Expression of the Staphylococcus aureus UDP-N-Acetylmuramoyl-
1-Alanyl-d-Glutamate:1-Lysine Ligase in Escherichia coli and
Effects on Peptidoglycan Biosynthesis and Cell Growth

DOMINIQUE MENGIN-LECREULX,1,* TIM FALLA,2† DIDIER BLANOT,1
JEAN VAN HEIJENOORT,1 DAVID J. ADAMS,2 AND IAN CHOPRA2

Laboratoire des Enveloppes Bactériennes, Centre National de la Recherche Scientifique, Université Paris-Sud,

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The monomer units in the Escherichia coli and Staphylococcus aureus cell wall peptidoglycans differ in the
nature of the third amino acid in the 1-alanyl-γ-d-glutamyl-X-D-alanyl-D-alanine side chain, where X is meso-
diaminopimelic acid or 1-lysine, respectively. The murE gene from S. aureus encoding the UDP-N-acetylmura-
moyl-1-alanyl-γ-d-glutamate:1-lysine ligase was identified and cloned into plasmid vectors. Induction of its over-
expression in E. coli rapidly results in abnormal morphological changes and subsequent cell lysis. A reduction of
28% in the peptidoglycan content was observed in induced cells, and analysis of the peptidoglycan composi-
tion and structure showed that ca. 50% of the meso-diaminopimelic acid residues were replaced by 1-lysine.
Lysine was detected in both monomer and dimer fragments, but the acceptor units from the latter contained
exclusively meso-diaminopimelic acid, suggesting that no transpeptidation could occur between the ε-amino
subgroup of 1-lysine and the ε-carboxyl group of D-alanine. The overall cross-linking of the macromolecule was
only slightly decreased. Detection and analysis of meso-diaminopimelic acid- and 1-lysine-containing pepti-
doglycan precursors confirmed the presence of 1-lysine in precursors containing amino acids added after the
reaction catalyzed by the MurE ligase and provided additional information about the specificity of the enzymes
involved in these latter processes.

Bacterial-cell-wall peptidoglycan (murein) is a giant macro-
molecule of periodic structure whose basic unit, a disaccha-
ride-pentapeptide, is polymerized linearly via the disaccha-
dride motif and cross-linked laterally via the peptide motif (for a
review, see reference 15). Any alteration of the basic unit thus
results in a global change of peptidoglycan structure and prop-
eties. Such global variations are encountered in nature as
conserved variations along phyletic lines (30) but have some-
times been acquired as a mechanism of resistance against cell-
wall-targeted antibiotics (5, 6, 8). The amino acid residue lo-
cated at the third position in the peptide chain plays a key role
in the integrity of the sacculus since it is directly involved in
peptide cross-linkages. This vital function is fulfilled by meso-
diaminopimelic acid (meso-A2pm) in Escherichia coli and 1-
lysine in Staphylococcus aureus.

In bacteria, free endogenous meso-A2pm is either irrevers-
ibly decarboxylated into 1-lysine (27) or used to form the
peptidoglycan precursor UDP-N-acetylmuramoyl-1-alanyl-γ-
d-glutamyl-meso-A2pm, the latter reaction being catalyzed by the
murE gene product (14, 24, 26). E. coli mutants altered in the
A2pm pathway require exogenous A2pm for growth and lyse if
lysine but not A2pm is supplied (18, 27). However, the A2pm
auxotrophy can be suppressed in some cases by endogenous
metabolic modifications (28) or, in the presence of lysine, by
the addition of certain A2pm analogs (7,18). The replacement
of A2pm by an analog thus appeared to be a very useful tool for
analyzing the specificity of the different enzymes involved in its
insertion into peptidoglycan metabolism and the complexity of
the transpeptidation reactions.

The E. coli UDP-MurNAc-1-Ala-γ-D-Glu-meso-A2pm ligase
(also named meso-A2pm-adding enzyme, EC 6.3.2.13) has
been previously purified, and its kinetic properties have been
investigated in detail (24, 26). The specificity of this enzyme for
both its nucleotide and amino acid substrates is very high but
not absolutely strict. Considering in particular the amino acid
site, 1-L-A2pm and many analogs of A2pm are substrates of the
reaction (3, 18, 21), but 1-lysine is not (18). The same was
observed with the A2pm-adding enzyme from other bacteria
(14). Less information is available on the UDP-MurNAc-1-
Ala-γ-D-Glu-1-lysyl ligase (1-lysine-adding enzyme, EC 6.3.2.7),
but Ito and Strominger showed that the enzyme from S. aureus
(13) and other bacterial species (14) does not accept meso-
A2pm as an alternative substrate. Since pools of lysine and
A2pm coexist in bacteria, the high (and inverse) specificities of
the MurE enzymes from E. coli and S. aureus clearly prevent
these strains from incorporating these nonspecific compounds
into cell wall peptidoglycan. It was thus tempting to speculate
that the expression of the E. coli murE gene in S. aureus or
inversely the S. aureus murE gene in E. coli could have dra-
natic effects on peptidoglycan metabolism and cell growth.
In the present study we describe the cloning of the murE gene
from S. aureus and show that its overexpression in E. coli re-
sults in a large and toxic recruitment of 1-lysine in the pathway
for peptidoglycan synthesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. E. coli BL21(DE3)pLYS8 (Promega)
was used as the host for the plasmids as well as for the overproduction of
the MurE enzyme. 2YT medium (25) was used for growing cells, and growth
was monitored at 600 nm with a Shimadzu UV-1601 spectrophotometer. Antibiotics

† Present address: Intrabiotics Pharmaceuticals, Inc., Mountain
View, CA 94043.
were used at the following concentrations: ampicillin (100 µg ml⁻¹), kanamycin (40 µg ml⁻¹), and chloramphenicol (30 µg ml⁻¹).

Cloning of the *S. aureus* murE gene and plasmid construction. Standard procedures for molecular biology were followed. The *S. aureus* murE gene was PCR amplified from strain RN4220 by using primers containing the start and the stop codons of the gene (5'-TTGTAGCAAGTACCTTTGT-3' and 5'-TTATGGATCAACAGGCAACC-3') (sequence data supplied by SmithKline Beecham Pharmaceuticals). A 1,485-bp product was amplified, cloned into pGEM-T Easy (Promega), and confirmed as *S. aureus* murE by sequencing. The resulting construct (pMuSa1) was then digested with EcoRI, and the excised *S. aureus* gene was ligated into EcoRI-digested pET30 (Novagen). The orientation of murE was confirmed by EcoRV digestion, and constructs containing the gene in the correct orientation (pMuSa2) were subsequently transformed into *E. coli* BL21 (DE3) :: pLysS for expression.

Extraction and quantitation of peptidoglycan precursors. Cells of *BL21(DE3)* pLysS::pMuSa2 (1-liter cultures) were grown exponentially at 37°C in 2YT medium. When an optical density (OD) of 0.4 (600 nm) was reached (approximately 2.5 × 10⁸ cells ml⁻¹), IPTG (isoprpyl-β-D-thiogalactopyranoside) was added to one culture at a final concentration of 1 mM. As soon as the first effects on cell growth were observed in induced cells (ca. 1 h after a final OD of 0.8), cultures were stopped by rapid chilling to 0 to 4°C, and cells were harvested in the cold. The extraction of peptidoglycan nucleotide precursors, as well as the analytical procedures used for their quantitation, were as described previously (9, 19, 20). Isolation, sacculi and quantitation of peptidoglycan. Cells of *BL21(DE3)* pLysS::pMuSa2 (0.5-liter cultures) were grown and induced with IPTG as described above. Harvested cells were washed with cold 0.85% NaCl solution and centrifuged again. Bacteria were then rapidly suspended under vigorous stirring in a solution consisting of 2 ml of water, and aliquots (100 µl) were hydrolyzed and analyzed with a Biotronic model LC2000 amino acid analyzer. The peptidoglycan content of the sacculi was expressed in terms of its muramic acid content (19, 22).

Purification of peptidoglycan and structure analysis. First, the crude preparations of *E. coli* sacculi were subjected to successive treatments with pronase, pronase, and trypsin to eliminate peptidoglycan-associated proteins (4, 19). After several washings with water, hydrolysis of an aliquot of this material showed that it contained only peptidoglycan constituents: muramic acid, glucosamine, alanine, and glycine (20). After the sacculi were subjected to successive treatments with pronase, pronase, and trypsin to eliminate peptidoglycan-associated proteins (4, 19). After several washings with water, hydrolysis of an aliquot of this material showed that it contained only peptidoglycan constituents: muramic acid, glucosamine, alanine, and glycine (20).

Preparation of crude protein extracts. Cells (0.5-liter cultures) grown as described above were harvested in the cold and washed with 40 ml of cold 20 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM MgCl₂ and 0.1% 2-mercaptoethanol. The wet cell pellet was suspended in 7.5 ml of the same buffer and disrupted by sonication in the cold (Bioblock Vibracell sonicator), and the resulting suspension was centrifuged at 4°C for 30 min at 200,000 × g. The supernatant was dialyzed overnight at 4°C against 100 volumes of the same buffer, and the resulting suspension was centrifuged at 4°C for 30 min at 200,000 × g. The supernatant was dialyzed overnight at 4°C against 100 volumes of the same buffer, and the resulting suspension was centrifuged at 4°C for 30 min at 200,000 × g. The supernatant was dialyzed overnight at 4°C against 100 volumes of the same buffer, and the resulting suspension was centrifuged at 4°C for 30 min at 200,000 × g.

Enzymatic assays. (i) *meso*-A2pm-adding activity. The standard assay mixture consisted of 100 mM Tris-HCl buffer (pH 8.0), 5 mM ATP, 100 mM MgCl₂, 0.1 mM meso-[¹⁴C]Apm (500 Bq), 0.2 mM UDP-MurNAc-t-Ala-t-Glu, and crude enzyme (5 µg of protein) in a final volume of 100 µl.

(ii) *meso*-A2pm-adding activity. The standard assay mixture contained 100 mM Tris-HCl buffer (pH 8.0, 5 mM ATP, 100 mM MgCl₂, 0.2 mM UDP-MurNAc-t-Ala-t-Glu), 0.1 mM meso-[¹⁴C]Apm (500 Bq), 0.2 mM UDP-MurNAc-t-Ala-t-Glu, and crude enzyme (1 to 125 µg of protein, depending on overexpression factor) in a final volume of 100 µl.

In both cases, mixtures were incubated at 37°C for 30 min, and reactions were stopped by the addition of 10 µl of acetic acid. Reaction products were separated by high-voltage electrophoresis on Schleicher & Schuell 3469 paper in 2% formic acid (pH 1.9) for 45 min at 40 V cm⁻¹ by using an LT36 apparatus (Savant Instruments). The radioactive spots corresponding to substrate and reaction products were detected by overnight autoradiography with type R2 films (3M, St. Paul, Minn.) or with a radioactivity scanner (Multi-Tracermaster LB285; Bert- hold France, Elancourt, France). The spots were cut out and counted in an Intertechnique SL30 liquid scintillation spectrophotometer with a solvent system consisting of 2 ml of water and 13 ml of Aquasol mixture (J.T. Baker Chemicals, Deventer, The Netherlands). One unit of enzyme activity was defined as the amount which catalyzed the synthesis of 1 µmol of UDP-MurNAc-t-Glu in 1 min.

Chemicals. The preparation of UDP-MurNAc-peptides and *meso*-Apm was previously described (9, 33). UDP-MurNAc-t-Ala-t-[¹⁴C]Glu was synthesized as described earlier (21) by using purified UDP-MurNAc-t-Ala-t-Glu ligase (2), and *meso*-¹⁴C]Apm was purchased from the CEA (Saclay, France). IPTG was obtained from Eurogentec (Seraing, Belgium). Lyszyme was from Sigma, and cellosyl was a gift from Hoechst Marie Roussel.

RESULTS AND DISCUSSION

Effect of overexpression of *S. aureus* murE in *E. coli* on cell survival. When the expression of the *S. aureus* murE gene was induced with IPTG in *E. coli* cells carrying the pMuSa2 plasmid, abnormal morphological changes of cell shape and size rapidly occurred, which were followed ca. 1 h later by an arrest of growth and finally by cell lysis (Fig. 1). Gram staining of the induced cells 2 h after induction revealed almost all cells to be lysed (data not shown), suggesting defective or greatly altered cell wall peptidoglycan biosynthesis. SDS-PAGE analysis of crude cell extracts showed that induced cells had greatly accumulated the MurE protein (Fig. 2). The latter was found in both the soluble and particulate fractions (Fig. 2) due to the formation of aggregates at such a high level of expression (inclusion bodies were effectively observed in induced cells by optical microscopy). Appropriate enzymatic assays confirmed the expression of the *l*-lysine-adding enzyme (*S. aureus* MurE) in pMuSa2 harboring cells (Table 1). A low but detectable activity observed in the absence of IPTG was due to a basal expression from the plasmid pMuSa2 since this activity was not detected in BL21(DE3) pLysS control cells (data not shown).

FIG. 1. Lytic effect of the expression of the *S. aureus* murE gene in *E. coli* cells. Cells of *BL21(DE3)* pLysS::pMuSa2 were grown exponentially at 37°C in 2YT-ampicillin medium. At the time indicated by the arrow (OD = 0.4), IPTG was added at a final concentration of 1 mM. Growth of cells induced (○) or not induced (●) with IPTG was monitored at 600 nm.
FIG. 2. Overproduction of *S. aureus* MurE enzyme in *E. coli* cells. Cells of BL21(DE3)pLysS/pMuSa2 were grown and induced for 1 h with IPTG as described in the legend to Fig. 1. Cells were harvested at an OD of 0.8 and were disrupted by sonication. The protein contents from both soluble and membrane fractions obtained after high-speed centrifugation of the crude extracts were analyzed by SDS-PAGE. Molecular weight standards (in thousands) indicated on the left are as follows: phosphorylase b, 94; bovine serum albumin, 67; ovalbumin 43; carbonic anhydrase, 30; and soybean trypsin. 20. Lanes: A and B, analysis of the soluble fractions from noninduced and IPTG-induced cells, respectively. C and D, analysis of the membrane fractions from noninduced and IPTG-induced cells, respectively. The arrow points to the overproduced *S. aureus* MurE enzyme.

The specific activity of the 1-lysine-adding enzyme was increased by a factor of 330 after IPTG induction, while that of the meso-A2pm-adding enzyme (*E. coli* MurE) was similar in noninduced and induced cells (Table 1). The ratio of *S. aureus* to *E. coli* MurE enzyme activities varied from 0.033 to 11 upon induction with IPTG.

**Effects of the expression of the *S. aureus* MurE enzyme on peptidoglycan metabolism.** Cells of BL21(DE3)pLysS/pMuSa2 induced with IPTG were harvested just before the first effects on cell growth were observed, and their peptidoglycan was extracted and quantified. In induced cells the peptidoglycan content was 28% lower than in noninduced cells (Table 1), suggesting that dysfunctioning of one (or more) step(s) in the pathway had occurred after overproduction of the *S. aureus* enzyme. The most likely explanation was a toxic recruitment to *S. aureus* noninduced and induced cells (Table 1). The pool level (for peptidoglycan precursors and peptidoglycan) is given in Table 1. The pool level of UDP-GlcNAc was 370, whereas the specific activity of UDP-MurNAc-tripeptide(lysine) was detected in induced cells at a very low concentration (at most a few nanomoles per gram of bacterial dry weight), suggesting that this compound was efficiently utilized by the enzyme MurF, which catalyzes the subsequent step of addition of d-alanyl-d-alanine in the pathway (23, 32).

**Incorporation of lysine into peptidoglycan.** To determine whether lysine was eventually incorporated at the place of A2pm in the macromolecule, peptidoglycan preparations were first made free of all traces of covalently associated proteins by successive treatments with proteases. Analyses showed that the material purified from noninduced cells contained only peptidoglycan constituents: muramic acid, glucosamine, alanine, glutamic acid, A2pm, and lysine in a ratio of 1/2.2/1/0.95/0.1, respectively. It was earlier established that some A2pm residues from *E. coli* peptidoglycan were covalently linked to C-terminal lysine residues of outer-membrane lipoprotein (4). Since these A2pm-lysine links (α-carboxyl-ε-amino amide bond) are not cleaved by proteases, the 10% of lysine found in the peptidoglycan purified from noninduced cells could consist of these residues but might also consist of lysine which had effectively replaced A2pm in the peptide chains. The latter was likely as it was shown above that a basal expression from pMuSa2 plasmid resulted in a small synthesis of lysine-containing peptidoglycan precursors. A similar analysis performed on the peptidoglycan from induced cells gave for the same constituents the following relative abundances: 1/1.7/1/0.51/0.6. It showed that a large incorporation of lysine had occurred in the macromolecule, half of A2pm residues in cell-wall peptidoglycan being now replaced by lysine.

**HPLC analysis of peptidoglycan structure.** The purified peptidoglycan preparations were subjected to prolonged digestion with specific N-acetylmuramidases, leading to the breakdown of glycan strands into monomer, dimer, and trimer fragments that could be separated by HPLC after reduction with NaBH4 (10, 11). The main monomer (tetra) and dimer (tetra-tetra), as well as the less-abundant monomer (tri), encountered in the solubilized material from noninduced cells were those

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**TABLE 1. Pool levels of peptidoglycan precursors, peptidoglycan, and specific activities of MurE enzymes in *E. coli* cells harboring the pMuSa2 plasmid**

<table>
<thead>
<tr>
<th>Peptidoglycan precursor, peptidoglycan, or enzyme</th>
<th>Pool level or sp act<strong>a</strong> in:</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Noninduced cells</td>
<td>Induced cells</td>
</tr>
<tr>
<td>Peptidoglycan precursors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDP-GlcNAc</td>
<td>160</td>
<td>370</td>
</tr>
<tr>
<td>UDP-MurNac</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>UDP-MurNAc-pentapeptide(A2pm)</td>
<td>1,230</td>
<td>400</td>
</tr>
<tr>
<td>UDP-MurNAc-pentapeptide(lysine)</td>
<td>150</td>
<td>1,710</td>
</tr>
<tr>
<td>Peptidoglycan<strong>b</strong></td>
<td>8,370</td>
<td>6,120</td>
</tr>
<tr>
<td>Enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2pm-adding enzyme</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>Lysine-adding enzyme</td>
<td>0.07</td>
<td>23.4</td>
</tr>
</tbody>
</table>

**a** Cells of BL21(DE3)pLysS(pMuSa2) were induced or noninduced with 1 mM IPTG for 1 h, as described in Materials and Methods. In each case, all of the parameters were tested from samples of the same culture.

**b** The pool level (for peptidoglycan precursors and peptidoglycan) is given in nanomoles per gram (dry weight) of bacteria. The specific activity (enzymes) is given in units per milligram of protein.

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(A2pm) is known to be very low in *E. coli* (19, 22). UDP-MurNAc-tripeptide(lysine) was detected in induced cells at a very low concentration (at most a few nanomoles per gram of bacterial dry weight), suggesting that this compound was efficiently utilized by the enzyme MurF, which catalyzes the subsequent step of addition of d-alanyl-d-alanine in the pathway (23, 32).
classically detected during analyses of peptidoglycan from wild-type E. coli cells (Fig. 3 and 4) (11, 18, 21). The nature of the compound in each peak was confirmed by analysis of its amino acid and hexosamine contents after acid hydrolysis (data shown in the legend to Fig. 4). Small additional peaks observed on the elution profile were identified as tri, tetra, and tetra-tetra fragments in which A2pm was replaced by lysine. As shown in Fig. 3, the retention time of these compounds was significantly higher than that of their A2pm counterparts, due to a great difference of polarity between lysine and A2pm residues. When the peptidoglycan from induced cells was analyzed in this way, the main difference was the large increase of the three peaks corresponding to lysine-containing monomers (tri and tetra) and dimer (tetra-tetra) (Fig. 3 and 4). Analysis of the latter dimer showed that it contained equimolar amounts of A2pm and lysine, and dinitrophenylation experiments further indicated that the ε-amino group of lysine was free (Fig. 4). This demonstrated that lysine was restricted to the donor unit in this dimer (designated DLA in Fig. 3 and 4) and that cross-linking was thus supported by A2pm. No other peaks of significant importance were observed in the elution profile that could consist of a hetero-dimer with lysine in the acceptor unit or a dimer containing exclusively lysine, suggesting that no transpeptidation could occur between the ε-amino group of lysine and the α-carboxyl group of D-alanine. However, a possibility exists that such dimers were formed but were too poorly represented to be detected by the technique employed here. It was noteworthy that the overall cross-linking of the macromolecule (as defined by the following ratio: Σ dimers/ [Σ monomers + 2 × Σ dimers] [reference 10]) was not significantly modified (Table 2).

Conclusions. Some bacteria contain meso-A2pm and others lysine at the third position of the peptide side chain in cell wall peptidoglycan (30). In each case, the MurE enzymes efficiently discriminate between the two amino acids in vitro, since they are only able to catalyze the addition of either meso-A2pm or lysine to UDP-MurNAc-L-Ala-D-Glu (13, 14, 18). As these two amino acids effectively coexist in bacterial cells (27), the high specificities of the MurE enzymes act as gatekeepers to ensure that only the specific substrate is incorporated in the peptidoglycan precursor. However, this specificity is not absolute since other A2pm analogs (lantionine, cystathionine, 3-hydroxy-A2pm, and dianinosuberic acid) were earlier shown to complement a A2pm auxotrophic strain and to totally replace meso-A2pm in E. coli peptidoglycan (7, 18). Enzymes catalyzing subsequent steps, from cytoplasmic synthetase MurF to membrane transglycosylases, are clearly less selective enzymes since they can accept a broader range of substrates (1, 12, 14, 31). In fact, the critical step after the incorporation of analogs of A2pm into E. coli peptidoglycan always appeared to be the final stage of transpeptidation, in which A2pm is directly in-
volved by its free amino group (15, 18, 21, 28). We observed that the pool levels of lysine-containing precursors (UDP-MurNAc-tripeptide and UDP-MurNAc-pentapeptide) in E. coli expressing S. aureus murE were quite similar to those of their Apm analogs in cells not expressing the staphylococcal enzyme. This suggested that in vivo the replacement of Apm by lysine in these precursors had little effect on their immediate subsequent use in the formation of peptidoglycan lipid intermediates. As demonstrated earlier with in vitro assays, the MurF, MraY, and MurG enzymes which catalyze these reactions utilized these alternative substrates with comparable kinetic parameters and lysis (7), the penicillin-sensitive transpeptidation pathway if the appropriate MurE ligase is supplied, but it is unable to fulfill the final essential role of Apm to ensure peptidoglycan cross-linking. This explains the toxic effect of a large incorporation of this amino acid in the macromolecule. Most likely a lower expression of S. aureus murE gene (a reduced level of lysine incorporated) could be tolerated by E. coli cells, and there is probably a critical ratio between Apm and lysine that is compatible with cell integrity.

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