Replacement of Diaminopimelic Acid by Cystathionine or Lanthionine in the Peptidoglycan of *Escherichia coli*

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In *Escherichia coli*, auxotrophy for diaminopimelic acid (A\(_5\)) can be suppressed by growth with exogenous cystathionine or lanthionine. The incorporation of cystathionine into peptidoglycan metabolism was examined with a dapA metC mutant, whereas for lanthionine, a dapA metA mutant strain was used. Analysis of peptidoglycan precursors and sacculi isolated from cells grown with epimeric cystathionine or lanthionine showed that meso-A\(_5\) was totally replaced in the same position by either sulfur-containing amino acid. Moreover, mainly l-allo-cystathionine (95%) or meso-lanthionine (93%) was incorporated into the precursors and sacculi. For this purpose, a new, efficient high-pressure liquid chromatography (HPLC) technique for analysis of the cystathionine isomers was developed. The formation of the UDP-MurNAc tripeptide appeared to be a critical step, since the MurE synthetase accepted meso-lanthionine or \(\text{d}-\text{allo-}\) or \(\text{l}-\text{allo-}\)cystathionine in vitro as good substrates, although with higher \(K_m\) values. Presumably, the 10-fold-higher UDP-MurNAc-L-Ala-D-Glu pool of cells grown with cystathionine or lanthionine ensured a normal rate of synthesis. The kinetic parameters of the MurF synthetase catalyzing the addition of d-alanyl-d-alanine were very similar for the meso-A\(_5\)-pm, l-allo-cystathionine-, and meso-lanthionine-containing UDP-MurNAc tripeptides. HPLC analysis of the soluble fragments resulting from 95% digestion by Chalaropsis *N*-acetylmuramidase of the peptidoglycan material in isolated sacculi revealed that the proportion of the main dimer was far lower in cystathionine and lanthionine sacculi.

The structure of the peptidoglycan of *Escherichia coli* has been studied extensively and is to a certain extent determined by the specificities of the various enzymes of its biosynthesis (11, 12, 32, 35). However, it has been shown to vary with growth conditions, antibiotic treatments, and mutations. These variations involve cellular aspects (size, shape, or thickness) as well as molecular characteristics (extent of peptide cross-linkages, size of the peptide subunit, length of the glycan strands, etc.). The reasons for most of these variations are still poorly understood and not readily accessible to biochemical analysis because of the complexity of the late steps of peptidoglycan metabolism and their correlation with growth and division (12, 24). The exact extent of these variations is also poorly defined. The study of the flexibility of peptidoglycan metabolism is essential not only for understanding cell physiology but also for understanding the mode of action of many antibiotics and possible resistance mechanisms.

The meso-diaminopimelic acid residue (meso-A\(_5\)) of the peptidoglycan monomer unit plays a key role in the integrity of the sacculus, since it is directly involved in peptide cross-linkages (11). In *E. coli*, free endogenous meso-A\(_5\) is either decarboxylated to yield lysine or used in peptidoglycan synthesis by its addition to UDP-MurNAc-L-Ala-D-Glu to yield UDP-MurNAc-L-Ala-\(\gamma\)-D-Glu-meso-A\(_5\) (25, 35). In the late steps of peptidoglycan metabolism, the A\(_5\) residue is subsequently used in various transpeptidation reactions, leading to the formation of the \(\text{d}-\text{Ala}\)→meso-A\(_5\)-pm and A\(_5\)-pm→A\(_5\)-pm cross-linkages (1, 11, 17, 24).

The replacement of A\(_5\) by an analog could be a useful tool for analyzing the specificity of the enzymes involved in its insertion into peptidoglycan metabolism and the complexity of the transpeptidation reactions. Mutants altered in the A\(_5\) pathway offer a possibility for this replacement. They generally require exogenous A\(_5\) for growth (25). However, A\(_5\) auxotrophy can be suppressed in some cases by endogenous metabolic modifications or compensated for, in the presence of lysine, by the addition of some exogenous A\(_5\) analogs. For instance, a dapA mutant of *E. coli* lacking A\(_5\) epimerase required no A\(_5\) for growth (30). It was found to have an unusually large pool of l-L-A\(_5\)-pm which was incorporated into peptidoglycan and its precursors (20). Nonetheless, a small pool of meso-A\(_5\)-pm was detected and found to be necessary for the formation of the d-alanyl-meso-A\(_5\)-pm cross bridges in the final material. It was speculated that an enzyme, other than the DapF epimerase, can catalyze the conversion of l-L-A\(_5\)-pm into the meso-isomer, at least at a rate sufficient to sustain growth (20). Another endogenous suppressing effect was observed with dapA metC mutant strains harboring a plasmid overexpressing the metB gene (31). They required only lysine for growth, and A\(_5\)-pm was totally replaced in peptidoglycan by endogenously formed cystathionine and lanthionine in a 2-to-1 ratio. Cystathionine is a normal metabolite of *E. coli*, but lanthionine is not. These specifically engineered strains undoubtedly possessed a high cystathionine pool and were able to synthesize lanthionine at a low rate by a yet unidentified mechanism (31).

Among the many analogs of A\(_5\)-pm that have been tested for their putative ability to suppress A\(_5\)-pm auxotrophy, only a few have been found to be more or less efficient. They were 4-methyl-A\(_5\)-pm (28, 29), 4-methylene-A\(_5\)-pm (9), 3-hydroxy-A\(_5\)-pm (28, 34), 4-hydroxy-A\(_5\)-pm (4), 4-fluoro-A\(_5\)-pm (4), diaminosuberic acid (26), dehydrodiaminosuberic acid (26), cystathionine (28, 31), djenkolic acid (28), and lanthionine (3, 4, 26, 28, 31). Analogs such as 4-oxo-A\(_5\)-pm and selenium-lanthionine were reported to be capable of partially substituting for A\(_5\)-pm (4). Interestingly, the peptidoglycans of some bacterial species

contain 3-hydroxy-A₂pm or lanthionine as a natural substituent for A₂pm (13, 14, 27, 38).

The aim of the present study was to test the total replacement of meso-A₂pm in E. coli dapA mutant strains by exogenous cystathionine or lanthionine. After examining the growth of dapA mutant strains with these sulfur-containing diamino-dicarboxylic acids in the presence of lysine, a biochemical analysis of this replacement was undertaken at the level of the UDP-MurNAc peptide precursors and of the sacculi. The close agreement between in vivo and in vitro data indicated that the same pathway was followed. Three steps appeared to be critical: the uptake of the analog by the cell, its addition to the nucleotide precursor UDP-MurNAc-L-Ala-d-Glu, and the formation of the cross bridges.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. E. coli K-12 strains B259 (dapA meta) and B243 (dapA metC) (31) were kindly provided by C. Richaud (31) and used throughout this study. Cells were grown as described previously (18) in LB medium or minimal medium M63 supplemented with glucose (0.2%), thymine (20 μg/ml), lysine (1 mM), and methionine (1 mM). The dapA meta mutant was grown with A₂pm, cystathionine, or lanthionine at 100 μg/ml. The dapA metC mutant was grown with cystathionine at 400 μg/ml. In all cases, 2-liter flasks containing 500 ml of medium were inoculated with 0.5 ml of overnight cultures, and growth was monitored by measuring the optical density at 600 nm (OD_600) in a Gilford model 240 spectrophotometer.

Chemicals and analytical procedures. The three isomers (dd, ll, and meso) of A₂pm, d-Ala-d-[¹⁴C]Ala, UDP-MurNAc-L-Ala-d-Glu, UDP-MurNAc-L-Ala-[¹⁴C]Glu, and UDP-MurNAc-L-Ala-L-Ala-d-Glu-meso-A₂pm were obtained by previously described methods (8, 20, 22, 36). dl-Lanthionine was obtained from Fluka (Buchs, Switzerland), and l-lanthionine was from Sigma (Saint Louis, Mo.). The mixture of l- and l-allo-cystathionine bought from Sigma was found to be in fact an epimeric mixture of the four stereoisomers (12). meso-Lanthionine, l-cystathionine, and a mixture of dl-allo- and l-cystathionine were kindly provided by S. Pochet (31). Chalaraopsis N-acetylmuramidase was kindly provided by G. Seibert (Hoechst, Frankfurt, Germany). 1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide was synthesized by the method of Marfey (16). High-pressure liquid chromatography (HPLC) was carried out as described previously (18, 19). Amino acid and amino sugar compositions were determined with the Biotronik model LC2000 amino acid analyzer (Frankfurt/Main, Germany) after hydrolysis of samples in 6 M HCl at 95°C for 16 h and by using o-phthalaldehyde and 2-mercaptoethanol as reagents. E. coli JMB30(pHE5) was the source of overproduced MurE and MurF (7, 23). The MurE activity was partially purified as described previously (23), and the dialyzed supernatant of the streptomycin-streptomyein sulfate step of this purification procedure was used as a source of MurF activity.

Pool levels of peptidoglycan precursors. Exponentially growing cells from 1-liter cultures at an OD of 0.7 (0.2% of 1 mg of bacteria [dry weight]) were rapidly chilled to 0°C and harvested in the cold. Extracts were prepared as described previously (18). For cells treated with d-cycloserine (Table 1), the antibiotic was added at a final concentration of 1 μg/ml to cultures of exponential-phase cells at an OD of 0.5, and cells were harvested 30 min later, 5 min before the normal onset of cell lysis. The analytical procedure used for the separation and quantification of the peptidoglycan nucleotide precursors was described earlier (18, 19).

Isolation and quantification of peptidoglycan. Exponentially growing cells from 1-liter cultures at an OD of 0.7 were rapidly chilled to 0°C and harvested in the cold. Pellets were washed with a cold aqueous 0.85% NaCl solution and centrifuged again. The bacteria were then rapidly suspended under vigorous stirring in 40 ml of a hot (95 to 100°C) aqueous 4% sodium dodecyl sulfate (SDS) solution for 30 min. After standing overnight at room temperature, the suspensions were centrifuged for 30 min at 200,000 × g, and the pellets were washed several times with water. The peptidoglycan content of sacculi was expressed in terms of the muramic acid content (21).

HPLC analysis of peptidoglycan structure. The structural analysis of E. coli peptidoglycan was carried out by the method of Glauner (10), slightly modified. Peptidoglycan from crude isolated sacculi was digested with Chalaraopsis N-acetylmuramidase. The resulting soluble fragments were reduced with sodium borohydride. The reduced compounds were separated by reverse-phase HPLC with a LiChrosorb RP-18 column (4 by
TABLE 1. Pool levels of the peptidoglycan nucleotide precursors in cells grown in the presence of A2pm, cystathionine, or lanthioninea

<table>
<thead>
<tr>
<th>Nucleotide precursor (retention time)</th>
<th>With A2pm</th>
<th>With cystathionine</th>
<th>With lanthionine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>With + d-Cycloserine</td>
<td>Control</td>
</tr>
<tr>
<td>UDP-GlcNAc (6 min)</td>
<td>315</td>
<td>270</td>
<td>320</td>
</tr>
<tr>
<td>UDP-MurNac (13 min)</td>
<td>23</td>
<td>21</td>
<td>24</td>
</tr>
<tr>
<td>UDP-MurNac-L-Ala (23 min)</td>
<td>3.6</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.1</td>
</tr>
<tr>
<td>UDP-MurNac-L-Ala-D-Glu (33 min)</td>
<td>5</td>
<td>18</td>
<td>73</td>
</tr>
<tr>
<td>UDP-MurNac tripeptide&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21</td>
<td>795</td>
<td>18</td>
</tr>
<tr>
<td>UDP-MurNac pentapeptide&lt;sup&gt;d&lt;/sup&gt;</td>
<td>170</td>
<td>27</td>
<td>140</td>
</tr>
</tbody>
</table>

<sup>a</sup> Nucleotide precursors were separated by reverse-phase HPLC on a μBondapak C18 column (7.8 by 300 mm). From 0 to 26 min, elution was performed with 50 mM ammonium formate (pH 3.7) at a flow rate of 3 ml/min. After 26 min, the pH and flow rate of the eluant were increased to 4.3 and 5 ml/min, respectively.

<sup>b</sup> ND, not determined.

<sup>c</sup> The retention times of the A2pm, cystathionine, and lanthionine-containing UDP-MurNac tripeptides were 21, 26, and 22 min, respectively.

<sup>d</sup> The retention times of the A2pm, cystathionine, and lanthionine-containing UDP-MurNac pentapeptide were 45, 54, and 47 min, respectively.

Enzymatic assay for MurF synthetase. MurF activity was assayed by monitoring the appearance of radioactive UDP-MurNac pentapeptide in a mixture (final volume, 50 μl) containing 0.1 M Tris-HCl (pH 8.6), 0.1 M MgCl₂, 5 mM ATP, 10 to 60 μM UDP-MurNac tripeptide, 100 μM D-Ala-D-[¹⁴C]Ala (0.53 kBq), and enzyme (15 μl in 20 mM potassium phosphate–1 mM 2-mercaptoethanol–0.1 mM MgCl₂ [pH 7.0]). The mixture was incubated for 30 min at 37°C, and the reaction was stopped by addition of 10 μl of glacial acetic acid. The amounts of product and unreacted D-Ala-D-Ala were then determined by high-voltage electrophoresis (pH 1.9, 40 V/cm, 1 h), autoradiography, and scintillation counting.

RESULTS

Complementation of A2pm auxotrophy by cystathionine or lanthionine. It has been shown that A2pm auxotrophy in E. coli can be complemented by exogenous cystathionine or lanthionine in the presence of lysine (3, 4, 15, 26, 28, 31). This was initially observed with W strain 173-25 (28) and more recently with K-12 strain β194 dapA (31). However, certain growth conditions must be fulfilled. Minimal medium is preferred, and preliminary growth with A2pm is essential before inoculation (28).

Strain β259 (dapA metC) grew with A2pm or lanthionine with a generation time of 65 or 70 min, respectively. Growth of this strain with cystathionine at 100 μg/ml was slow and led to clumping and lysis. Most likely this effect was due to the too intensive conversion of internalized cystathionine into homocysteine, leading to a decreased cystathionine pool, to an insufficient rate of incorporation into peptidoglycan, and to cells highly susceptible to lysis. This could be overcome by a higher exogenous cystathionine concentration (400 μg/ml) and by using a strain with a metC mutation in which the conversion to homocysteine was blocked. Strain β243 (dapA metC) grew with cystathionine with a generation time of 90 min. Similar effects were not observed with lanthionine, perhaps because it was more efficiently internalized than cystathionine or because it was metabolized at a slower rate in pathways other than that of peptidoglycan. Lysis of an A2pm auxotroph grown with lanthionine at 20 μg/ml has been observed at the onset of the stationary phase (3). No such effect was observed here with lanthionine at 100 μg/ml.

Incorporation into peptidoglycan. Sacculi were prepared from cells grown with A2pm, cystathionine, or lanthionine. Growth with exogenous epimeric cystathionine or lanthionine led to the total replacement of A2pm in isolated sacculi by...
either amino acid. As observed previously (31), sacculi from a dapA metC mutant strain grown with cystathionine contained no lanthionine, in agreement with the fact that it is not normally synthesized in E. coli. Conversely, the lanthionine sacculi from strain β243 (dapA metC) grown with lanthionine contained no cystathionine, in agreement with the blocking of its endogenous synthesis. It was observed previously that strain β243 (dapA metC) grown with lanthionine in its peptidoglycan (31), although the blocking of the formation of homocysteine presumably led to an increase in the cystathionine pool. This suggested that exogenous lanthionine leads to a cell level far higher than that of endogenous cystathionine or that it is far better incorporated in the biosynthesis of peptidoglycan. Since exogenous epimeric mixtures were used for growth, it was essential to determine which isomers were in fact incorporated into peptidoglycan. Like A2pm sacculi, which contained only meso-A2pm, the cystathionine or lanthionine sacculi contained mainly l-allo-cystathionine (95%) or meso-lanthionine (93%), respectively.

Cystathionine- and lanthionine-containing precursors. In E. coli, the biosynthesis of the peptidoglycan unit proceeds in the cytoplasm by the sequential addition of L-alanine, d-glutamic acid, meso-A2pm, and d-alanyl-d-alanine to UDP-N-acetylmuramic acid (32). The incorporation of cystathionine or lanthionine into the metabolism of peptidoglycan was investigated first by analyzing the series of UDP-MurNAc peptide precursors of cells grown with exogenous A2pm, cystathionine, or lanthionine (Table 1). In the three cases, the same nucleotide precursors up to UDP-MurNAc-L-Ala-d-Glu were encountered on the HPLC profiles. Modifications appeared at the level of the UDP-MurNAc tripeptide and UDP-MurNAc pentapeptide precursors. They were initially identified on the HPLC profiles by examining variations in peak size after treating cells with d-cycloserine at a sublytic concentration (Table 1). It is well established that this drug leads to a sharp increase in the UDP-MurNAc tripeptide pool and to the depletion of the UDP-MurNAc pentapeptide pool because of the inhibition of the formation of d-alanyl-d-alanine (reference 5 and references therein). In this way, the cystathionine-containing precursors were found to have somewhat higher retention times than the A2pm- or lanthionine-containing precursors (Table 1). These identifications were further substantiated by amino acid analyses of the recovered purified products. For convenience, the cystathionine and lanthionine UDP-MurNAc tripeptide precursors were recovered from d-cycloserine-treated cells, whereas corresponding UDP-MurNAc pentapeptide precursors were secured from untreated cells. Analysis of the isomer contents of the isolated nucleotides revealed that they contained mainly either l-allo-cystathionine (95%) or meso-lanthionine (93%), in agreement with the isomer contents of the final peptidoglycan material. The pool levels of the peptidoglycan nucleotide precursors of cells grown in the presence of A2pm, cystathionine, or lanthionine (Table 1) were very similar except for the UDP-MurNAc-L-Ala-d-Glu pool, which was 10- to 15-fold higher in cells grown with either sulfur-containing analog.

Specificity of MurF synthetase. In E. coli, the addition of meso-A2pm to UDP-MurNAc-L-Ala-d-Glu is catalyzed by the MurF synthetase, which has been partially purified (25). Prizes from the addition of l-cystathionine or lanthionine to UDP-MurNAc-L-Ala-d-Glu in cells grown with cystathionine or lanthionine. To ascertain this point and to account for the isomer specificity of the in vivo incorporation, in vitro assays were carried out with UDP-MurNAc-L-Ala-[14C]Glu, purified MurE, and A2pm analogs (Table 2). meso-Lanthionine and l-allo- and l-allo-cystathionine appeared to be good substrates, whereas other isomers of these amino acids were poorly accepted. Except for l-allo-cystathionine, these results correlated well with the isomer contents of the isolated UDP-MurNAc peptide precursors and sacculi, suggesting that MurE was also the cystathionine- or lanthionine-adding enzyme in vivo. Small amounts of UDP-MurNAc tripeptide precursors containing l-allo-cystathionine or meso-lanthionine were prepared enzymatically in vitro.

Specificity of MurF synthetase. The addition of d-alanyl-d-alanine to UDP-MurNAc tripeptide is the next step in the biosynthesis of the peptidoglycan unit (35). It is catalyzed by the MurF gene product, which has been purified to homogeneity (7). The Km and Vmax of the crude overproduced MurF activity for meso-A2pm, l-allo-cystathionine, and meso-lanthionine-containing UDP-MurNAc tripeptide precursors did not differ greatly (Table 3). These results suggested that the MurF enzyme also catalyzes the formation of cystathionine- and lanthionine-containing UDP-MurNAc pentapeptide precursors in vivo. Furthermore, the similar pool levels of these precursors confirmed not only that the replacement of A2pm by cystathionine or lanthionine had little effect on their formation but also that it had little effect on their immediate subsequent use in the formation of the peptidoglycan lipid intermediates.

### Table 2. Substrate specificity of the MurE synthetase for meso-A2pm and various analogs

<table>
<thead>
<tr>
<th>Analog</th>
<th>Relative sp act</th>
</tr>
</thead>
<tbody>
<tr>
<td>meso-A2pm</td>
<td>100</td>
</tr>
<tr>
<td>DD-A2pm</td>
<td>6.8</td>
</tr>
<tr>
<td>L-A2pm</td>
<td>2.6</td>
</tr>
<tr>
<td>meso-Lanthionine</td>
<td>52</td>
</tr>
<tr>
<td>LL-Lanthionine</td>
<td>1.5</td>
</tr>
<tr>
<td>l-allo-Cystathionine</td>
<td>28</td>
</tr>
<tr>
<td>d-allo-Cystathionine + l-cystathionine</td>
<td>26</td>
</tr>
<tr>
<td>L-Cystathionine</td>
<td>0.5</td>
</tr>
<tr>
<td>D-Lysine</td>
<td>0.6</td>
</tr>
<tr>
<td>l-Lysine</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

* A2pm and analogs were tested at 10 mM except as noted.

* Assayed at a 20 mM concentration of an equimolecular mixture.

### Table 3. Kinetic constants of the MurE synthetase for A2pm, lanthionine, and cystathionine and of the MurF synthetase for the UDP-MurNAc tripeptide precursors

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km (mM)</th>
<th>Vmax (nmol min⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MurE synthetase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>meso-A2pm</td>
<td>0.04</td>
<td>32</td>
</tr>
<tr>
<td>meso-Lanthionine</td>
<td>1.5</td>
<td>23</td>
</tr>
<tr>
<td>l-allo-Cystathionine</td>
<td>3.9</td>
<td>19</td>
</tr>
<tr>
<td>d-allo-Cystathionineb</td>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td>MurF synthetase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precursor with meso-A2pm</td>
<td>0.016</td>
<td>13</td>
</tr>
<tr>
<td>Precursor with meso-lanthionine</td>
<td>0.013</td>
<td>7.3</td>
</tr>
<tr>
<td>L-Lanthionine</td>
<td>0.11</td>
<td>6.7</td>
</tr>
</tbody>
</table>

* Km and Vmax values were determined by the method of Wilkinson (39).

b Assayed with a 10 mM concentration of an equimolecular mixture of d-allo- and l-cystathionine.

The Km for the d-allo-isomer was determined by considering that the synthetase had very low affinity for the l-isomer (Table 2).
were also found to be dominant in the lanthionine and cystathionine profiles. The retention times of the lanthionine fragments were the same as those of the corresponding A2pm fragments, whereas those of the cystathionine fragments were higher. This was in agreement with the differences in retention time observed between the nucleotide precursors (Table 1) and was presumably due to the longer hydrophobic chain between the two asymmetric centers of cystathionine. However, the most notable difference concerned the relative amounts of the main monomer and dimer fragments (Table 4). The relative amount of the main monomer was higher in the cystathionine and lanthionine sacculi than in the A2pm sacculi, whereas that of the main dimer was far lower. Interestingly, the replacement of A2pm by the sulfur-containing amino acids had no effect on the relative amounts (\%) of the dimer fragments that were second in importance and eluted before the main dimer (Fig. 2). In A2pm sacculi, these dimers have been characterized as tetra-tri, tetra-tri(A2pm), and tetra-tetra (Gly4) (10). The degrees of cross-linkage evaluated with the main dimer alone or by including these other dimers (Table 4) were much lower in the cystathionine and lanthionine sacculi.

**DISCUSSION**

Auxotrophy for A2pm can be suppressed in a *dapA* mutant strain by growth with exogenous cystathionine or lanthionine. Analysis of the UDP-MurNAc peptide precursors and of the isolated sacculi clearly showed that A2pm was totally replaced at the same position in peptidoglycan by either sulfur-containing diamino-dicarboxylic acid. In comparing cystathionine and lanthionine, the latter appears to be a better analog, more closely mimicking A2pm; presumably this is because its structure is strictly isosteric with A2pm, whereas cystathionine has a longer chain between its two asymmetric centers.

The first critical step involved in the replacement of A2pm by an analog is its uptake by an efficient transport system. *E. coli* possesses two transport systems for the uptake of cystine, one shared with A2pm (the cystine general system) and the other not (the cystine-specific system) (2). It has been observed that certain A2pm analogs (3-hydroxy-A2pm, diaminosuberic acid, lanthionine, and cystathionine) were efficient inhibitors of the uptake of cystine by the general system, suggesting that these analogs could also be taken up by it (2). It was noteworthy that

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**TABLE 4. Extent of peptidoglycan cross-linkage in A2pm, lanthionine, and cystathionine sacculi**

<table>
<thead>
<tr>
<th>Sacculi</th>
<th>Molar amt* (%)</th>
<th>Cross-linkage%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monomer(^a)</td>
<td>Dimer(^a)</td>
</tr>
<tr>
<td>A2pm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lanthionine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystathionine</td>
<td></td>
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</tbody>
</table>

\(^a\) Molar amounts are expressed as percentages of the molar amounts of the main monomer plus the main dimer (top number) and of the main monomer plus the main dimer and the faster-eluting dimers (bottom number) (Fig. 2).

\(^b\) Main monomer (tetra).

\(^c\) Main dimer (tetra-tetra).

\(^d\) Dimers eluting before the main dimer (Fig. 2).

\(^e\) The extent of cross-linkage was determined as the ratio of dimer(s) to the monomer plus 2x dimer(s) (32).
these inhibitory analogs could all support growth of A₂pm auxotrophs. However, in these experiments, no distinction between stereoisomers was made. The isomer distribution in the pool of cystathionine or lanthionine after uptake from the exogenous epimeric mixture was not examined in this study. Considering the high exogenous concentrations used, it is not an easy matter to analyze the isomer contents of such pools. It is therefore difficult to state whether there is any stereospecificity of the transport system.

Once internalized, the addition of an A₂pm analog to UDP-MurNAc-L-Ala-D-Glu was the next critical step in its use because of the specificity of the MurE synthetase. In vivo, meso-lanthionine and D-allo-cystathionine are preferentially incorporated into the peptidoglycan precursors. For lanthionine, this result was in agreement with the high specificity of the MurE synthetase for its meso-isomer and implies that the pool of the L,l-isomer is not overwhelmingly higher than that of the meso-isomer. It was found that, in vitro, the DapF epimerase readily catalyzed the interconversion of L,l- and meso-lanthionine (31). This suggested that there is an equilibrium between the two isomers in vivo, as in the case of A₂pm (references 18 and 20 and references therein), and therefore the specificity of the transport system for them is of little importance. If internalized to any extent, D,D-lanthionine is presumably not metabolized.

The situation is more complex with cells grown with the epimeric mixture of the four cystathionine isomers. In vivo, the D-allo-isomer is predominantly incorporated into the peptidoglycan metabolism. However, in vitro, the D-allo-isomer is good a substrate as the L-allo-isomer (Table 2), in agreement with the similar kinetic constants of the MurE synthetase for them (Table 3). This suggested either that D-allo-cystathionine is poorly internalized or that, if well internalized, it is consumed at a high rate in some yet unspecified pathway. The availability of the pure D-allo-isomer should enable us to determine which possibility is correct. It was previously observed that L-cystathionine alone could sustain growth of strain β243 (dapA metC) (31). Equivalent amounts of L- and D-allo-isomers were found in its peptidoglycan, indicating an in vivo conversion of the L-isomer into the D-allo-isomer. Considering that the specificity of the MurE synthetase for the D-allo-isomer is far greater than for the L-isomer (Table 2), the pool of the D-allo-isomer should be much lower than that of the L-isomer. This in turn implies a limited rate of conversion of the L-isomer into the D-allo-isomer. If internalized, the fate of D-cystathionine remains unknown.

The Kₘ values of the MurE synthetase for D-allo-cystathionine and meso-lanthionine were much higher than for A₂pm, whereas the Vₘₐₓ values were similar (Table 2). In order to sustain the rate of peptidoglycan synthesis necessary for similar rates of cell growth, the pool levels of the substrates (amino acid or nucleotide) of the synthetase must be higher than in cells grown with A₂pm. This was clearly the case for UDP-MurNAc-L-Ala-D-Glu. The pool of the A₂pm analog could therefore be limiting for the use of the nucleotide and the rates of peptidoglycan synthesis and cell growth. Considering the specificity of the MurE synthetase and its kinetic constants (Tables 2 and 3), it is also possible to conclude that the distribution of L,l-lanthionine in vitro is not due to uptake of L,l-cystathionine in the peptidoglycan of strain β253 (dapA metC) (pMB42[metB]) (31) grown without A₂pm or any analog indicates that the L-cystathionine pool was in this case much higher than that of the D-allo-isomer or of meso-lanthionine. This would again assess the poor in vivo conversion of L-cystathionine into its D-allo-isomer.

In E. coli peptidoglycan, the A₂pm residue is linked to the γ-carboxyl of the D-Glu residue by the amino function adjacent to its L-asymmetric carbon (references 6 and 37 and references therein). Assuming an analogy with the D-Glu-A₂pm linkage and considering that cystathionine is an unsymmetrical molecule formed by the condensation of serine and homocysteine residues, it can be speculated that D-allo-cystathionine is added in vivo to the D-Glu residue by its homocysteine moiety, whereas the L-allo-isomer is added in vivo and in vivo by its serine moiety. More detailed structural analyses would be necessary to ascertain this point. If this were the case, the position of the sulfur atom would be unessential, and it would be interesting to carry out assays with the cystine isomers.

After the formation of the UDP-MurNAc tripeptide, the replacement of A₂pm by cystathionine or meso-lanthionine had little effect on the next step in the biosynthesis of peptidoglycan, as judged from the in vitro results with MurF, or on the subsequent steps of the pathway up to the polymerization steps included. However, two types of reaction are involved in the transpeptidation of peptidoglycan: the first concerns the formation of the glycan strands by transglycosylation, and the second concerns the formation of the peptide cross bridges by transpeptidation (17, 32). In lanthionine and especially cystathionine sacculi, the amount of the main dimer was lower and that of the main monomer higher than in A₂pm sacculi. This suggested that in the transpeptidation reactions leading to the formation of the dimer, there was a discrimination between A₂pm and its analogs. This was not surprising, since the amino group of the diaminodicarboxylic acid (A₂pm or analog) is directed in the ester bond linking the two subunits of the dimer (32). It is noteworthy that the low extent of cross-linkage was still sufficient to ensure normal cell growth and division. A similar situation was observed with the incorporation of L-lanthionine into the peptidoglycan of dapF mutant strains (20).

Some A₂pm analogs support poorly the growth of A₂pm auxotrophs and lead to morphological alterations and lysis (4). In particular, elongated cells with bulges in the middle were observed with 4-hydroxy-A₂pm (4). This was reminiscent of the effects of penicillin G on E. coli (33) and suggested that the penicillin-sensitive transpeptidation reactions involved in septation are a critical step for proper growth with an A₂pm analog. Perhaps in this case the replacement of A₂pm by an analog is more critical for the transpeptidation reactions than for its addition to UDP-MurNAc-L-Ala-D-Glu. The mechanisms involved in the antibacterial action of certain A₂pm analogs could be analyzed by the same biochemical approach followed here for cystathionine and lanthionine.

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