, 30). As the acceptor substrate for the DD transpeptidation reaction is the D center of *m*-dap, it seems conceivable that some D-aa could mimic the natural substrate and interfere with the activity of the enzyme. Therefore, D-aa apparently affect peptidoglycan metabolism at least at two levels; first, D-aa compete for the normal function of an enzyme with LD-transpeptidase activity, and second, D-aa partially inhibit the activity of at least one high-molecular-weight PBP.

Interestingly, the effect of D-aa on the adaptive changes of peptidoglycan at the end of the active growth phase was moderate, with the exception of the variation in the proportions of dap-dap bridge-cross-linked and lipoprotein-bound muropeptides (Table 3).

The nature and physiological function of the enzyme responsible for the incorporation of D-aa into peptidoglycan remain unknown. In this respect, our results argue against previous proposals postulating the involvement of this enzyme in the binding of lipoprotein to peptidoglycan (28). In fact, this reaction is supposed to occur through the transfer of the *meso*-diaminopimelyl-D-alanine peptide bond to the ε-NH_2 group of the C-terminal L-lysine of lipoprotein (2). However, according to our results, neither the lysine enantiomers and derivatives tested nor the achiral amino acids (ε-NH_2 is achiral in lysine) were incorporated into peptidoglycan. Furthermore, lysine and lysine derivatives had no effect on the proportion of lipoprotein-bound muropeptides. In our opinion, the reduction in the proportion of bound lipoprotein could be due either to an additional inhibitory effect on the genuine lipoprotein-binding enzyme or to substrate depletion if modified muropeptides were not acceptable as substrates for lipoprotein binding.

An attractive alternative hypothesis would be to assume that the physiological role of the enzyme incorporating D-aa into peptidoglycan was the synthesis of dap-dap bridge-cross-linked muropeptides. As the dap-dap bridge peptide bond has an LD conformation (6), it seems reasonable to expect the participation of an LD-transpeptidase, the enzyme anticipated for the incorporation of D-aa. The large reduction in the proportion of dap-dap bridge-cross-linked muropeptides in the peptidoglycan of cells treated with D-aa supports this idea; however, more information is needed for a definite conclusion. Work directed to the unambiguous determination of the structures of a number of modified muropeptides, to the design of practical in vitro assays, and to the exploration of possible applications of modified muropeptides is under way.

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