

Peptidoglycan Hydrolases of *Escherichia coli*

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INTRODUCTION	637
<i>E. COLI</i> PEPTIDOGLYCAN GLYCOSIDASES	637
<i>N</i> -Acetylglucosaminidase	637
Lytic Transglycosylases	638
Slt70 lytic transglycosylase	638
MltA lytic transglycosylase	639
MltB lytic transglycosylase	639
MltC lytic transglycosylase	640
MltD lytic transglycosylase	640
MltE lytic transglycosylase	640
MltF lytic transglycosylase	641
Enzymatic properties of <i>E. coli</i> LTs	642
<i>E. COLI</i> PEPTIDOGLYCAN PEPTIDASES	642
Penicillin-Binding Peptidases	642
DD-Peptidase PBP4	643
DD-Carboxypeptidase PBP4b	643
DD-Carboxypeptidase PBP5	643
DD-Carboxypeptidase PBP6	643
DD-Carboxypeptidase PBP6b	644
DD-Peptidase AmpH	644
DD-Endopeptidase PBP7/PBP8	644
Penicillin-Insensitive Peptidases	644
DD-Endopeptidase MepA	644
EnvC endopeptidase	645
LD-Endopeptidase releasing D-alanyl-D-alanine from UDP-MurNAc-pentapeptide	645
LD-Carboxypeptidases cleaving the tetrapeptide (L)- <i>meso</i> -A ₂ pm→D-Ala bond	645
MpaA amidase cleaving the γ-D-Glu→(L)- <i>meso</i> -A ₂ pm bond	646
L-Alanyl-D/L-glutamate dipeptide epimerase	646
DdpX dipeptidase cleaving D-alanyl-D-alanine	646
Enzymatic properties of <i>E. coli</i> PG peptidases	646
<i>E. COLI</i> N-ACETYLMURAMOYL-L-ALANINE AMIDASES	647
AmiA, AmiB, and AmiC <i>N</i> -Acetylmuramoyl-L-Alanine Amidases	647
AmpD Anhydro- <i>N</i> -Acetylmuramoyl-L-Alanine Amidase	648
AmiD Anhydro- <i>N</i> -Acetylmuramoyl-L-Alanine Amidase	648
39-Kilodalton Amidase Cleaving MurNAc-Triptide	648
Enzymatic Properties of the <i>E. coli</i> Amidases	648
HYDROLASE-DEFECTIVE MUTANTS	649
Multiple Deletions of the LTs	649
Multiple Deletions of the LMM PBPs	650
Multiple Deletions of the <i>N</i> -Acetylmuramoyl-L-Alanine Amidases	650
Multiple Deletions of Hydrolases of Different Enzymatic Specificities	650
HYDROLASES AND PEPTIDOGLYCAN MATURATION	650
Tetrapeptide Subunits	650
Triptide Subunits	651
Dipeptide Subunits	652
1,6-Anhydro- <i>N</i> -Acetylmuramic Acid	652
<i>N</i> -Acetylmuramoyl-L-Alanine Linkage	652
HYDROLASES AND PEPTIDOGLYCAN TURNOVER	652
HYDROLASES AND LATERAL WALL PEPTIDOGLYCAN	653
HYDROLASES AND SEPTAL PEPTIDOGLYCAN	653
HYDROLASES AND PEPTIDOGLYCAN RECYCLING	654

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HYDROLASES AND AUTOLYSIS654
Autolysis of Growing Cells by Inhibition of PG Synthesis654
Autolysis of Growing Cells by Depletion of the Lipid II Pool.....654
Autolysis of Growing Cells by Inhibition of PG Polymerization.....655
Morphological Changes Accompanying Autolysis of Growing Cells655
Resistance of Nongrowing Cells to Autolysis.....655
Autolysis of Harvested Cells.....656
Inhibitory Effect of Mg²⁺ Ions on Autolysis656
CONCLUDING REMARKS.....656
ACKNOWLEDGMENTS657
REFERENCES657

INTRODUCTION

Peptidoglycan (PG) (or murein) is a continuous covalent heteropolymer extending on the outside the cytoplasmic membrane of most eubacteria (194, 220, 232, 294). Its main functions are to preserve cell integrity by withstanding internal osmotic pressure and to maintain the shape of the bacterial cell. PG has been extensively studied owing to its importance as an essential structural cell wall component, its involvement in cellular morphogenesis, and the fact that steps of its biosynthesis are specific targets for well-known antibiotics and potential ones for novel antibacterials (71).

PG consists of linear glycan chains, which contain alternating β-1,4-linked units of GlcNAc and MurNAc-peptide and which are interconnected by the cross-linking of their peptide subunits (Fig. 1). Its biosynthesis is a two-stage process. First, the PG monomer unit is assembled in the form of a lipid intermediate by enzymes located in the cytoplasm or at the inner side of the cytoplasmic membrane (7, 26, 286, 288). In the second stage, extracytoplasmic glycosyltransferases catalyze the formation of the glycan chains using the lipid intermediate as a substrate (76, 229, 287). Finally, cross-linking between peptide subunits by transpeptidases leads to the PG network (77, 229).

Besides the enzymes of the PG biosynthesis pathway, eubacteria generally harbor a more or less important set of endogenous hydrolases that specifically cleave PG linkages (238, 295). A hydrolytic activity has been identified for each bond linking the PG amino acid and sugar components. It has long been speculated that PG hydrolases are involved in many different aspects of PG metabolism and bacterial cell physiology (238, 240, 295). In particular, the uncontrolled action of PG hydrolases operating as autolysins plays a major role in the bacterial autolysis observable under various conditions (215).

The discovery of PG hydrolases in *Escherichia coli* paralleled the structural characterization of its cell wall PG (304). Initially, they were elicited by incubating hydrolase-containing cell extracts with PG muropeptides and analyzing the reaction products (198, 199). Later, isolated radiolabeled PG (sacculi) was used as substrate (89). Another approach entailed the use of the zymogram method initially developed for Gram-positive organisms (137) and later adapted to Gram-negative organisms (16). Nine PG hydrolases from *E. coli* were visualized in this way. Finally, sequence comparisons by database analysis became a powerful way to identify new hydrolases. Data on the *E. coli* PG hydrolases have concerned mainly but not exclusively strain K-12.

In *E. coli*, at least 35 PG hydrolases have been identified and

classified into 12 distinct families (Table 1). As in many other eubacteria, they include mainly glycosidases, peptidases, and *N*-acetylmuramoyl-L-alanine amidases. So far, only the PG hydrolases of *Bacillus subtilis* have been studied to a similar extent (240). The multiplicity and redundancy of the *E. coli* hydrolases raise the question of their respective functions in various aspects of PG metabolism. Work up to 1995 has been reviewed (99, 100, 102). More recent reviews have concerned specific hydrolase families (72, 73, 92, 103, 185, 196, 230, 295).

The aim of the present work is to bring together the past and present abundant information on the PG hydrolases of *E. coli* and to attempt a critical assessment of their functions in PG maturation, turnover, elongation, septation, and recycling as well as in cell autolysis. Except for a few examples, comparisons with the abundant literature concerning PG hydrolases of other organisms were not attempted. The present review should be helpful for developing future comparative studies.

***E. COLI* PEPTIDOGLYCAN GLYCOSIDASES**

In *E. coli*, several glycosidases cleave the GlcNAc-(1→4)-MurNAc (β-*N*-acetylglucosaminidase) or MurNAc-(1→4)-GlcNAc (*N*-acetylmuramidase) bonds of PG glycan chains (Fig. 2). In contrast with other bacterial organisms, no *N*-deacetylases acting on the GlcNAc or MurNAc units have been reported for *E. coli* (296). However, an *N*-acetylglucosamine-6-phosphate deacetylase acting on GlcNAc-6-phosphate (306) and a D-lactyletherase acting on MurNAc-6-phosphate (119) were characterized.

***N*-Acetylglucosaminidase**

A β-*N*-acetylglucosaminidase removing GlcNAc from the disaccharide-tripeptide muropeptide (Fig. 2) was initially identified in a cell extract from *E. coli* (198). Using monomer and dimer muropeptides or *p*-nitrophenyl-β-*N*-acetylglucosaminide as a substrate, it was purified as a cytoplasmic enzyme (155, 310). An activity-deficient mutant showed normal growth and morphology (311). Later, the encoding *nagZ* gene was identified (37), and an overproduced recombinant form was purified (298). A null mutant completely lacked NagZ activity, thereby establishing that NagZ was the only β-*N*-acetylglucosaminidase present in *E. coli* (37). The findings that this mutant grew normally in rich medium and had normal morphology proved that NagZ is not essential for cell growth. However, it is required for the PG recycling process (196). NagZ is specific for the β linkage and is active on monomer and dimer muropeptides (155, 310) but not on the tetrasaccharide muropeptide

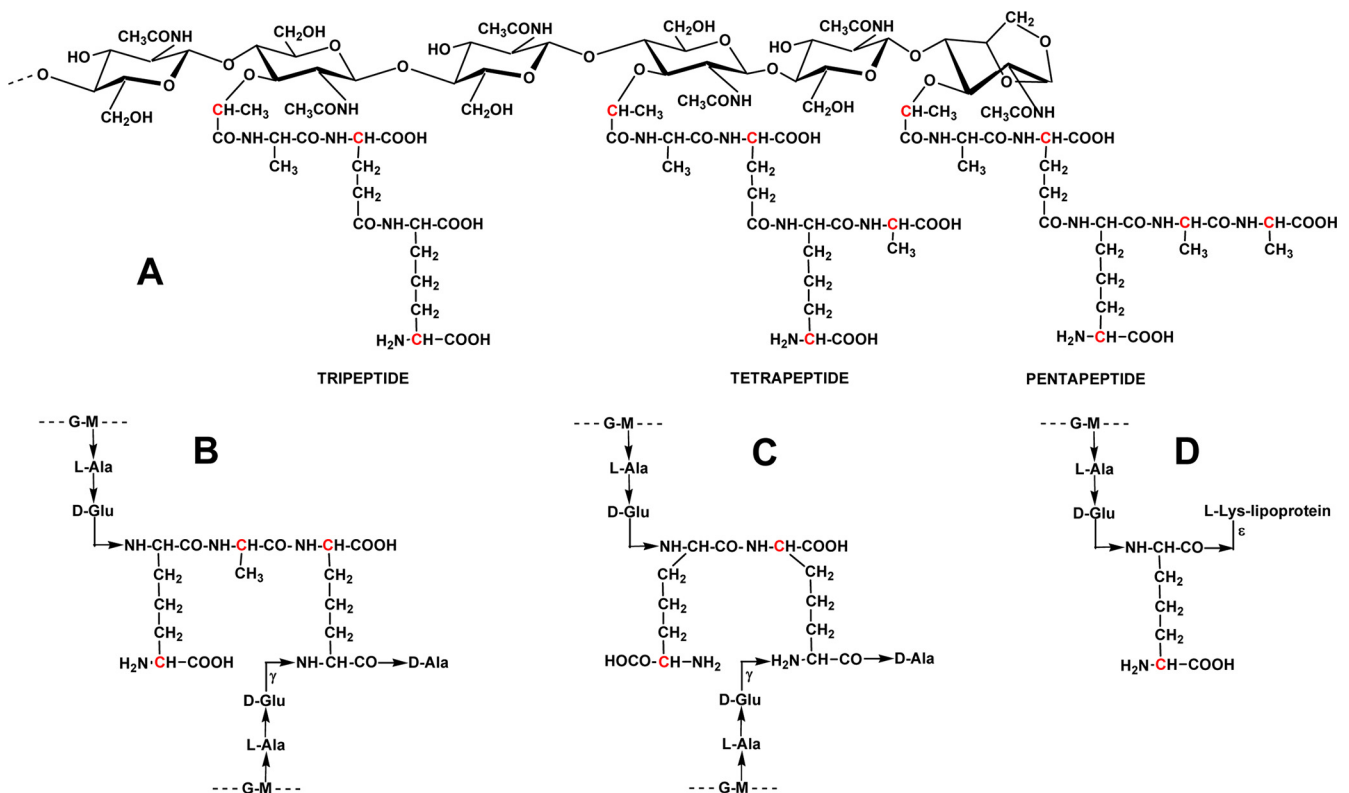


FIG. 1. Structure of *E. coli* peptidoglycan. (A) Glycan chain with monomer peptide subunits. (B) PG dimer with the D-Ala \rightarrow (D)-*meso*-A₂pm cross-linkage. (C) PG dimer with the (L)-*meso*-A₂pm \rightarrow (D)-*meso*-A₂pm cross-linkage. (D) Tripeptide subunit with linked Lpp lipoprotein. Abbreviations: G, *N*-acetyl- β -D-glucosamine (GlcNAc); M, *N*-acetyl- β -D-muramic acid (MurNAc); red C, carbon of the D-configuration; \rightarrow , CO-NH linkage.

(155). It cleaves the GlcNAc-(1 \rightarrow 4)-1,6-anhydro-MurNAc disaccharide (271). Its cytoplasmic location excludes any *in vivo* effect on PG, but NagZ releases GlcNAc from isolated radiolabeled PG, presumably by acting as an exoenzyme at the nonreducing end of the glycan chains (310).

Lytic Transglycosylases

Lytic transglycosylases (LTs) are *N*-acetylmuramidases that not only cleave the β -1,4 glycosidic bond between the MurNAc and GlcNAc units but also concomitantly catalyze an intramolecular glycosyl transfer reaction whereby a 1,6-anhydro bond is formed between C₁ and C₆ of *N*-acetylmuramic acid (Fig. 2 and see Fig. 4). The LT activity producing 1,6-anhydro-MurNAc mucopeptides was initially characterized with phage λ and phage ViII endolysins (253, 254). The first *E. coli* LT was designated Slt70, for soluble lytic transglycosylase (98). Subsequently, six other identified *E. coli* LTs were designated MltA, MltB, MltC, MltD, MltE, and MltF, for membrane-bound lytic transglycosylases (Table 1). LTs were found in many other organisms (230, 295) and were classified into four families on the basis of consensus sequence motifs (23). Slt70, MltC, MltD, and MltE belong to family 1, which are homologous to goose-type lysozyme, whereas MltA and MltB are classified as belonging to families 2 and 3, respectively. Phage λ endolysin belongs to the fourth LT family.

Slt70 lytic transglycosylase. Slt70 was identified in the soluble fraction of disrupted cells by its action on isolated diaminopimelic acid (A₂pm)-radiolabeled PG yielding 1,6-anhydro-MurNAc mucopeptides and was purified (98, 135). By immunoelectron microscopy, it was shown to be localized mainly in the cell wall, with only 20 to 30% remaining in the cytoplasm. Tight binding to the outer surface of isolated sacculi was demonstrated as well (299). The sequence of the structural *slt* gene (21) codes for a polypeptide with a typical signal sequence cleaved off during maturation, confirming a periplasmic localization (66). The deletion of *slt* led to no changes in growth, cell morphology, or PG mucopeptide composition but had an effect on the response toward various β -lactams (255). Similarly, bulgecin A, which reversibly and noncompetitively inhibits isolated Slt70, also modified the response to β -lactams. Growth inhibition without extensive lysis was observed with overproduced Slt70 (66). However, the inhibition of protein synthesis induced the rapid autolysis of the overproducing strains (21). Affinity chromatography studies (223, 297) revealed protein-protein interactions between Slt70 and different penicillin-binding proteins (PBPs) (PBP2, PBP3, and PBP7/8), suggesting a possible participation of Slt70 in multienzyme complexes.

The structures of native Slt70 and its complex with a 1,6-anhydro-MurNAc mucopeptide were solved at 1.65-Å and 1.90-Å resolutions, respectively (257, 258, 276). Slt70 has α -he-

TABLE 1. *E. coli* peptidoglycan hydrolases^a

Hydrolase	Gene	Localization	3D structure reference	Substrate(s)
β -N-Acetylglucosaminidase NagZ	<i>nagZ</i>	C		SC + PG
Lytic transglycosylases				
Slt70	<i>slt</i>	P	257	PG
MltA	<i>mltA</i>	OM	289	SC + PG
MltB	<i>mltB</i>	OM	277	SC + PG
MltC	<i>mltC</i>	OM		PG
MltD	<i>mltD</i>	OM	9	PG
MltE	<i>mltE</i>	OM	3	SC + PG
MltF	<i>yfhD</i>	OM		PG
DD-Carboxypeptidases				
PBP4b	<i>yfeW</i>	CM		SC
PBP5	<i>dacA</i>	CM	46	SC + PG
PBP6	<i>dacC</i>	CM	36	SC
PBP6b	<i>dacD</i>	CM		PG
DD-Endopeptidases				
PBP7	<i>pbpG</i>	P		PG
MepA	<i>mepA</i>	P	161	SC + PG
EnvC	<i>envC</i>	CM/P		
DD-Peptidases				
PBP4	<i>dacB</i>	P	125	SC + PG
AmpH	<i>ampH</i>	CM		SC + PG
LD-Carboxypeptidases				
LdcA	<i>ldcA</i>	C		SC
12 kDa				SC
86 kDa		P		SC
LD-Transpeptidases				
ErfK	<i>erfK</i>	P		PG
YbiS	<i>ybiS</i>	P		PG
YcbB	<i>ycbB</i>	P		PG
YcfS	<i>ycfS</i>	P		PG
YnhG	<i>ynhG</i>	P		PG
LD-Endopeptidase, 34 kDa				
				SC
Endoamidase MpaA				
	<i>mpaA</i>	C		SC
MurNAc-L-Ala amidases				
AmiA	<i>amiA</i>	P		PG
AmiB	<i>amiB</i>	P		PG
AmiC	<i>amiC</i>	P		PG
AmpD	<i>ampD</i>	C	147	SC
AmiD	<i>amiD</i>	OM	124	SC + PG
39 kDa		P		SC
L-Ala-D/L-Glu epimerase				
YcjG	<i>ycjG</i>	C	84	SC
D-Ala-D-Ala dipeptidase				
DdpX	<i>ddpX</i>	C	144	SC

^a DD-Peptidases show both DD-carboxypeptidase and DD-endopeptidase activities. C, cytoplasm; CM, cytoplasmic membrane; P, periplasm; OM, outer membrane; SC, soluble component (precursor, muropeptide, peptide, or glycan chain); PG, isolated PG.

lices assembled into three distinct domains, generating a doughnut-like shape (Fig. 3a). The N-terminal and central domains form a closed ring with a large central hole. The catalytic site is located in the C-terminal domain and shows close structural similarity to that of goose-type lysozyme (258). It has a bilobal structure with a long substrate-binding groove and a catalytic acid/base glutamic acid. The crystal structure of Slt70 complexed with a 1,6-anhydro-MurNAc muropeptide showed that the recognition site for the peptide moiety is located at the interface of the three domains (276). The structure of Slt70 complexed with bulgecin A was determined at a

2.8-Å resolution and revealed the binding of one inhibitor molecule to the active site of the enzyme (259).

MltA lytic transglycosylase. In an *slt* deletion mutant, an LT was detected in the membrane fraction, solubilized with detergents, and purified (221, 275). The membrane enzyme differed from Slt70 by accepting soluble peptide-free poly-[GlcNAc-(1→4)-MurNAc] glycan chains as substrate besides cross-linked PG (221, 275). Initially referred to as Mlt38 (275), it was redesignated MltA (64), and its encoding gene was identified (149). Surprisingly, MltA shares no region of sequence similarity with Slt70, MltB, or MltC. It contains a typical lipoprotein consensus sequence. Its lipoprotein nature was demonstrated by [³H]palmitate labeling, and its localization in the outer membrane was established by membrane fractionation. In an MltA deletion mutant, growth was unaffected, and no change of morphology was observed (149). In contrast, the overproduction of MltA resulted in the formation of spheroplasts and lysis at 30°C but not at 37°C, which is consistent with the purified enzyme having a temperature optimum at 30°C (149). Affinity chromatography experiments using purified proteins revealed protein-protein interactions with PBPs and periplasmic proteins (293). In particular, the periplasmic protein MipA mediates the MltA-PBP1b interaction, suggesting the participation of MltA in a PG-synthesizing complex.

The crystal structure of a functional soluble form of MltA lacking its membrane anchor was determined at a 2.0-Å resolution (289). It revealed two domains separated by a large groove (Fig. 3b), which can accommodate a six-residue-long glycan chain and which has a centrally located aspartic acid residue conserved in all known MltAs (206, 289). This first structure of a member of LT family 2 showed a fold different from those of the other known LTs. While the latter proteins share a catalytic core with a lysozyme-like fold, the catalytic domain of MltA contains a double-psi β-barrel fold similar to that of members of the barwin-like endoglucanase superfamily.

MltB lytic transglycosylase. Soluble LT activity was also detected in a strain carrying a chromosomal *slt* deletion. The LT was purified as a fully soluble 35-kDa enzyme distinct from Slt70 and was named Slt35 (67). The identified encoding gene was designated *mltB* (56, 64). The coding region displayed a consensus lipoprotein signal at the N-terminal end, and the presumed prolipoprotein form accumulated in the presence of globomycin (64). The soluble LT activity associated with MltB-overproducing cells increased with time as the result of proteolytic degradation. The lipoprotein character of MltB was proven by [³H]palmitate labeling, and the localization of the enzyme at the outer membrane was demonstrated (64). Two overproduced soluble forms of MltB were fully active (64, 249). In particular, one form was able to cleave the middle MurNAc-(1→4)-GlcNAc bond of a synthetic soluble tetrasaccharide muropeptide, yielding disaccharide muropeptides (249). An MltB deletion mutant showed no significant change in growth or in PG muropeptide composition, whereas the overproduction of MltB led to spherical cells and subsequent lysis (64).

The structure of the Slt35 soluble form of MltB was determined at a 1.7-Å resolution (277, 278) and revealed an ellipsoid molecule with three domains, called α, β, and core (Fig. 3c). The fold of the core domain resembles that of lysozyme and that of the catalytic domain of Slt70. The active site is located in a deep groove formed by residues from the α and

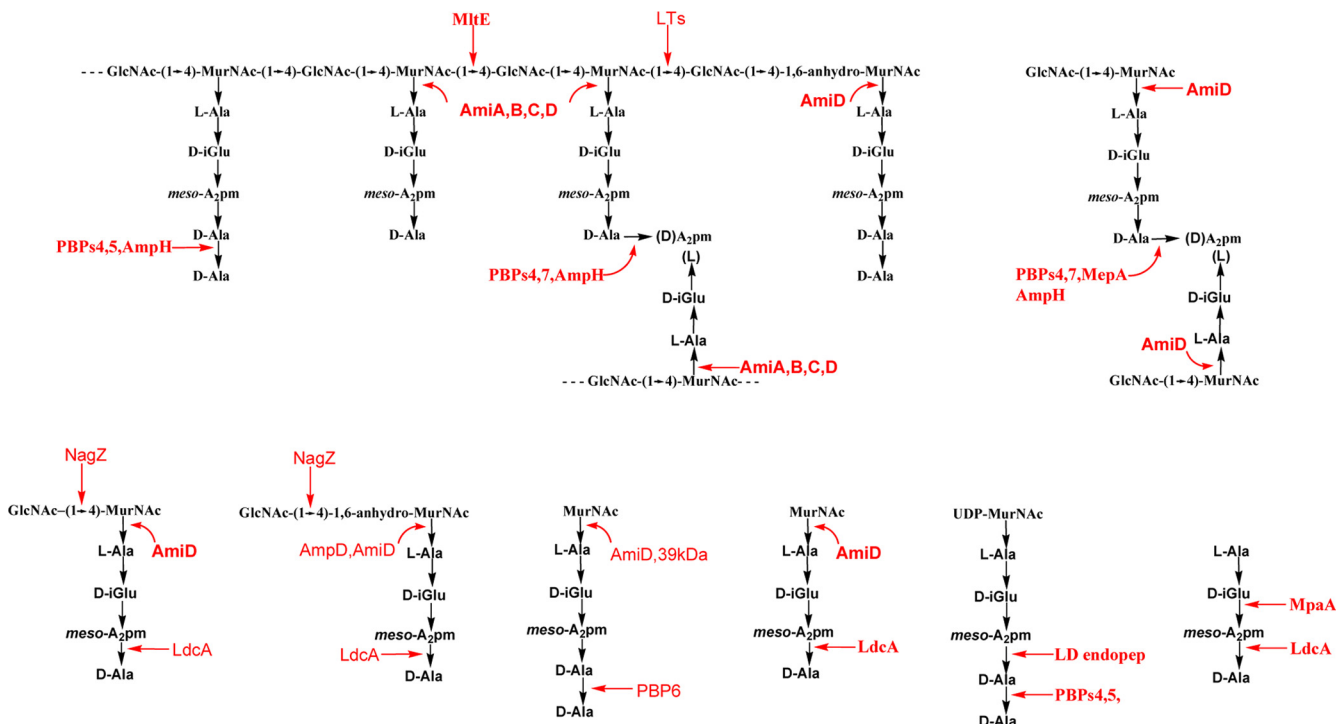


FIG. 2. Sites of action of hydrolases in PG, precursor, and mucopeptide.

core domains and has a catalytic acid/base glutamic acid. A study of Slt35 complexed with chitobiose, the mucopeptide GlcNAc-(1→4)-MurNAc-L-Ala-D-Glu, or bulgecin A revealed the location of four sugar-binding sites and two peptide-binding sites, all close to the catalytic glutamic acid residue (278).

MltC lytic transglycosylase. The search for LTs in the *Haemophilus influenzae* genome revealed an open reading frame having *slt*-derived fingerprints and a strong homology with an *E. coli* open reading frame at centisome 66.8 containing a putative consensus lipoprotein signal sequence (57). The newly discovered *E. coli* LT was designated MltC, and three forms were overproduced without lysis and were analyzed by zymography. The full-length sequence with the putative lipoprotein signal sequence as well as the one without it showed significant PG hydrolase activity. The form with only the C-terminal domain also had a high level of activity. All forms produced 1,6-anhydro-MurNAc mucopeptides from isolated PG (57).

MltD lytic transglycosylase. The sequence of the *E. coli yafG* open reading frame at centisome 5.1 contains the three *slt* fingerprints indicative of muramidase activity (57), and the gene was designated *mldD* (58). The N-terminal end of MltD displayed a lipoprotein signal and an LT domain, whereas the C-terminal end had two repeats of the LysM PG-binding motif (9, 58). The LysM domain is a widespread protein module, and there is evidence suggesting that it is a general PG-binding module. Often encountered in cell wall-degrading enzymes, it probably acts to anchor the catalytic domain to PG (9). The PG hydrolase activities of full-length MltD and of a recombinant form lacking the N-terminal signal peptide and the membrane-anchoring residues were revealed by zymography (58, 249). The overproduction of

MltD led to spheroplast formation and lysis (58).

Structural studies of MltD have been limited to its C-terminal domain containing the two LysM repeats (9). The peptide encompassing the two motifs was overproduced, and assignments were obtained by using a range of two-dimensional (2D) and three-dimensional (3D) heteronuclear nuclear magnetic resonance (NMR) experiments. The LysM domain has a $\beta\alpha\alpha\beta$ secondary structure, with the two helices packing onto the same side of an antiparallel β sheet. The structure showed no similarity to other bacterial cell surface domains. A shallow groove on the protein surface was identified as a potential binding site.

MltE lytic transglycosylase. An LT accepting unsubstituted PG glycan chains as a substrate was detected in membrane extracts from an *mldA* mutant and was named MltE. Partial purification led to a 22-kDa protein (132). In a second step, an open reading frame at centisome 26.7 was identified by using a motif common to Slt70 and MltC. Initially named *emtA*, for endo-type membrane-bound lytic transglycosylase (132), it was later redesignated *mldE* (92, 249). Its coding region started with a signal peptide followed by a lipoprotein-processing site. It was cloned, and the lipoprotein character of the *mldE*-encoded protein was demonstrated by [3 H]palmitate labeling (132). The PG hydrolase activity of MltE on isolated PG was revealed by zymography (132, 249). However, no products were released when isolated radiolabeled PG was used as a substrate (132). Membranes from cells overproducing MltE degraded isolated PG glycan chains having seven disaccharide units (132), and the specificity of an endotransglycosylase activity was revealed by the cleavage of internal glycosidic bonds, leading to shorter chains with 1,6-anhydro-MurNAc ends. The overproduction of

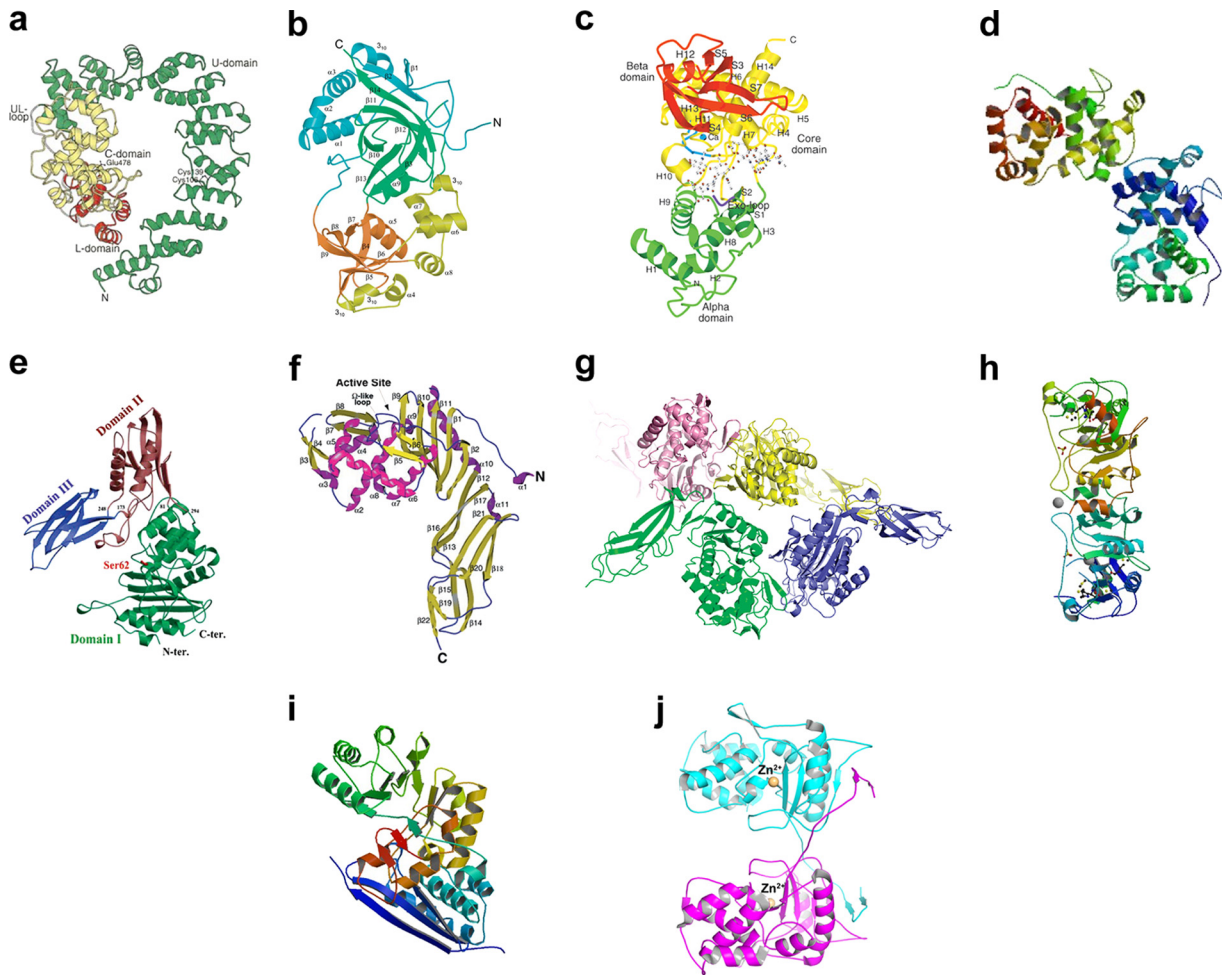


FIG. 3. Three-dimensional structures of 10 *E. coli* PG hydrolases. (a) Slt70. (Reprinted from reference 276 with permission of Elsevier.) (b) MltA. (Reprinted from reference 289 with permission of Elsevier.) (c) MltB. (Reprinted from reference 277 with permission of Elsevier.) (d) MltE. (Reprinted from reference 3 with permission from the publisher. Copyright 2011 American Chemical Society.) (e) PBP4. (Reprinted from reference 125 with permission from the publisher. Copyright 2006 American Chemical Society.) (f) PBP5. (Reprinted from reference 46 with permission of the publisher.) (g) PBP6. (Reprinted from reference 36 with permission from the publisher. Copyright 2009 American Chemical Society.) (h) MepA. (Reprinted from reference 161 with permission of the publisher.) (i) YcjG epimerase. (Reprinted from reference 84 with permission of the publisher. Copyright 2001 American Chemical Society.) (j) AmiD dimer. (Reprinted from reference 124 with permission of Elsevier.)

MltE led to a shortening of the PG glycan chains and to an increased (L)-*meso*-A₂pm→(D)-*meso*-A₂pm cross-linkage content. These changes occurred without rapid cell lysis (132).

The crystal structure of MltE was determined at a 2.0-Å resolution (3) and revealed a two-lobe ellipsoid shape (Fig. 3d). Its overall fold contains 10 α-helices and resembles that of goose-type lysozyme. The active site containing eight saccharide-binding subsites is sequestered in a deep groove spanning the two lobes and is similar to that of Slt70. The absence of a peptide-binding subsite, which allows processivity in the functioning of exolytic Slt70, explains the endolytic specificity of MltE.

MltF lytic transglycosylase. A potential LT was recognized in the sequence of the *yfhD* open reading frame of *E. coli* containing the three *slt* fingerprints indicative of muramidase activity (57). Based on sequence alignments and the presence of consensus motifs, the hypothetical YfhD protein was the

archetype of LT family 1B (23). It had a predicted cleavable signal sequence and a transmembrane helix. Experimental proof confirmed its LT activity, and it was named MltF (231). Full-length overproduced MltF was associated with the outer membrane (231). An overproduced soluble truncated form lacking the N-terminal signal sequence and the membrane anchor catalyzed the release of 1,6-anhydro-MurNAc muropeptides from isolated PG. The deletion of *mltF* led to no change in cell growth or morphology, whereas the overproduction of MltF had a lytic effect.

MltF appeared to be a bimodular structure composed of an N-terminal non-LT domain and a C-terminal LT domain (231). To confirm this modular structure, the *mltF* gene was engineered to encode each domain separately. The overproduced C-terminal domain retained full LT activity, whereas the N-terminal domain was devoid of PG-binding capacity and LT activity. A prediction of the 3D structures of the N-terminal

and C-terminal domains was made by using a protein fold recognition server.

Enzymatic properties of *E. coli* LTs. The seven identified *E. coli* LTs are all associated with the cell envelope and are presumably located close to their PG substrates. However, Slt70 is tightly bound to PG, whereas the six other LTs are bound to the outer membrane as lipoproteins (MltA to MltE) or by a membrane anchor (MltF). The seven LTs are active on isolated PG, but the release of 1,6-anhydro-disaccharide-peptide units was established only with Slt70, MltA, MltB, and MltF (67, 98, 231, 275). These four LTs are thus exoenzymes processively degrading PG glycan chains at one end. Unfortunately, it is not clear at which end degradation proceeds. The crystal structure of Slt70 suggests that binding occurs at the 1,6-anhydro-MurNAc end (276), whereas the kinetics of the release of mucopeptides from PG with specifically radiolabeled GlcNAc ends show that degradation proceeds at the nonreducing end of the glycan chain (12). Among the other specificity particularities, MltA accepts the peptide-free poly-(GlcNAc-1,4-MurNAc) glycan chains as substrate (275), and MltB is active on a short synthetic glycan fragment (249). MltE acts only with endo activity, cleaving internal MurNAc-(1→4)-GlcNAc bonds with the formation of shorter chains (132). The functioning of MltC as an LT is based on the release of 1,6-anhydro-MurNAc-containing mucopeptides (57), whereas that of MltD is based on sequence comparisons with other LTs (58). It should be stressed that the use of isolated PG as an insoluble substrate has not favored extensive studies of LT enzymatic properties.

Analysis of the crystal structures of Slt70, MltA, MltB, and MltE as well as inhibition studies led to the same two-step reaction mechanism (Fig. 4) valid for different LTs (3, 206, 213, 221, 230, 255, 258, 259, 276, 277, 278, 289, 290). The first step entails the cleavage of the glycosidic bond between MurNAc and GlcNAc by the action of a glutamic or aspartic acid residue functioning as a single-acid catalyst donating a proton. This results in the formation of an oxocarbenium ion-like transition state stabilized by the formation of an oxazolinium intermediate. In the second step of the reaction, the deprotonated catalytic residue acts as a general base to abstract a proton from the OH on C₆ of MurNAc, thereby allowing for an intramolecular nucleophilic attack at C₁, collapsing the oxazolinium intermediate with the concomitant formation of the 1,6-anhydro-MurNAc-peptide (230). Experimental evidence for this reaction mechanism was obtained by studies of the inhibitory effect of bulgecin A and *N*-acetylglucosamine thiazoline functioning on LTs as structural analogs of the putative oxazolinium intermediate (213, 221, 255, 258, 259). Site-directed mutagenesis confirmed the crucial role played by the glutamic or aspartic acid residue in the mechanism (213, 276, 289).

E. COLI PEPTIDOGLYCAN PEPTIDASES

In *E. coli*, there are at least 20 peptidases (Table 1) cleaving peptide bonds in isolated PG, mucopeptides, or precursors (Fig. 1 and 2). Their high enzymatic specificities are due to the presence of nonprotein amino acids (D-Ala, D-Glu, and *meso*-A₂pm) in the peptide subunits and to the unusual stereochemistry [DD, DL, LD, and γ -D-Glu-(L)-*meso*-A₂pm] of the peptide

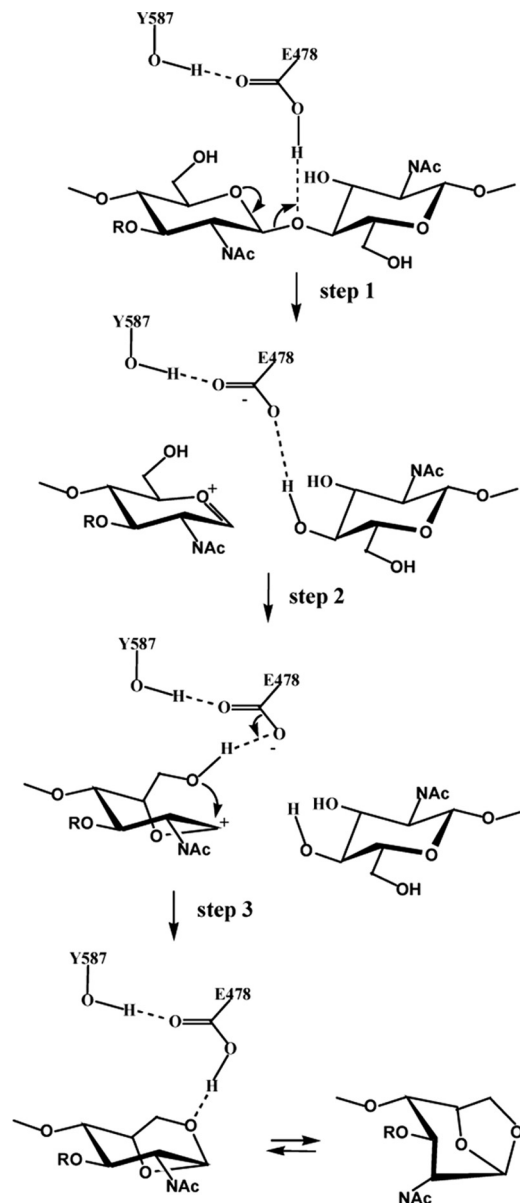


FIG. 4. Reaction mechanism of the Slt70 LT proposed by Thunnissen et al. (259). R, D-lactoyl peptide. Step 1 is the cleavage of the MurNAc-(1→4)-GlcNAc bond with the formation of an oxocarbenium ion intermediate. Step 2 is an intramolecular nucleophilic attack on the C₁ carbon of the oxocarbenium ion. Step 3 is the extraction of a proton by catalytic Glu478 and the formation of the 1,6-anhydro-MurNAc-peptide.

bonds (Fig. 1). The *E. coli* PG peptidases are either penicillin-binding or penicillin-insensitive enzymes.

Penicillin-Binding Peptidases

Penicillin-binding proteins (PBPs) are a family of bacterial acyl-serine transferases of common evolutionary origin specifically inhibited by the covalent binding of β -lactam antibiotics to their active sites (77, 156, 229, 312). *E. coli* PBPs are visualized by polyacrylamide gel electrophoresis after the treatment of cells with radiolabeled or fluorescence-labeled β -lac-

tams and are designated according to their electrophoretic migrations (6, 49, 72, 95, 103, 186, 243, 291, 315). Most PBPs are anchored to the cytoplasmic membrane with their active site in the periplasm (73). The five high-molecular-mass (HMM) PBPs (PBP1a, PBP1b, PBP1c, PBP2, and PBP3) catalyze the extracytoplasmic reactions of PG polymerization and are devoid of hydrolytic activity (76, 229). Among the eight low-molecular-mass (LMM) PBPs (PBP4, PBP4b, PBP5, PBP6, PBP6b, PBP7/8, AmpC, and AmpH), only the β -lactamase AmpC has no recognized DD-peptidase activity (95). The *E. coli* LMM PBPs belong to four subcategories of the class C subdivision of the PBP classification (229): type 4 (PBP4), type 5 (PBP5, PBP6, and PBP6b), type 7 (PBP7/8), and type AmpH (PBP4b and AmpH). The description of the LMM PBPs will follow the order of increasing electrophoretic mobility.

DD-Peptidase PBP4. The PBP4 amino acid sequence encoded by the *dacB* gene is similar to those of class C β -lactamases and other LMM PBPs (130, 181). PBP4 was purified from wild-type and overproducing strains (39, 130, 146, 182). The *dacB* gene encodes a signal peptide, which directs the protein to the periplasm and which is lacking in purified PBP4. Isolated without detergents, PBP4 behaves as a soluble protein. Its primary structure reveals no evidence of a transmembrane helix, an amphiphilic membrane anchor, or a lipid anchor (181). Nevertheless, PBP4 is associated with the membrane, as established by its presence in inner membrane vesicles (117, 143) and its purification from the membrane fraction (130). This behavior supports the view that PBP4 is bound to some membrane protein. Affinity chromatography experiments suggested a possible interaction with MltB (297).

PBP4 shows DD-endopeptidase activity (Fig. 2) by hydrolyzing the D-Ala \rightarrow (D)-*meso*-A₂pm bond in the soluble dimer mucopeptide (130) and in the dimer units of isolated PG (39, 130, 146). PBP4 is identical to the soluble and periplasmic forms of the D-alanine carboxypeptidase IB activity, cleaving the D-Ala \rightarrow D-Ala bond of the UDP-MurNAc-pentapeptide precursor and the cross-linkage of the dimer mucopeptide (111, 165, 252). PBP4 thus exhibits both DD-carboxypeptidase and DD-endopeptidase activities (Fig. 2).

The high-resolution (1.6-Å) structures of PBP4 and covalent complexes with different antibiotics are related to those of other PBPs and β -lactamases (125). The overall structure shows three distinct domains, with the C terminus ending close to the N terminus in domain 1 (Fig. 3e). More recently, a mutational analysis of the substrate specificity of PBP4 revealed the importance of a subsite in accommodating the *meso*-A₂pm residue of the UDP-MurNAc-pentapeptide substrate (39).

The loss of PBP4 fails to induce growth defects or detectable morphological alterations (49, 165, 170). The changes in PG structure accompanying PBP4 overproduction indicated that PBP4 acts *in vivo* as a DD-endopeptidase and a DD-carboxypeptidase but not as a DD-transpeptidase (130). Moreover, the decrease in D-Ala \rightarrow (D)-*meso*-A₂pm cross-linkage contents was partially compensated for by an increase in the (L)-*meso*-A₂pm \rightarrow (D)-*meso*-A₂pm cross-linkage content, possibly explaining the tolerance of *E. coli* for high-level PBP4 overproduction.

DD-Carboxypeptidase PBP4b. PBP4b was recognized as an LMM PBP DD-carboxypeptidase by a study of *E. coli* open

reading frame *yfeW*, encoding a 51-kDa polypeptide which had the three PBP motifs and strong homology with AmpH (291). Its N-terminal signal peptide serving for the anchoring to the cytoplasmic membrane had no putative signal peptidase sequence. An overproducing strain showed binding to radiolabeled β -lactam, as visualized by polyacrylamide gel electrophoresis. Overproduced PBP4b displayed DD-carboxypeptidase activity with N^α,N^ε-diacetyl-L-lysyl-D-alanyl-D-alanine. Gene inactivation demonstrated the nonessential character of PBP4b and led to viable mutants with unchanged morphologies. Interestingly, the *yfeW* gene encoding PBP4b is in an operon together with the *murP* and *murQ* genes, which are required for growth on MurNAc (118).

DD-Carboxypeptidase PBP5. As the major PBP of *E. coli* (60, 243), PBP5 has been extensively studied (72, 205, 280, 301). It was identified with D-alanine carboxypeptidase IA activity encoded by the *dacA* gene (30, 166, 191, 242). Its primary structure shows sequence similarity with PBP4, PBP6, and β -lactamases (30, 163). Synthesized by the removal of a hydrophobic N-terminal signal sequence, PBP5 localizes in the cytoplasmic membrane, with the bulk of the protein extending into the periplasm (208, 209). The 18-amino-acid-long amphiphilic helix at the C-terminal end is essential for tight anchoring to the membrane (114, 115), and the mechanism of this attachment has been discussed (187).

Initially, PBP5 was purified to homogeneity from wild-type *E. coli* by affinity chromatography (1). Various N- and C-terminally truncated forms were produced (68, 205, 280). The soluble and membrane-bound forms of PBP5 recognize the same range of substrates. They display a broad substrate specificity by catalyzing the cleavage of the C-terminal D-Ala \rightarrow D-Ala bond (Fig. 2) in many soluble substrates (UDP-MurNAc-pentapeptide, lipid II, muropentapeptides from different sources, pentapeptides, and synthetic compounds) and in uncross-linked and cross-linked PG (1, 188, 205, 246, 281). In cross-linked PG, PBP5 recognizes D-Ala \rightarrow D-Ala bonds in both the monomer and dimer subunits (205).

The crystal structure of a soluble form of PBP5 (Fig. 3f) revealed two distinct, roughly perpendicular domains (46, 189). The active site located in domain 1 showed high structural similarity with those of other PBPs and β -lactamases. Domain 2, which has a hydrophobic surface, is of unknown function but is essential for stabilizing the protein (280) and for the proper *in vivo* functioning of PBP5 (186).

PBP5-defective mutants (166, 191, 244) show normal cell growth and an increased pentapeptide subunit content (50, 134, 146, 226). This finding indicates that PBP5 is acting *in vivo* on PG. Morphological defects accompanying the loss of PBP5 are visible by microscopic inspection (185), and shape changes with slight abnormalities at the poles are detectable by fluorescence-activated cell sorting (170). The overproduction of PBP5 is lethal, causing *E. coli* to grow as spherical cells before lysing (162, 247). The proposed function of PBP5 is to regulate the availability of pentapeptide subunits for the formation of the cross-linkages by transpeptidation (48, 205).

DD-Carboxypeptidase PBP6. PBP6, encoded by *dacC*, has a high level of sequence homology with PBP5 (30). The cleavage of the signal sequence yields a mature protein anchored to the cytoplasmic membrane by its C-terminal amphiphilic helix, with the bulk of the protein extending into the periplasm (73,

208). PBP6 was purified to homogeneity at the same time and in the same way as PBP5 (1, 2). With UDP-MurNAc-pentapeptide as a substrate, it shows a much lower level of DD-carboxypeptidase activity than PBP5. A later report asserted that both native and overproduced soluble forms of PBP6 have no detectable DD-carboxypeptidase activity with the nucleotide substrate (279). However, a soluble form of PBP6 cleaves the D-Ala→D-Ala bond of MurNAc-pentapeptide (36).

The crystal structure of PBP6 lacking its signal peptide and membrane anchor was determined at a 2.1-Å resolution, while the structures of complexes with ampicillin and MurNAc-pentapeptide were determined at a 1.8 Å-resolution (36). Like PBP5, PBP6 has two perpendicular domains: a large N-terminal domain harboring the active site and a smaller β-sheet-rich C-terminal domain (Fig. 3g).

The deletion of PBP6 has no effect on cell growth (28, 49) and, unlike PBP5, leads to no increase of the PG pentapeptide subunit content (226). The overproduction of the native or soluble form is not bacteriolytic (279). The expression of *dacC* mRNA is far more dependent on the BolA transcriptional regulator than that of *dacA* mRNA (226). Despite homology, the marked differences between PBP5 and PBP6 point to distinct biological functions. Owing to its unusually high level in stationary-phase cells (31), PBP6 could play a role in the stabilization of PG at the end of the growth phase (279).

DD-Carboxypeptidase PBP6b. PBP6b was identified by a study of an *E. coli* open reading frame showing the capacity to encode a polypeptide with a predicted amino acid sequence strikingly similar to those of PBP5 and PBP6 (6). The gene was designated *dacD*. Its product bound penicillin and showed DD-carboxypeptidase activity *in vivo* on PG pentapeptide subunits. As with PBP5 and PBP6, PBP6b is associated with the cytoplasmic membrane by its C-terminal end, which has the capacity to form the amphiphilic α-helical secondary structure required for anchoring to the membrane (73). The *dacD* gene is expressed at a very low level, and PBP6b is not essential for cell growth (6).

DD-Peptidase AmpH. A PBP different from the previously known ones was observed in *E. coli* cells infected with λ phages from the Kohara genomic library (95). After cloning, the established sequence was identical to that of the open reading frame *yaiH* at centisome 8.5. Owing to its relationship to other *amp* genes of the class C family, the gene was named *ampH*. In wild-type *E. coli*, *ampH* is expressed at a low level, and its deletion leads to no morphological alterations. It is nonessential under normal laboratory conditions. However, morphological alterations are observable when an *ampH* deletion is associated with PBP1a and PBP5 deficiencies, indicating a role in PG metabolism (95).

Initially, AmpH had no recognized enzymatic activity (95). The recently reported cloning and purification of overproduced forms have enabled a better characterization of its enzymatic specificities (79). Like the other PBPs, AmpH shows a very low level of β-lactamase activity. On the other hand, it displays a wide spectrum of high-level DD-peptidase activities on monomer, dimer, and oligomer muropeptides as well as on isolated PG (Fig. 2). AmpH DD-carboxypeptidase activity hydrolyzes disaccharide-pentapeptide rapidly but the synthetic

N^{α},N^{ϵ} -diacetyl-L-lysyl-D-alanyl-D-alanine tripeptide slowly. Like PBP4, AmpH shows both DD-carboxypeptidase and DD-endopeptidase activities.

An inspection of the amino acid sequence revealed an N-terminal sequence peptide but no hydrophobic membrane-anchoring sequence (95). The mature overproduced 42-kDa form remained membrane associated and was solubilized by detergent (79). The removal of the signal peptide and the analysis of spheroplasts suggested a localization at the outer surface of the inner membrane. Like PBP4, the attachment of AmpH implies interactions with other membrane constituents.

DD-Endopeptidase PBP7/PBP8. The identification and cloning of the *pbpG* gene encoding PBP7 were key to an understanding of the initially confused status of PBP7 and PBP8 (94), characterized by the inconsistency of their appearance in PBP assays and by differences in their assignments (222). PBP8 is an artifact of sample preparation due to the proteolytic truncation of the C-terminal end of PBP7 by the protease OmpT (93). Together, PBP7 and PBP8 make up a significant fraction of the total PBP pool and are as abundant as PBP5 (60). PBP7 displays some sequence identity with PBP4, PBP5, and PBP6. PBP7 and PBP8 have a common N-terminal end and are processed in the same way as the other LMM PBPs (222). The amino acid sequence of PBP7 revealed no transmembrane helix, amphiphilic helix, or lipid anchor (94). PBP7 and PBP8 are loosely membrane-associated periplasmic proteins, since substantial amounts are released upon spheroplast formation or by osmotic shock and by salt treatment of a membrane fraction (222).

Purified PBP7 and PBP8 are DD-endopeptidases (Fig. 2) with a strict requirement for intact PG as a substrate. They both cleave D-alanyl→(D)-*meso*-A₂pm cross-linkages in isolated PG but cleave (L)-*meso*-A₂pm→(D)-*meso*-A₂pm cross-linkages poorly (222). Moreover, they have no activity on isolated dimer muropeptides, and they are devoid of any DD-carboxypeptidase activity (222). PBP7 and PBP8 thus differ from the DD-endopeptidases PBP4, AmpH, and MepA, which cleave the D-Ala→(D)-*meso*-A₂pm cross-linkage both in soluble dimer muropeptides and in isolated PG. These results emphasize the unusually narrow substrate specificity of PBP7/PBP8.

E. coli grows normally when *pbpG* is inactivated or overexpressed (94). A protein-protein interaction between PBP7/8 and Slt70 was demonstrated by affinity chromatography (223). Furthermore, PBP8 stabilizes and stimulates the activity of Slt70 *in vivo*. It was speculated that Slt70, PBP7/8, and PBP3 could form a multienzyme complex *in vivo* (223). PBP7 also plays a role in the penem-induced lysis of nongrowing cells (266).

Penicillin-Insensitive Peptidases

DD-Endopeptidase MepA. Early work on *E. coli* PBP DD-peptidases revealed the presence of a penicillin-insensitive peptidase degrading isolated PG and cleaving the D-Ala→(D)-*meso*-A₂pm cross-linkage of the dimer muropeptide (89, 252). Purification to homogeneity yielded a 30-kDa soluble enzyme, which bound no known β-lactam antibiotic and which remained fully active in their presence (122, 123, 260). It operated exclusively as an endopeptidase (Fig. 2) and displayed no detectable DD-carboxypeptidase activity with UDP-MurNAc-pentapeptide as a substrate (122). Single-stranded DNA and

polynucleotides had an inhibitory effect (122, 260). Two deficient mutants, named *mepA* and *mepB*, for murein endopeptidase, were isolated (107). Comparison with the N-terminal sequence of purified endopeptidase showed that *mepA* is the structural encoding gene and that MepA is synthesized initially with an N-terminal signal peptide, indicating a periplasmic location (123).

The crystal structure of MepA (Fig. 3h) was determined at a 1.4-Å resolution (161). The C-terminal catalytic domain contains a zinc-binding site, in agreement with the sensitivity of MepA to metal chelators. The active site resembles that of the LAS metallopeptidases, which include lysostaphin, D-alanyl-D-alanine carboxypeptidase, and sonic hedgehog protein, their initials forming the term LAS. The MepA sequence has two conserved LAS-characteristic motifs but no PBP Ser-Xaa-Xaa-Lys motif. The reaction mechanism of MepA thus differs from that of the LMM PBPs (123). Furthermore, there is no significant sequence homology with the endopeptidases PBP4 and PBP7, and overproduction leads to no change in the normal degree of cross-linking (123). Mutational analysis revealed that the metal ligands conserved in MepA and its homologs are required for folding and catalysis (69).

EnvC endopeptidase. A mutant forming chains of cells of irregular lengths was isolated from *E. coli*, and the mutated gene was designated *envC* (219) and mapped (120). As septation was partially blocked, some cells became longer than normal, and daughter cell poles remained fused to each other for several generations. The mutant showed hypersensitivity to dyes, a leakage of periplasmic proteins, and other defects indicating the perturbation of the outer membrane (121). Later, *envC* was recognized as the *yibP* open reading frame, and the single-residue substitution causing the mutation was identified (88).

The cleavage of the signal sequence yields a mature protein anchored to the cytoplasmic membrane, and a 43-kDa form is released upon spheroplast formation (88). The N-terminal half of the mature form is composed mostly of sequences with a repetitive pattern characteristic of a coiled-coil structure possibly involved in the oligomerization of EnvC or in its interaction with another protein (88). The activation of AmiA and AmiB by EnvC (273) suggests a possible protein-protein interaction between EnvC and these amidases. The C-terminal domain of EnvC resembles a zinc metallopeptidase and has homology with the staphylococcal LytM lysostaphin, which is known to degrade PG cross-linkages (88). The PG hydrolase activity of EnvC was assessed by zymography (20). Furthermore, EnvC hydrolyzes β-casein (106) and binds to PG in cosedimentation assays (273). These facts are consistent with PG endopeptidase activity. However, this finding is disputed by the absence of the zinc-chelating residues of the LytM active site and by the fact that different forms of EnvC have no effect on isolated dye-labeled PG (273). Therefore, it is difficult to decide whether EnvC truly has peptidase activity.

The disruption of chromosomal *envC* causes the same defects as the *envC* point mutation, indicating that the gene is dispensable for growth but important for normal septation and cell envelope integrity. The overexpression of *envC* leads to growth inhibition, morphological aberrations, and cell lysis (88, 273). EnvC, together with three other LytM

factors (NlpD, YgeR, and YebA) discovered in *E. coli*, is required for septal PG splitting and daughter cell separation (272).

LD-Endopeptidase releasing D-alanyl-D-alanine from UDP-MurNac-pentapeptide. A peptidase cleaving the (L)-*meso*-A₂pm→D-Ala bond of UDP-MurNac-pentapeptide (Fig. 2) with the release of UDP-MurNac-tripeptide and D-alanyl-D-alanine was identified in *E. coli* (78). Partially purified as a 34-kDa soluble enzyme, this LD-endopeptidase was devoid of any MurF synthetase activity catalyzing the addition of D-alanyl-D-alanine onto UDP-MurNac-tripeptide. Conversely, purified MurF had no hydrolytic effect on UDP-MurNac-pentapeptide. However, in *B. subtilis*, purified MurF catalyzes the removal of D-alanyl-D-alanine from UDP-MurNac-pentapeptide but only in the presence of Co²⁺ ions (63). This is not strictly a reverse reaction, since no ATP is formed in the presence of ADP and phosphate.

Furthermore, it was suggested that the *E. coli* LD-endopeptidase could be the LD-transpeptidase catalyzing the formation of the (L)-*meso*-A₂pm→(D)-*meso*-A₂pm cross-linkages (145). This proposal is consistent with the finding that the LD-transpeptidase from *Enterococcus faecalis*, which catalyzes the formation of dimers by the removal of D-alanyl-D-alanine from the branched disaccharide-heptapeptide monomer muropeptide, also displays LD-endopeptidase activity catalyzing the removal of D-alanyl-D-alanine from the same substrate (158).

LD-Carboxypeptidases cleaving the tetrapeptide (L)-*meso*-A₂pm→D-Ala bond. LD-Carboxypeptidase activity cleaving the (L)-*meso*-A₂pm→D-Ala bond of the disaccharide-tetrapeptide muropeptide or of the UDP-MurNac-tetrapeptide (Fig. 2) with the release of D-alanine was initially detected in *E. coli* (112, 198) and was later detected in many other organisms (179, 256). The use of UDP-MurNac-tetrapeptide as a substrate allowed the characterization of three *E. coli* LD-carboxypeptidases (14, 180, 274). Two of the enzymes were released by Tris-EDTA treatment and were purified as 86-kDa and 12-kDa proteins (14, 180). The osmotic shock treatment confirmed the periplasmic localization of the 86-kDa enzyme (13, 14), but that of the 12-kDa enzyme remained uncertain (180).

The third enzyme was the best-studied LD-carboxypeptidase. Its inhibition by nocardicin A allowed its purification by affinity chromatography as a 32-kDa enzyme found exclusively in the cytoplasm (274). The identified encoding gene was designated *ldcA* (256). The substrate specificity of LdcA was examined with various derivatives carrying the L-Ala-γ-D-Glu-(L)-*meso*-A₂pm-(L)-D-Ala tetrapeptide (142, 256, 274). Only monomeric muropeptides (with either a MurNac or a 1,6-anhydro-MurNac residue), free tetrapeptides, and UDP-MurNac-tetrapeptide are substrates. LdcA interacts with neither isolated PG nor cross-linked muropeptides (274). No LD-carboxypeptidase activity was detectable in soluble extracts from an *ldcA* deletion mutant (256). Moreover, the growth and morphology of the mutant are severely affected at the onset of the stationary phase, and it is prone to lysis. The high-level overproduction of LdcA has no effect on the growth rate or cell viability (256). LdcA plays a major role in the recycling process by converting the potentially harmful L-Ala-γ-D-Glu-(L)-*meso*-A₂pm-(L)-D-Ala tetrapeptide into the L-Ala-γ-D-Glu-(L)-*meso*-A₂pm tripeptide (196).

Among the five LD-transpeptidases (ErfK, YcfS, YbiS,

YcbB, and YnhG) recently discovered in *E. coli*, three (ErfK, YcfS, and YbiS) anchor the Lpp lipoprotein to PG, whereas two (YcbB and YnhG) are responsible for the formation of the (L)-*meso*-A₂pm→(D)-*meso*-A₂pm cross-linkages (157, 159). They also catalyze the exchange of D-alanine with glycine in PG tetrapeptide subunits. All five LD-transpeptidases show LD-carboxypeptidase activity catalyzing the formation of tripeptide subunits from tetrapeptide subunits. They appear as periplasmic proteins possessing a sole cysteine residue essential for activity (54), like other LD-transpeptidases (160). The identification of either one of these *E. coli* LD-transpeptidases with the purified 12-kDa and 86-kDa LD-carboxypeptidases or with the D-alanyl-D-alanine-releasing LD-endopeptidase remains an open question.

MpaA amidase cleaving the γ -D-Glu→(L)-*meso*-A₂pm bond. In the PG peptide subunits, the γ -D-Glu→(L)-*meso*-A₂pm bond is not *sensu stricto* a peptide bond, and the hydrolases cleaving it are designated γ -D-Glu→(L)-*meso*-A₂pm amidases. Among the various reported examples, sporulation-related endopeptidase 1 (or ENP1 amidase) from *Bacillus sphaericus* has been well studied, and its C-terminal catalytic domain was shown to contain a zinc-binding site (105). The release of the dipeptide (L)-*meso*-A₂pm-D-Ala into the culture medium of growing cells was the first indication of a similar activity in *E. coli* (82). Later, the presence of the dipeptide L-Ala-D-Glu was demonstrated in an *mpl* mutant lacking the UDP-*N*-acetylmuramate:tripeptide ligase and accumulating large amounts of the L-Ala- γ -D-Glu-(L)-*meso*-A₂pm tripeptide (176).

Regions of the *E. coli ycjI* open reading frame displayed significant homology with the catalytic domain of ENP1 and its zinc-binding triad (269). The encoding gene was named *mpaA*, for murein peptide amidase A, and its *in vivo* activity was assessed by the accumulation of the L-Ala- γ -D-Glu-(L)-*meso*-A₂pm tripeptide in an *mpl mpaA* double mutant (269). MpaA recognizes tri- and tetrapeptides (Fig. 2) but not disaccharide-peptides or UDP-MurNAc-peptide precursors (269). The finding of an absence of a signal sequence in *mpaA* was in favor of a cytoplasmic localization (269). However, the release of (L)-*meso*-A₂pm-D-Ala in the growth medium as a PG turnover product does not exclude the possible presence of some MpaA activity in the periplasm (82).

L-Alanyl-D/L-glutamate dipeptide epimerase. The search for members of the enolase superfamily in the *E. coli* and *B. subtilis* genomes led to the discovery of the L-alanyl-D/L-glutamate epimerases YcjG and Ykfb, respectively, which convert L-Ala-D-Glu to L-Ala-L-Glu (233). Their crystal structures (Fig. 3i) were determined at 2.6- to 2.7-Å resolutions, and their reaction mechanism was studied (84, 128). The location of the gene encoding the *E. coli* YcjG epimerase downstream of *mpaA* suggested that it could play a role in PG metabolism. Its likely cytoplasmic localization excludes any action on PG. In *E. coli*, the L-Ala-L-Glu reaction product of the epimerase is a substrate for the broad-specificity PepD dipeptidase (234). Thus, the sequential actions of MpaA, YcjG, and PepD can release all of the amino acids from the PG tripeptide. However, this is a minor pathway, since in the recycling process, the Mpl ligase converts most of the cell tripeptide to UDP-MurNAc-tripeptide (196).

DdpX dipeptidase cleaving D-alanyl-D-alanine. In PG-containing bacteria, D-alanyl-D-alanine is an essential cytoplasmic metabolite used in the formation of UDP-MurNAc-pentapeptide (286). The Ddl ligase catalyzes the ATP-driven condensation of two D-alanine molecules to form D-alanyl-D-alanine, and MurF synthetase catalyzes the addition of the dipeptide onto UDP-MurNAc-tripeptide. In the mechanism of resistance to vancomycin in enterococci, the dipeptidase VanX is responsible for the hydrolysis of the dipeptide (214). Analyses of the *E. coli* genome database revealed the presence of a VanX homolog with 27% similarity, originally referred to as EcoVanX and later renamed DdpX (144, 145). DdpX was overproduced and purified, and its activity was validated. DdpX conserves the active-site residues and auxiliary residues maintaining the active-site topology of VanX, and a kinetic analysis showed the same substrate specificity (144). Homology modeling of DdpX with the crystal structure of VanX from *Enterococcus faecium* (EntVanX) revealed a striking similarity in the overall structures, with the highest identity seen within the key catalytic residues (144).

DdpX has a potential lethal activity owing to the hydrolysis of the D-alanyl-D-alanine necessary for PG biosynthesis. However, DdpX is present preferentially at the onset of the stationary phase (144, 145). Therefore, in growing cells, DdpX plays no role in the regulation of the dipeptide pool controlled by the feedback inhibition of the Ddl ligase (286). The tight regulation of the D-alanyl-D-alanine pool is required to avoid the depletion of the alanine pool. In the stationary phase, DdpX could hydrolyze D-alanyl-D-alanine and supply D-alanine for use as metabolic fuel (145).

Enzymatic properties of *E. coli* PG peptidases. The identified PG peptidases (Table 1) are characterized by their narrow specificity for a given peptide bond, except for PBP4 and AmpH, which recognize both D-Ala→D-Ala and D-Ala→(D)-*meso*-A₂pm bonds. Some are unique for a given bond (MepA and YcjG). Redundancies concern the D-Ala→D-Ala bond (PBPs 4, 5, and 6; AmpH; and DdpX), the cross-bridges (PBP4, PBP7, AmpH, MepA, and EnvC), and the (L)-*meso*-A₂pm→D-Ala bond (LD-carboxypeptidases and LD-transpeptidases), but there are great differences in substrate specificity between isoenzymes of a same family. Structural data (Fig. 3) are now available for six peptidases (PBPs 4, 5, and 6; MepA; DdpX; and YcjG), and reaction mechanisms have been proposed for some of them.

LMM PBPs have a common two-step reaction mechanism (77). The initial acylation step (Fig. 5a) involves the nucleophilic attack of the D-Ala→D-Ala or the D-Ala→(D)-*meso*-A₂pm bond by a serine at the carbonyl carbon of the penultimate D-Ala residue, which results in a tetrahedral high-energy intermediate. The collapse of this species gives rise to an acyl enzyme intermediate and the release of the C-terminal D-Ala or of the acceptor peptide unit. In the subsequent deacylation step, a water molecule attacks the acyl enzyme to generate another tetrahedral high-energy species, which in turn collapses to yield the shortened peptide subunit and the regenerated PBP.

Several penicillin-insensitive *E. coli* PG peptidases (MepA, EnvC, MpaA, and DdpX) bind Zn²⁺ ions and have no PBP active-site sequence motifs, indicating that their reaction mechanisms differ from that of the PBPs (70). The strong

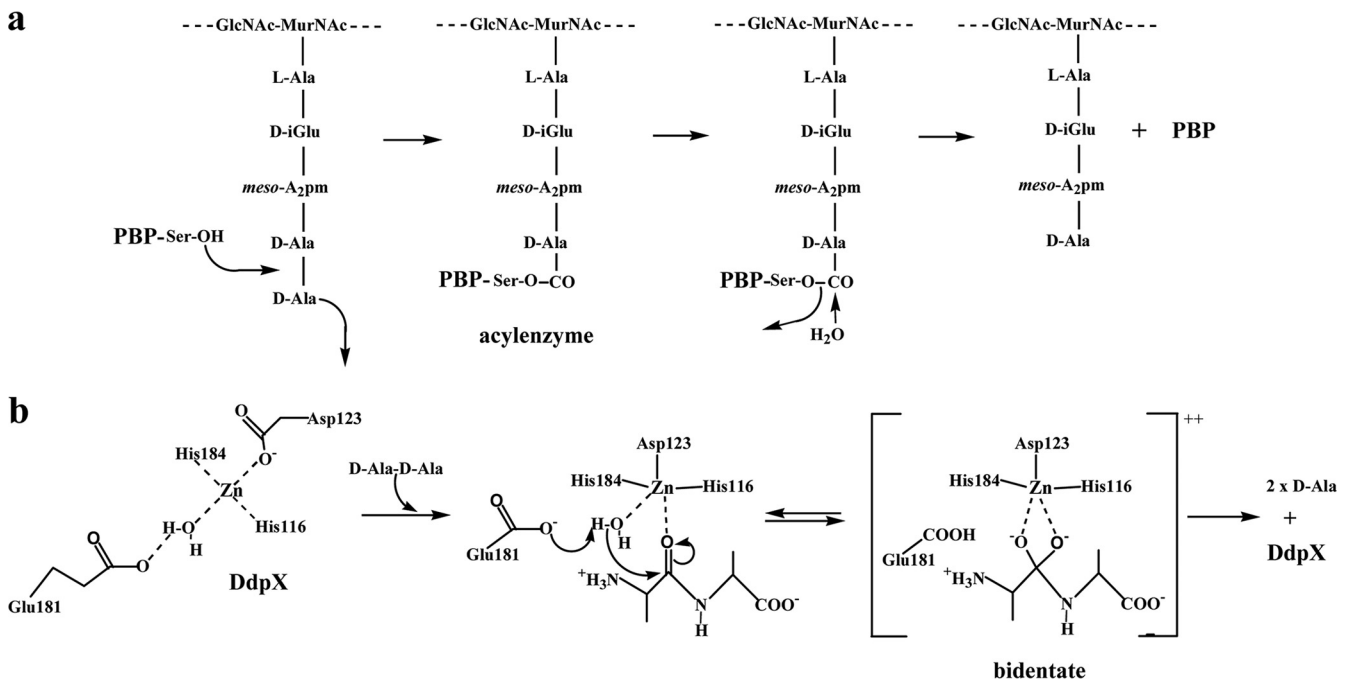


FIG. 5. Reaction mechanisms of the LMM PBPs and D-Ala-D-Ala dipeptidases. (a) Two-step catalytic mechanism with the formation of an acyl enzyme intermediate and its attack by a water molecule. (b) Attack of the dipeptide carbonyl by a water molecule and formation of a bidentate complex with the catalytic Zn^{2+} .

structural similarity of DdpX with VanX and the conservation of the active-site topology (144) suggest that DdpX could follow the same reaction mechanism as that of VanX (32, 167). This implies (Fig. 5b) that the incoming D-alanyl-D-alanine dipeptide displaces a zinc-bound water molecule toward Glu181. The water molecule, activated by the influence of the catalytic zinc and Glu181, attacks the polarized carbonyl to form a tetrahedral intermediate, yielding a bidentate complex with the catalytic zinc. Subsequently, Glu181 donates the proton to the scissile nitrogen, completing the reaction with the release of D-alanine.

E. COLI N-ACETYLMURAMOYL-L-ALANINE AMIDASES

In bacterial PG, the peptide subunits are bound to the glycan chains by an amide bond between their N-terminal L-alanine residue and the D-lactoyl moiety of the MurNAc units (Fig. 1). Amidases cleaving the N-acetylmuramoyl-L-alanine bond are present in many eubacteria (238, 240, 295). In *E. coli*, an N-acetylmuramoyl-L-alanine amidase acting on monomer and dimer mucopeptides was first detected in a crude cell extract (198). So far, six *E. coli* N-acetylmuramoyl-L-alanine amidase activities (Table 1) have been reported: five (AmiA, AmiB, AmiC, AmpD, and AmiD) were genetically well defined, while another one (39-kDa amidase) was only biochemically characterized. Based on the similarity of their amino acid sequences, AmiA, AmiB, and AmiC are members of the amidase_3 superfamily of the Pfam database, whereas AmiD and AmpD belong to the amidase_2 superfamily of the same database (200).

AmiA, AmiB, and AmiC N-Acetylmuramoyl-L-Alanine Amidases

Homology studies led to the identification of AmiA, AmiB, and AmiC as N-acetylmuramoyl-L-alanine amidases. Upstream of the *hemF* gene at centisome 55, an open reading frame codes for a 32-kDa protein homologous to the CwIB amidase from *B. subtilis* (262). The designation *amiA* is now used for the encoding gene (91) and must not be confused with the same previously proposed designation (261) for the 39-kDa amidase (197, 283). A second open reading frame at centisome 94.7 encodes a 48-kDa protein with homology to AmiA and other bacterial amidases, and the gene was designated *amiB* (263). Finally, an open reading frame at centisome 63.4 showed significant sequence similarity to other amidases, and the gene encoding a 45-kDa protein was named *amiC* (91). The overexpression of *amiB* or *amiC* led to cell lysis (91).

The presence of a signal sequence in each amidase indicated their periplasmic localization (19, 91). AmiA and AmiC are exported to the periplasm by the Tat system (19, 113), whereas AmiB is supposedly exported by the more general Sec pathway (19). The subcellular periplasmic localization of AmiA and AmiC was studied by use of a functional fusion to green fluorescent protein (GFP) (19). AmiA-GFP remains dispersed in the periplasm throughout the cell cycle, and AmiC-GFP remains dispersed only in nondividing cells. In contrast, AmiC-GFP was concentrated mostly at the septal ring in dividing cells, as confirmed by an electron microscopy analysis of the metallothionein-tagged protein (55).

The release of the L-Ala- γ -D-Glu-(L)-*meso*-A₂pm tripep-

tide and the L-Ala- γ -D-Glu-(L)-*meso*-A₂pm-(L)-D-Ala tetrapeptide was observed after the incubation of isolated PG with overproduced AmiA and AmiC (91). An analysis of changes in PG structure revealed the amidase activity of AmiB. The release of soluble material upon incubation with isolated dye-labeled PG confirmed the activity of all three amidases (273). AmiA, which is a zinc enzyme, cleaves uncross-linked PG fragments having at least a tetrasaccharide glycan chain (154). Neither amidase cleaves 1,6-anhydro-MurNac \rightarrow L-Ala bonds (270). Mutants devoid of any one of the three amidases were constructed and showed no major changes in growth (91). Morphological changes appeared mostly in the late stationary phase, with the AmiA and AmiC mutants growing in chains of 3 to 6 cells. None of the single mutants showed any major change in PG structure.

AmpD Anhydro-*N*-Acetylmuramoyl-L-Alanine Amidase

The *ampD* gene of enterobacteria was first shown to encode a cytoplasmic protein acting as a negative regulator of β -lactamase induction (148). It was later established that AmpD is an *N*-acetylmuramoyl-L-alanine amidase exhibiting an exclusive specificity for substrates containing 1,6-anhydro-MurNac residues (101, 116). In the cytoplasm, AmpD rapidly cleaves 1,6-anhydro-MurNac-peptides but has no effect on UDP-MurNac-pentapeptide (196). This high specificity is confirmed by the cleavage of the 1,6-anhydro-MurNac \rightarrow L-Ala bond of the 1,6-anhydro-MurNac-peptide located at the end of isolated PG chains (100). GlcNac-(1 \rightarrow 4)-1,6-anhydro-MurNac-tetrapeptide is also recognized as a substrate (97). The accumulation of 1,6-anhydro-MurNac-tripeptide in an AmpD-deficient mutant suggests that it is the *in vivo* substrate of AmpD in the PG-recycling process (196). AmpD from *Citrobacter freundii* is 90% identical in sequence to the *E. coli* enzyme, and its 3D structure determined by NMR revealed a deep substrate-binding pocket (147). To explain the specificity of AmpD for low-molecular-mass substrates, a model of the enzyme-substrate complex was established.

AmiD Anhydro-*N*-Acetylmuramoyl-L-Alanine Amidase

A second enzyme with 1,6-anhydro-MurNac-L-alanine amidase activity was identified when a *nagZ ampD* mutant was found to accumulate not only GlcNac-(1 \rightarrow 4)-1,6-anhydro-MurNac-tripeptide, as expected, but also a significant amount of the GlcNac-(1 \rightarrow 4)-1,6-anhydro-MurNac disaccharide in the cytoplasm (270). A search of the genome revealed a paralog, YbjR, having 40% identity with a 123-amino-acid sequence of AmpD, and it was named AmiD. The presence of a short signal sequence and a lipobox motif in *ybjR* suggested that AmiD is a lipoprotein, and it was demonstrated to be in the outer membrane (270). Membrane and soluble forms of AmiD were overproduced, purified, inactivated by chelators, and shown to bind a zinc ion required for activity (200, 270).

A null mutation had no effect on cell growth or morphology (270). The deletion of *amiD* from the *nagZ ampD* double mutant produced a strain totally lacking 1,6-anhydro-MurNac-L-Ala amidase activity, demonstrating that AmpD and AmiD were the only enzymes in *E. coli* able to cleave the 1,6-anhydro-MurNac \rightarrow L-Ala bond (270). AmiD has a broader substrate

specificity than AmpD. It can cleave both MurNac \rightarrow L-Ala and 1,6-anhydro-MurNac \rightarrow L-Ala bonds in mucopeptides and in MurNac-tri- or tetrapeptides, with the disaccharide-tetrapeptide mucopeptide being the best substrate (200, 270). AmiD presumably corresponds to the MurNac-L-Ala amidase activity detected previously by using radiolabeled disaccharide-tetrapeptide as a substrate (80). Moreover, the digestion of isolated PG leads to the release of free tri-, tetra-, and octapeptides, whereas AmiD has no effect on MurNac-L-Ala-D-Glu, UDP-MurNac-tripeptide, UDP-MurNac-pentapeptide, lipid I, or lipid II (200, 270).

The crystal structure of AmiD devoid of its lipid anchorage was determined at a 1.8-Å resolution (124). The overall shape is a 45-Å-long and 30-Å-thick cylinder, with the zinc ion critical for activity located at the intersection of the two branches of the L-shaped groove of the active site (Fig. 3j). The structures of AmiD in complexes with 1,6-anhydro-MurNac-tripeptide or the tripeptide reaction product were also determined. AmiD shares a common fold with AmpD from *Citrobacter freundii*, bacteriophage T7 lysozyme zinc amidase, and PG recognition proteins (124).

39-Kilodalton Amidase Cleaving MurNac-Tripeptide

An *N*-acetylmuramoyl-L-alanine amidase activity from *E. coli* was biochemically characterized by use of radiolabeled MurNac-tripeptide as a substrate (197, 261, 282, 283, 308). After cell disruption by sonication, it appeared mainly (261, 283) but not exclusively (308) in the soluble fraction. Most of the activity was released upon spheroplast formation, but none was released by osmotic shock, suggesting binding to a periplasmic structure or an outer membrane location (197).

The enzyme was purified to near homogeneity from the spheroplast supernatant as a 39-kDa protein (283) and was shown to have a narrow substrate specificity limited to MurNac-peptides (197). The enzyme had practically no effect on the disaccharide-tetrapeptide mucopeptide and 1,6-anhydro-MurNac-containing mucopeptides or on isolated PG. A mutant showing 95% reduced amidase activity with MurNac-tripeptide was isolated and showed no distinct physiological changes (261). However, it was not established whether the mapped gene, named *amiA*, was truly the structural gene of the 39-kDa amidase. Owing to clear differences in specificities, the identification of the 39-kDa amidase with any of the five amidases genetically characterized so far is unlikely. It could play a role in the processing of MurNac-peptides originating from the turnover reaction products or from exogenous PG fragments used as metabolic fuel.

Enzymatic Properties of the *E. coli* Amidases

The substrate specificities of the *E. coli* PG amidases differ considerably from one another. Some act on isolated PG (AmiA, AmiB, and AmiC), while others recognize only low-molecular-mass PG fragments (AmpD and 39-kDa amidase). The amidase AmiD has the broadest specificity by acting on both types of substrates. Several amidases (AmiA, AmpD, and AmiD) are zinc metalloenzymes. So far, the biochemical properties of only AmiD (200) and the 39-kD amidase (197) have been studied to some extent. In particular, AmiD exhibits a

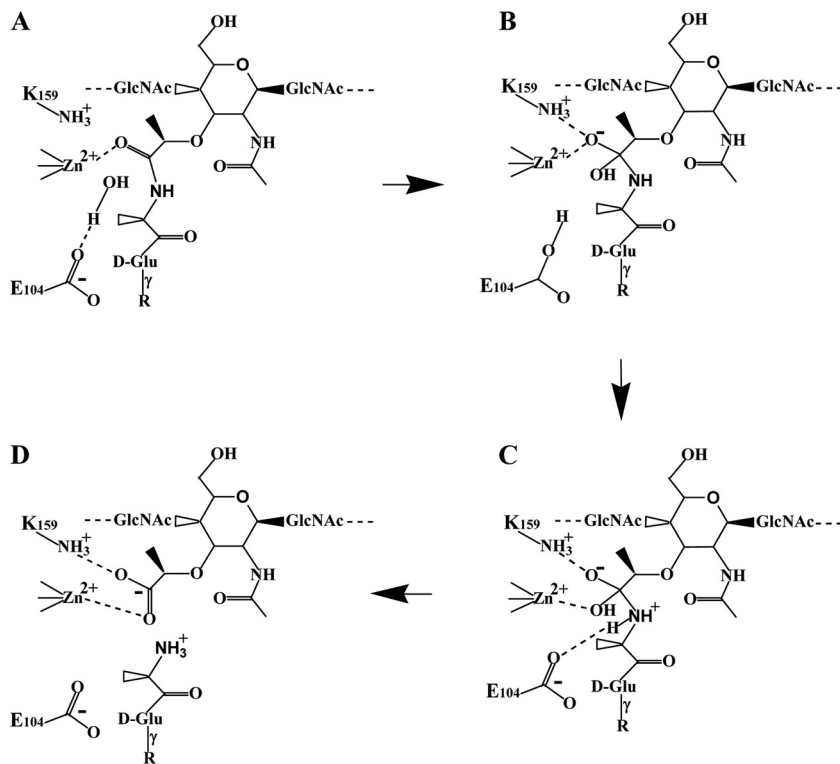


FIG. 6. Reaction mechanism of the AmiD amidase proposed by Kerff et al. (124). The scheme describes the catalytic mechanism for the AmiD hydrolysis of the MurNAc-L-Ala amide bond. (A) Activation of the catalytic water molecule by Zn^{2+} and Glu104 upon binding of the substrate; (B) formation of the tetrahedral intermediate stabilized by Zn^{2+} and Lys159; (C) rotation of the amide bond leading to an ideal tetrahedral conformation of the amide bond nitrogen; (D) transfer of the hydrogen from the protonated Glu104 to the amide nitrogen and cleavage of the amide bond. R, remaining peptide moiety.

biphasic time course, which is explained by the presence of a branched pathway involving a substrate inactivation process (200). It is unknown whether this unusual kinetic behavior has an *in vivo* regulatory significance.

The determination of the crystal structure of AmiD led to the proposal of a reaction mechanism (Fig. 6) similar to that of the thermolysin-related zinc peptidases (124). Upon the approach of the substrate, the water molecule bound to active-site Zn^{2+} is shifted toward residue Glu104, which favors the nucleophilic attack of the amide bond, producing a tetrahedral conformation of the amide carbon and a transient pentameric coordination of the zinc ion. Hydrogen interactions involving the carbonyl oxygen of the scissile bond stabilize the tetrahedral intermediate. The next step is proton transfer from the protonated glutamic acid to the leaving nitrogen. Mutation analysis demonstrated the critical role played by Glu104 in the catalytic mechanism.

HYDROLASE-DEFECTIVE MUTANTS

To determine possible functional roles, single defective mutants were constructed for most *E. coli* PG hydrolases. Except for the deletions of the DD-carboxypeptidase PBP5 (170, 185), the LD-carboxypeptidase LdcA (256), the endopeptidase EnvC (219), and the amidases AmiA and AmiC (92), most mutants grew well and displayed no morphological changes. Therefore, many hydrolases are not individually essential, at least under

conditions of laboratory culture. This is in sharp contrast with the functionally well-defined enzymes of the PG biosynthesis pathway leading to lipid II, which are unique and essential (5, 224, 286). For this reason, the construction of mutants with multiple deletions of PG hydrolases was undertaken. Since access to mutants with all possible combinations of deletions is not readily feasible, only a limited number of multiple mutants of the same or different enzymatic specificities have been considered so far.

Multiple Deletions of the LTs

Several multiple-deletion mutants of the LTs were constructed (92, 149). The double mutants lacking Slt70, MltA, or MltB as well as the triple mutants lacking all three LTs displayed no obvious phenotype and no effect on morphology (149). The triple mutant showed a reduced rate of PG turnover and a low level of β -lactamase induction, but neither the rate of PG synthesis nor the cell PG content varied (133). A triple mutant lacking MltC, MltD, and MltE and a hexuple mutant with deletions of six LTs, not including MltF, grew with 30% to 50% of the cells in short chains containing three to eight cells (92). Only minor changes in the PG structure were detected, and no significant variation in the 1,6-anhydro-MurNAc content was observed (92). An attempt to isolate a multiple mutant deficient for all seven identified LTs failed (231).

Multiple Deletions of the LMM PBPs

Mutants with multiple deletions of the LMM PBPs were constructed (6, 29, 49, 62, 92, 226, 250, 291). In particular, mutants lacking all possible combinations of PBP4, PBP5, PBP6, PBP6b, and PBP7 (49) as well as a mutant lacking six LMM PBPs (291) were generated. All multiple deletions had little or no effect on growth under laboratory conditions. However, some multiple-PBP deletions produced cells with random shapes, lengths, and diameters (49, 62, 170, 185, 291). The minor alterations observable for some simple mutants were enhanced in multiple mutants. In particular, the modifications in the PBP5 single mutant were also present in the different multiple mutants lacking PBP5. They were exacerbated by the additional deletion of PBP4 or/and PBP7, with the greatest alterations being observed with the triple mutant (170). PBP4 and PBP7 are thus endopeptidases influencing cell shape, but the absence of shape abnormalities in mutants lacking only PBP4 and PBP7 stresses the importance of PBP5. A PBP4 PBP5 double mutant showed a higher increase of the PG pentapeptide subunit content than did the PBP5 single mutant (134). However, the triple deletion of the endopeptidases PBP4, PBP7, and MepA had no effect on morphology and only minor effects on the PG structure (92). Cells with a double deletion of PBP5 and PBP6 grew as filaments failing to construct and divide efficiently (226).

Multiple Deletions of the *N*-Acetylmuramoyl-L-Alanine Amidases

Mutants lacking two or all three AmiA, AmiB, and AmiC amidases showed normal growth (91). In the three double mutants, cell separation took place, but 5 to 20% of the cells had a tendency to grow in chains. The triple mutant grew in long chains, and the change in morphology was accompanied by an increase of trimer and tetramer contents as well as a higher 1,6-anhydro-MurNAc content. The double deletion of *amiA* and *amiC* rendered the integrity of the cell envelope highly defective, resulting in an increased permeability of the outer membrane to hydrophobic antibiotics and detergents (113).

Multiple Deletions of Hydrolases of Different Enzymatic Specificities

An early study concerned mutants with multiple deletions in the amidases AmiA, AmiB, and AmiC combined with deletions in the endopeptidases PBP4, PBP7, and MepA or in LTs (92). The average number of cells in the chains increased significantly compared to the average number of cells in chains of amidase mutants. A multiple-amidase mutant also lacking the endopeptidases MepA, PBP4, and PBP7 formed chains containing 6 to 80 cells. A mutant with deletions in the three amidases, the three endopeptidases, and the LT Slt70 formed the longest chains, consisting of 20 to 100 cells. The conclusion was that all three types of activities are involved in the splitting of the PG septum (92). Some mutants showed lower outer membrane permeabilities and greater susceptibilities to antibiotics, whereas LT activity was essential for β -lactamase induction (131).

Recently, mutants combining multiple deletions of the LMM PBPs and the AmiA, AmiB, AmiC amidases were constructed to determine which hydrolases facilitate cell separation (210, 211). Particular combinations of PBPs and amidases affected the overall septum geometry in that certain mutants formed highly abnormal cell chains. Amidase mutants lacking PBP4 grew as longer chains, identifying a specific biological role for this endopeptidase in cell separation. PBP7 appeared to play a minor role. The deletion of PBP5 from a mutant lacking both AmiA and AmiC led to twisted chains (210).

Several mutants with multiple deletions of the LdcA, AmiD, AmpD, and NagZ hydrolases involved in the recycling process were constructed (196, 256, 270). In particular, a mutant lacking AmpD, AmiD, NagB, and NagZ was used to establish the important role of AmiD in the recycling process (270).

HYDROLASES AND PEPTIDOGLYCAN MATURATION

If PG metabolism was restricted to the formation of the glycan chains by transglycosylation and their cross-linking by transpeptidation using pentapeptide subunits, mature PG would contain only tetrapeptide subunits in the donor position of the DD-cross-linkages and unused pentapeptide subunits. However, the analysis of the mucopeptide composition of isolated *E. coli* PG (74) revealed a far greater structural complexity (Fig. 1) characterized by the presence, in particular, of various truncated peptide subunits, LD-cross-linkages, glycan chains with nonreducing 1,6-anhydro-MurNAc ends, and substitutions by the Lpp lipoprotein (61) or glycine (74). These modifications are due to maturation reactions taking place in the periplasm during the formation of new PG. Pulse and pulse-chase labeling experiments carried out with radiolabeled A_2pm revealed that the various reactions follow different kinetics from incipient to mature PG (33, 47, 51, 75). Several envelope-associated PG hydrolases are involved in the process.

Tetrapeptide Subunits

The most obvious feature of the mucopeptide composition of PG from growing *E. coli* cells is the predominance of tetrapeptide subunits, which amount to ca. 60% of the total peptide subunits, and the presence of less than 1% pentapeptide subunits (74). Short pulse experiments showed the rapid formation of tetrapeptide subunits from pentapeptide subunits very early in the synthesis of nascent PG (47, 75). The pentapeptide subunits are used as donors in the formation of the D-Ala \rightarrow (D)-*meso*- A_2pm cross-linkages by DD-transpeptidation, and the unused ones are converted to tetrapeptide subunits by the removal of their C-terminal D-alanine residue. Pulse-chase experiments revealed a complex time course for the formation and use of the tetrapeptide subunits, which are at the crossroads of several reactions (33, 75). Tetrapeptide subunits are used as acceptors in the DD-transpeptidation reactions but also as energy donors in the formation of (L)-*meso*- A_2pm \rightarrow (D)-*meso*- A_2pm cross-linkages by LD-transpeptidation, the linking of the Lpp lipoprotein, and the replacement of D-alanine residues by glycine. At the same time, tetrapeptide units are formed as a PG turnover product by the DD-endopeptidase cleavage of D-Ala \rightarrow (D)-*meso*- A_2pm cross-linkages.

PBP5 appears to be the major DD-carboxypeptidase respon-

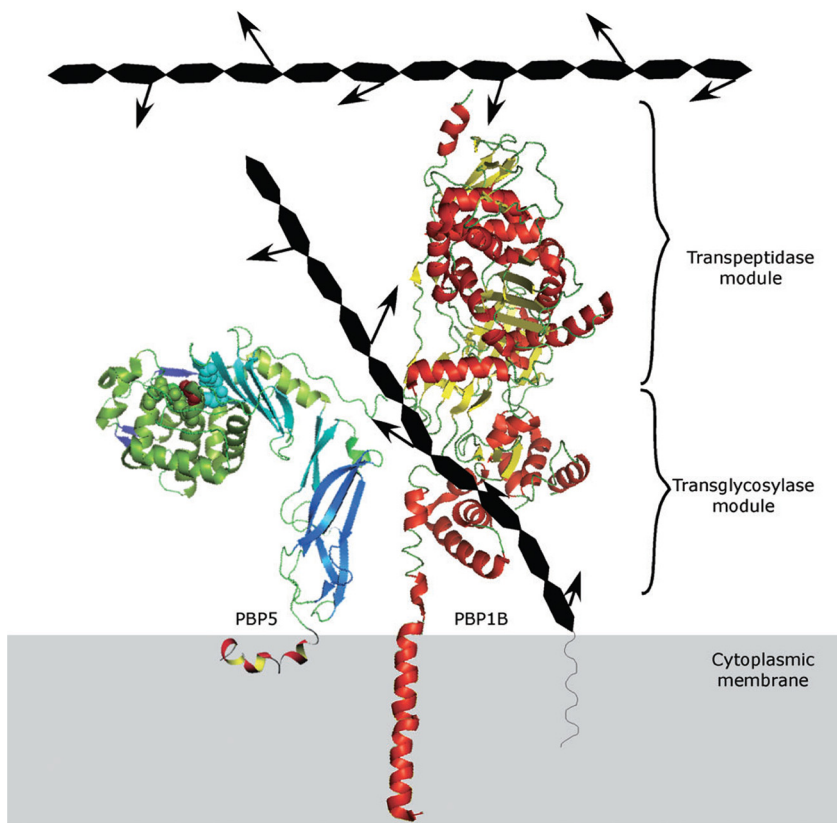


FIG. 7. Model showing the concomitant actions of PBP5 and PBP1b on the pentapeptide subunits of nascent PG. Arrows represent the pentapeptide subunits linked to the glycan chains (in black). (Reprinted from reference 205 with permission of John Wiley & Sons.)

sible for the conversion of the pentapeptide subunits not used in transpeptidation to tetrapeptide subunits. The mutational loss of PBP5 leads to an increased pentapeptide subunit content (50, 134, 146). PBP5 cleaves the D-Ala→D-Ala bonds of un-cross-linked and cross-linked PG (1, 205). The additional deletion of PBP4 enhances the pentapeptide content, as observed for a double-defective mutant (134). The deletion of PBP4 alone leads to no great increase in numbers of pentapeptide subunits. This finding suggests that both PBPs cooperate in some way in controlling the amount of pentapeptide subunits available for transpeptidation and regulating the degree of PG cross-linkage (48, 170, 185, 205). The known 3D structures of PBP5 and bifunctional PBP1 have given rise to a model (Fig. 7) for the coordinated removal of D-alanine from PG by hydrolytic cleavage and transpeptidation (205). The close attachment of PBP5 to the outer face of the cytoplasmic membrane restricts its *in vivo* action to pentapeptide subunits of incipient PG.

Tripeptide Subunits

In the tripeptide subunits of *E. coli* PG, the L-carboxyl of the C-terminal *meso*-A₂pm residue is either free or engaged in an (L)-*meso*-A₂pm→(D)-*meso*-A₂pm cross-linkage or linked to the Lpp lipoprotein or glycine (74). The unsubstituted and substituted forms account for ca. 13% and 10%, respectively, of the total PG peptide subunits. In growing cells, their formation follows a slow time course. Cells harvested without

rapid cooling lead to higher free tripeptide subunit contents (303). In the stationary phase (74) or during amino acid starvation (268), a considerable increase of the tripeptide subunit content is observed in parallel with those of (L)-*meso*-A₂pm→(D)-*meso*-A₂pm cross-linkages and bound Lpp lipoprotein.

The formation of tripeptide subunits by the cleavage of the (L)-*meso*-A₂pm→D-Ala bond of tetrapeptide subunits by the LdcA LD-carboxypeptidase is unlikely, considering its cytoplasmic localization and the absence of an effect on isolated PG (256, 274). Some tripeptide subunits could originate from the use of the UDP-MurNAc-tripeptide precursor in parallel with UDP-MurNAc-pentapeptide in the biosynthesis pathway (15, 285). However, recent results point to the major role played by LD-transpeptidases. A sharp decrease of the tripeptide subunit content was observed with a quadruple mutant lacking the ErfK, YcfS, YbiS, and YcbB LD-transpeptidases (157), and a total loss of tripeptide subunits accompanied their deletion together with that of the YnhG LD-transpeptidase (159). Thus, these enzymes are not only acting as LD-transpeptidases in the formation of (L)-*meso*-A₂pm→(D)-*meso*-A₂pm cross-linkages and the binding of the Lpp lipoprotein but are also responsible for the formation of the PG tripeptide subunits by the LD-carboxypeptidase cleavage of tetrapeptide subunits. Furthermore, the overexpression of each LD-transpeptidase gene led to the accumulation of tripeptide subunits. Biochemical data con-

firming their LD-carboxypeptidase activity are still lacking. The large increases in amounts of bound Lpp lipoprotein and (L)-*meso*-A₂pm→(D)-*meso*-A₂pm cross-linkages in the stationary phase parallel the increase in amounts of tripeptide subunits (74), indicating a close connection between LD-transpeptidase and LD-carboxypeptidase activities. The balance between the two activities is presumably dependent on the availability of the acceptors (D-amino-*meso*-A₂pm groups and free Lpp lipoprotein). The location of free Lpp lipoprotein at the outer side of the outer membrane (42) could be a factor limiting its use. An enzyme showing both LD-transpeptidase and LD-carboxypeptidase activities was reported previously for *E. faecalis* (158).

Dipeptide Subunits

The presence of 3% of dipeptide subunits in isolated PG (74) could result from the cleavage of the γ -D-Glu→(L)-*meso*-A₂pm bond in tri- and tetrapeptide subunits by the MpaA endoamidase. However, having no effect on disaccharide muropeptides and being located in the cytoplasm, its *in vivo* action on PG is unlikely (196, 269). Another possibility is that dipeptide subunits originate to a small extent from the use of the UDP-MurNac-dipeptide precursor by the biosynthesis pathway in parallel with UDP-MurNac-pentapeptide. The ratio of dipeptide/pentapeptide precursor pool levels is in agreement with the observed 3% of dipeptide subunits (172). A high PG dipeptide subunit content was observed previously for a *Staphylococcus aureus* mutant accumulating large amounts of UDP-MurNac-dipeptide (192).

1,6-Anhydro-N-Acetylmuramic Acid

An important structural feature of *E. coli* PG is the presence of a 1,6-anhydro-MurNac residue at the reducing end of the glycan chains, as substantiated by the extremely low reducing power of isolated PG (304) and the presence of 1,6-anhydro-MurNac muropeptides accounting for 3 to 4% of the total muropeptide composition (74). This percentage is a measure of the average length of the glycan chains. By pulse and pulse-chase experiments, it was established that the glycan chains of nascent PG undergo a shortening from 50 to 60 disaccharide units to 31 disaccharide units in less than 5 min after formation, implying the action of LTs (75).

The formation of the glycan chains in the periplasm proceeds by the repetitive addition of the disaccharide units, with the reducing end of the growing chain remaining bound to undecaprenyl pyrophosphate (287). It was proposed that the termination of the polymerization reaction entails the release of the newly synthesized glycan chains from undecaprenyl pyrophosphate and the formation of 1,6-anhydro-MurNac ends (132). Owing to their enzymatic activity, LTs should in some way be responsible for this process. However, a multiply defective mutant lacking six (Stl70, MltA, MltB, MltC, MltD, and MltE) out of the seven identified LTs exhibited no significant decrease of the 1,6-anhydro-MurNac muropeptide content (92). Moreover, the PG material synthesized *in vitro* by purified glycosyltransferases remained bound to undecaprenyl pyrophosphate (8, 300). Thus, neither glycosyltransferases nor most LTs appear to be directly involved in the termination

step. The enzymes catalyzing the release of undecaprenyl pyrophosphate, the formation of the 1,6-anhydro ends, and the shortening of the glycan chains during maturation remain to be clearly identified.

N-Acetylmuramoyl-L-Alanine Linkage

The muropeptide composition of isolated *E. coli* PG showed that MurNac residues and peptide subunits were present in roughly equivalent amounts, and no muropeptide lacking the GlcNac-(1→4)-MurNac disaccharide was reported (74, 304). Therefore, if not nil, the number of cleaved MurNac→L-Ala bonds in PG is low. The free N-terminal L-alanine amino groups in PG from cells grown under different conditions accounted for 1 to 2.5% of the total muramic acid content and were presumably those of truncated oligomer units (197). Cells harvested without rapid cooling have more cleaved MurNac→L-Ala bonds, and their muropeptide composition shows the presence of free disaccharide and a truncated dimer (197, 283). Supposedly, only the amidase AmiA, AmiB, AmiC, or AmiD, having a known action on isolated PG (91, 200, 270, 273), is responsible for the *in vivo* cleavage of MurNac→L-Ala bonds, which has also been observed for many other organisms.

HYDROLASES AND PEPTIDOGLYCAN TURNOVER

As PG is an essential continuous structural component of the cell envelope, its synthesis is correlated with cell growth and division. The incorporation of new PG material has been studied by various labeling and microscopy approaches (48, 313). The results are consistent with the presence of different PG-synthesizing machineries in a given organism. In *E. coli*, one is associated with cell elongation, while another one is involved in cell septation (48). The muropeptide composition is the same in lateral and septal PG, suggesting that the maturation reactions are also the same throughout the cell cycle (47, 183).

In growing *E. coli* cells, PG synthesis is associated not only with maturation reactions but also with a 40 to 50% turnover per generation of the cell wall PG (83, 196). It is possible to study lateral wall and septal PG turnovers independently from one another by specifically blocking septation or elongation (48, 271) or by using synchronously growing cells (47). Lateral wall and septal PGs undergo 60 and 30% turnovers, respectively, but cell pole PG does not and remains stable (33, 52). In the process, PG is continuously degraded in the periplasm to GlcNac-(1→4)-1,6-anhydro-MurNac-peptide monomers, GlcNac-(1→4)-1,6-anhydro-MurNac disaccharides, and free peptides (83, 196). Owing to size exclusion by the pores of the outer membrane, only some free peptides are released into the medium (82), whereas the greater part of the turnover products is reused in a recycling process (83, 196). The formation of mostly truncated monomer PG fragments is evidence for the combined action of LT, endopeptidase, and amidase activities. It is presently difficult to say which specific hydrolases play a role in lateral wall or septal PG turnover and whether they function randomly or in a defined order.

HYDROLASES AND LATERAL WALL PEPTIDOGLYCAN

The formation of nascent lateral wall PG proceeds by the incorporation of precursors at a limited number of discrete sites, which are highly mobile and distributed over the cell surface (48). Since nascent PG does not exist as free material in *E. coli* (33, 81), the extension of the growing glycan chains must proceed simultaneously with cross-linking to preexisting PG. Furthermore, the enlargement of lateral wall PG implies the cleavage of old bonds to enable the intercalation of the new glycan chains while preserving PG integrity. Different models describing the insertion of new glycan chains into lateral wall PG have been proposed (34, 41, 47, 103, 193).

In the cut-and-insert model (193), the insertion of a new single glycan chain is associated with the simultaneous action of an endopeptidase opening cross-linkages in preexisting PG (34). To avoid any rupture in the stress-bearing PG layer, the process implies a tight coordination between the endopeptidase activity and the cross-linkage-forming transpeptidases. In this model, the insertion of incipient PG and PG turnover do not appear to be necessarily linked processes (196, 271).

The three-for-one model is an elaborate two-step process (103). First, a triplet of new glycan chains is cross-linked to preexisting glycan chains, and this addition occurs adjacently to the cytoplasmic membrane. In the second step, the removal of a nearby preexisting chain by the endopeptidase cleavage of its cross-linkages leaves space for the incoming new material. As a result, three new glycan chains replace the preexisting one with a preservation of PG integrity, hence the name of the model. The released chain is degraded at the outer side of the multilayered PG (75). According to this inside-to-outside growth mechanism, insertion and turnover are two closely coupled processes (75).

It has not yet been determined which specific endopeptidases or LTs are involved in the enlargement process. Although all possible mutational combinations of hydrolase have not yet been considered, many of the single or multiple mutants of the endopeptidases and LTs constructed so far show normal growth, and it has been questioned whether these hydrolases are truly essential for the synthesis of lateral wall PG (92, 211). *In vitro* protein-protein interactions between Slt70 or MltA and PBP1b or PBP2 have been described (293, 297). An association of PG hydrolases with the multienzyme complex (elongase) responsible for lateral wall PG synthesis has been considered (48), but evidence is still lacking.

HYDROLASES AND SEPTAL PEPTIDOGLYCAN

During cell division, it is obvious that septal PG must be split to promote the separation of the daughter cells and that hydrolases are involved in the process. The formation and turnover of septal PG were investigated by the specific PG labeling of growing cells (47, 48, 52, 271). The septum is made exclusively of 40% of the newly synthesized cell PG, over 30% of which is rapidly degraded during septation, while the remaining hydrolysis-resistant part ends up in the stable poles formed after cell separation. Although quantitatively less important than that of lateral wall PG, septal PG turnover proceeds at a much higher rate, since it is functioning over a shorter time of the cell cycle. It was proposed that the rapid removal of excess

septal PG is responsible for the separation of the new poles of the daughter cells (271). The involvement of LTs, endopeptidases, and amidases in septal turnover was substantiated by an analysis of the degradation products originating from radiolabeled septal PG (271). How the degradation of septal PG correlates with the splitting of the septum remains a matter of debate (92, 271). Supposedly, cell division follows an ordered sequence of events (initiation, invagination, septation, and cell separation), with different hydrolases functioning at different stages of the process.

Studies of certain defective mutants and subcellular localizations have given a few hints as to how hydrolases are involved in cell division. The role of the AmiA, AmiB, and AmiC amidases in septal PG turnover is illustrated by a triple-defective amidase mutant characterized by long chains of cells connected by both complete and incomplete septa, as revealed by electron microscopy analysis of whole cells and isolated sacculi (91, 211). Fluorescence microscopy confirmed the existence of rings on sacculi (211). The septal rings are material accumulating by continued PG synthesis after the arrest of turnover (211). They are observable in any of the three possible double amidase mutants but not in any of the single amidase mutants, indicating that all three amidases have a function in septal PG turnover (211). During the cell cycle, AmiC physically localizes mostly to the septal ring in the periplasm. The N-terminal nonamidase domain of AmiC is both necessary and sufficient for the targeting of the enzyme to the septal ring (19). Moreover, AmiC requires FtsN for recruitment to the septal ring and appears localized at the time of constriction initiation (20).

Presently, there are limited data on the role played by the PBP4, PBP7, and MepA endopeptidase activities in septal PG turnover. Their deletion has no morphological effect except when made in the triple-amidase-defective mutant (91, 210). On the other hand, the deletion of the putative EnvC endopeptidase alone or in combination with the NlpD LytM factor leads to morphological changes similar to those observed for the amidase mutants (272). Experiments with fluorescent fusion proteins revealed that both EnvC and NlpD are recruited to the septal ring during cell division, indicating that they play a role in septation (272). EnvC functions only at a very early stage of cell septation, since it localizes to the nascent division site prior to the start of active invagination and disappears from the septal ring in deeply constricted cells (20, 91, 92). A striking finding is the activation of the AmiA and AmiB activities by EnvC and that of AmiC by NlpD observed in *in vitro* assays with purified proteins and dye-labeled PG (273). EnvC and NlpD thus appear as regulators of the amidases involved in septal PG turnover. The mechanism of this activation remains to be determined.

The DD-carboxypeptidase PBP5 is involved in both elongation and septation, as established by immunolocalization and the use of fusions with green fluorescent protein (48, 205). When septation is inhibited, PBP5 shows a random and dispersed membrane localization, whereas it localizes almost exclusively at the septum in spherical cells obtained after the inhibition of elongation and even prior to the appearance of visible constriction. This indicates that PBP5 is where the peptidoglycan-synthesizing activity is highest, meaning that its interaction with pentapeptide subunits of incipient PG determines its localization. Interactions of PBP5 with proteins of the

elongation or septation complexes are not required (48, 205). The importance of PBP5 in determining and maintaining cell morphology by controlling the availability of pentapeptide subunits for transpeptidation has been stressed (170, 185), but many aspects of the process remain unknown (205).

HYDROLASES AND PEPTIDOGLYCAN RECYCLING

In *E. coli*, over 90% of the products generated by PG turnover return to the cell for reuse (196). The pathways of this recycling have been worked out and reviewed in detail (196, 204). Most enzymes of the process were characterized, and the effect of their deletion was studied. Briefly, recycling proceeds according to three successive steps: (i) transport of the turnover degradation products generated in the periplasm into the cytoplasm, (ii) further intracellular degradation, and (iii) reuse of the final products.

After PG degradation in the periplasm, the resulting GlcNAc-(1,4)-1,6-anhydro-MurNAc-peptides are transferred into the cytoplasm by the highly specific AmpG permease (38), while the free PG peptides not released into the medium are taken up by peptide permeases (83, 195). The incorporated fragments are further degraded by the cytoplasmic AmpD, NagZ, and LdcA hydrolases into the L-Ala- γ -D-Glu-(L)-*meso*-A₂pm tripeptide, D-alanine, GlcNAc, and 1,6-anhydro-MurNAc (196). The tripeptide is ligated into UDP-MurNAc by the Mpl ligase, and the resulting tripeptide nucleotide precursor returns to the biosynthesis pathway (96, 176). GlcNAc and 1,6-anhydro-MurNAc are both converted into GlcNAc-6-phosphate, which is available for reuse (119, 196, 204). The kinase NagK produces GlcNAc-6-P directly from GlcNAc, whereas the conversion of 1,6-anhydro-MurNAc is a two-step process. The kinase AnmK produces the MurNAc-6-phosphate converted to GlcNAc-6-phosphate by the MurQ etherase. A structural model of the *E. coli* MurQ etherase was generated, and a reaction mechanism for the cleavage of the D-lactylether bond by MurQ yielding MurNAc-6-phosphate was proposed (119).

The deletion of AmpD or NagZ has no effect on cell growth (196). However, the LD-carboxypeptidase LdcA is essential at the onset of the stationary phase, since an LdcA-deficient mutant forms thick oval cells, many of which lyse (256). A similar phenotype was observed with dithiazoline-inhibited LdcA (10). In the absence of LdcA activity due to mutation or inhibition, the L-Ala- γ -D-Glu-(L)-*meso*-A₂pm-(L)-D-Ala tetrapeptide accumulates and is used by Mpl to form UDP-MurNAc-tetrapeptide (96, 256). Tetrapeptide subunits are incorporated into PG instead of pentapeptide subunits, leading to a cross-linkage deficiency and lysis. The effect due to the absence of LdcA activity suggests that at the onset of the stationary phase, recycling may be of critical importance for sustaining the prolonged synthesis of structurally modified PG resistant to autolysis (24).

Besides recycling its own PG, *E. coli* is able to grow on PG fragments, GlcNAc, and MurNAc as the sole carbon and nitrogen sources (118, 197, 306). Presumably, hydrolases of the recycling process, together with other cell enzymes, have an important role in the uptake and use of these exogenous products as metabolic fuel. In particular, this is exemplified by the action of the MurQ etherase in connection with growth on MurNAc (119).

HYDROLASES AND AUTOLYSIS

Being responsible for the mechanical resistance of the bacterial cell wall, PG is essential for preserving cell integrity. Its degradation by the uncontrolled action of endogenous PG hydrolases is potentially dangerous for cell survival, leading to the rupture of the cell envelope and lysis (238, 295). Although not as readily prone to autolysis as other bacterial organisms (248), *E. coli* cells lyse under a wide variety of conditions not always explicitly correlated with PG degradation. Here, attention is limited to autolysis induced by the inhibition of PG synthesis in growing cells, to the resistance of nongrowing cells to autolysis, and to the autolysis of harvested cells. Various aspects of the role of *E. coli* PG hydrolases functioning as autolysins have been reviewed (53, 99, 100, 102, 103, 264, 265).

Autolysis of Growing Cells by Inhibition of PG Synthesis

In growing *E. coli* cells, the steady formation and insertion of new material in the lateral wall and septum compensate for the loss of PG material by turnover. It can be speculated that any abrupt slowdown or arrest of the polymerization reactions will result in an unbalanced state where hydrolases presumably continue to cleave PG bonds unabated, leading to excessive PG degradation and cell lysis. In this case, autolysis is not due to any specific induction of autolysins but rather to an uncoupling between synthesis and degradation owing to an absence of a control of the turnover hydrolases. Autolysis by the inhibition of PG synthesis is thus a two-step process (203), with the initial one being a deficiency in PG polymerization due to the depletion of the lipid II pool or to the inhibition of the HMM PBPs. In the second step, the hydrolases involved in turnover are presumably responsible for PG degradation. This simple model is supported by the similar release of monomer PG fragments during autolysis (127) and turnover (83, 196), indicating the same combined action of LT, endopeptidase, and amidase activities. However, this does not exclude the action of hydrolases other than those of the turnover process. Another way of promoting lysis is not to inhibit PG synthesis but to favor an increase of the rate of PG degradation. This could be one cause of the lytic effect observed with the overproduction of certain hydrolases (Slt70, MltA, MltB, MltD, MltF, EnvC, AmiB, and AmiC).

Autolysis of Growing Cells by Depletion of the Lipid II Pool

In *E. coli*, most enzymes of the linear biosynthesis pathway leading to lipid II are unique and essential (5, 224, 286). Interference with any step has an effect on the downstream precursors and, consequently, on the lipid II pool level. This is illustrated by the autolytic effect of specific inhibitors such as DON (6-diazo-5-oxo-L-norleucine), fosfomycin, or D-cycloserine (40, 129, 153, 173). Vancomycin, which complexes lipid II, can also bring about the lysis of *E. coli* at high concentrations (136). Many temperature-sensitive mutations specifying enzymes of the biosynthesis pathway and auxotrophic conditional-lethal mutations involving essential metabolites (Table 2) have a phenotype of osmotic fragility. The shift of the mutants to nonpermissive conditions brings about cell lysis. More generally, the depletion of virtually any metabolite essential for a

TABLE 2. Examples of autolysis of *E. coli* by inhibition of lipid II synthesis

Metabolic target	Inhibitor	Mutant ^a	Reference(s)
GlmS synthetase	DON	<i>glmS</i>	227, 309 40
GlmM mutase		<i>glmM</i>	177
GlmU synthetase		<i>glmU</i>	175
MurA transferase	Fosfomycin Protein A2		173, 292 18
D-Ala racemase	D-Cycloserine Alaphosphin	<i>alr</i>	307 45 4
D-Ala-D-Ala ligase	D-Cycloserine	<i>ddl</i>	153 45
D-Glu racemase		<i>murI</i>	59
A ₂ pm synthesis		<i>a₂pm</i>	169
MurC synthetase	Excess L-serine	<i>murC</i>	151 314
MurE synthetase		<i>murE</i>	152, 178
MurF synthetase	Diarylquinoline	<i>murF</i>	151 11
Promoter <i>mra</i>		<i>mra</i>	87
MraY transferase	Protein E Colicin M	<i>mraY</i>	27 17 65
MurG transferase	Vancomycin Colicin M	<i>murG</i> <i>murG</i>	225 174 136 65

^a Mutant refers to a point mutation or a gene deletion.

step of the biosynthesis pathway has an autolytic effect. This explains why autolysis occurs under many different circumstances. The 40 to 50% PG breakdown observed prior to cell lysis (174, 177) is consistent with the lowest PG content found to be compatible with cell viability (207). Table 2 summarizes examples of autolysis due to interference with the early steps of PG biosynthesis.

Autolysis of Growing Cells by Inhibition of PG Polymerization

E. coli HMM PBPs are responsible for the formation of the glycan chains by transglycosylation and of the D-Ala→(D)-*meso*-A₂pm cross-linkages by DD-transpeptidation (109, 110, 202, 251). PBPs 1a, 1b, and 1c catalyze both types of reactions, whereas PBP2 and PBP3 are limited to transpeptidation (48, 229). The inhibition of transglycosylation by moenomycin (287, 305) has a lytic effect on *E. coli* (129, 140, 141, 284). In particular, the PG degradation of growing cells treated with moenomycin does not exceed 30% at the onset of lysis, accompanied by an irregular attack of the PG layer (141).

The inhibition of DD-transpeptidation by β-lactams like penicillin, which has similar affinities for all five HMM PBPs, leads to lysis (44, 241). The highest rate of lysis is observable only when all PBPs are β-lactam saturated (228). The specific targeting of PBPs 1a and 1b by cefsulodin also has a lytic effect, whereas the preferential binding of amdinocillin or aztreonam to PBP2 or PBP3, respectively, has morphological effects (spherical or filamentous cells), with later lysis, at least in fast-growing cells (44, 202, 228, 241, 245). Similar results were found when singly and multiply defective mutations specifying the HMM PBPs were considered (49, 245, 250). Furthermore, the simultaneous use of two β-lactams targeting different HMM PBPs has a synergistic effect that also causes rapid lysis, even though each drug is at a sublytic concentration (85). Yet another kind of interference with PG polymerization leading to lysis was reported when inactive variants of the polymerase PBP1b were overproduced in cells with a normal level of wild-type PBP1b (171).

Morphological Changes Accompanying Autolysis of Growing Cells

Phase-contrast or electron microscopy of whole cells or isolated sacculi revealed the important morphological changes observed for *E. coli* prior to the onset of autolysis (53, 86, 126, 138, 141, 150, 212, 237). These changes include an increase in size, a loss of regular morphology, the appearance of protrusions (usually in the middle of the cell), filamentation, and spheroplasting.

The penicillin-induced formation of spheroplasts initially revealed the correlation between the loss of PG integrity and autolysis in *E. coli* (86, 138). When growing cells are treated with penicillin, the rate of PG synthesis is reduced by 55 to 75% prior to the onset of lysis (129, 184), and the rate of the loss of PG with different β-lactams does not exceed 20 to 35% (126, 141). The correlation between the β-lactam inhibition of PG transpeptidation, PG degradation by endogenous hydrolases, and the induction of autolysis was established (127, 235, 236, 237).

In penicillin-treated cells, bulges appear at the septum or at the presumed septum location, and sacculi are split at the midcell precisely into two halves (237). The lytic responses to β-lactam treatment correlate with the inhibition of lateral wall or septal PG synthesis (53, 237). An electron microscopy study of sacculi from cefsulodin-treated cells showed that lysis started earlier and was clearly faster in a strain with septal PG synthesis than in one without septal PG synthesis, and cells were cut at the septum (53). In contrast, strains with only lateral wall PG synthesis showed a general weakening of the PG network with random breakpoints. These results are consistent with the far-higher rate of septal turnover (271).

Resistance of Nongrowing Cells to Autolysis

The rate of autolysis of *E. coli* is highest for fast-growing cells but low or nil for nongrowing cells (24, 25, 86, 139, 164, 264). During entry into stationary phase, PG progressively undergoes considerable structural modifications. It becomes more cross-linked and has more covalently bound Lpp lipoprotein, more (L)-*meso*-A₂pm→(D)-*meso*-A₂pm cross-link-

ages, and shorter glycan chains (201, 267). It was suggested that these changes are responsible for the greater resistance to autolysis observed in the stationary phase (268). In particular, nongrowing cells are generally not prone to lysis when treated with most β -lactams and are characterized by the phenomenon of phenotypic tolerance (265).

The inhibition of the protein synthesis of growing cells by amino acid deprivation gives rise to resistance to antibiotic-induced autolysis after the arrest of growth (265). The rapid modifications of the PG structure taking place after amino acid starvation could be responsible for the resistance to autolysin attack (268). The stringent response (35) also has a role in resistance to autolysis via the inhibition of phospholipid synthesis, which is essential for both PG synthesis and autolysin activity (216, 217). However, when protein synthesis is inhibited in the absence of stringent control (the use of chloramphenicol or a *relA* mutant), the observed β -lactam-induced lysis is due to a too-low level of PG polymerase activity (218).

Certain β -lactams (mostly penems) can induce the lysis of amino-acid-starved cells although retaining an intact stringent response (264, 265). They all bind to PBP7, and conversely, β -lactams without a lytic effect do not bind well to PBP7 (43, 266). It can be speculated that by its enzymatic specificity for D-Ala \rightarrow (D)-*meso*-A₂pm cross-linkages (222), PBP7 endopeptidase activity has a role in the PG structural changes generating autolysis resistance at the onset of the stationary phase. Inhibition by penems could favor the maintenance of growing-phase D-Ala \rightarrow (D)-*meso*-A₂pm cross-linkages and a PG structure sensitive to lytic attack. However, the importance of the binding of penems to PBP7 is disputed by the fact that PBP7 mutants are fully viable (94). Thus, it is unclear what the binding of penems to PBP7 means as far as cell lysis is concerned. Another possibility is that penems inhibit the LD-transpeptidases responsible for forming (L)-*meso*-A₂pm \rightarrow (D)-*meso*-A₂pm cross-linkages and thereby contribute to the maintenance of an autolysin-sensitive PG structure. A penem-sensitive LD-transpeptidase from *Enterococcus faecium* has been reported (160).

Autolysis of Harvested Cells

Wild-type *E. coli* cells generally do not lyse spontaneously at a high rate after harvesting and resuspension in a NaCl solution or buffer (90, 140, 248). Obviously, after the arrest of cell growth, PG hydrolases undergo tight control with the setup of a barrier between them and PG by an unknown mechanism. Supposedly, the harvesting of cells leads to a change in the dynamic status of the cell envelope and to the segregation of the autolysins from PG. Some kind of triggering is necessary to promote the breakdown of the barrier and the attack of PG by the autolysins (90). This is brought about by submitting harvested cells to osmotic shocks or by treating them with high-salt concentrations, sucrose, trichloroacetic acid (TCA), EGTA, EDTA, or chaotropic agents (90, 108, 139, 239). These treatments disorganize the cell envelope and lead to high rates of extensive cell lysis, with over 60 to 80% PG breakdown, as visualized by the disappearance of the PG material over the whole cell surface (141). During TCA-induced autolysis, monomer muropeptides and free peptides are released, indi-

ating that, like with growing cells, LTs, endopeptidases, and amidases are operating (127).

Inhibitory Effect of Mg²⁺ Ions on Autolysis

The dramatic effect of preventing lysis by the addition of 20 mM Mg²⁺ was initially reported for penicillin-treated cells (168) and can be observed for many types of autolyses of both growing and harvested *E. coli* cells (90, 140). The mechanism of the Mg²⁺ effect is unknown. The importance of divalent cations in the organization of the outer membrane and their role in the stabilization of the lipopolysaccharide (LPS) monolayer portion of the outer membrane has been stressed (190). It can be speculated that the binding of Mg²⁺ ions to LPS induces a change of the outer membrane structure favoring the segregation of the autolysins from the normally closely associated PG layer. On the other hand, the removal of the Mg²⁺ ions by chelators will lead to the destabilization of the LPS monolayer, the disorganization of the outer membrane, and the uncontrolled action of autolysins.

CONCLUDING REMARKS

In *E. coli*, there is a hydrolytic activity for each bond linking the PG amino acids and sugars. Owing to redundancy, PG hydrolases far outnumber the enzymes of the biosynthesis pathway. The presence of two sets of highly specific enzymes acting on the same bonds raises the question of possible functional correlations or interferences. When cytoplasmic and cell envelope localizations are considered, few hydrolases, if any, are found simultaneously in both compartments, at least in nonoverproducing strains (Table 1).

The cytoplasmic hydrolases appear to act independently from the enzymes responsible for the assembly of the PG monomer unit, which are cytoplasmic or operating at the inner side of the cytoplasmic membrane (286). No interaction between them has been reported, and interferences are avoided in different ways. The hydrolases NagZ, AmpD, and MpaA, involved in recycling, have little or no action on the nucleotide precursors (196). The LD-carboxypeptidase LdcA, which prevents the deleterious accumulation of UDP-MurNAc-tetrapeptide, also has no effect on the precursors owing to a specificity limited to tetrapeptide-containing monomer derivatives (142, 256, 274). Furthermore, the dipeptidase DdpX is without an effect on the D-alanyl-D-alanine pool in growing cells produced in the stationary phase. Finally, a possible hydrolytic activity associated with an enzyme of the biosynthesis pathway has been seldom considered (63, 78) and has not been reported for *E. coli*. The reversibility of biosynthesis steps has been observed in a few cases but only in the presence of the reaction products (7). Thus, PG hydrolases and biosynthesis enzymes have both distinct reaction mechanisms and no overlapping functions.

The envelope-bound hydrolases localize to the outer membrane, in the periplasm, or at the outer side of the inner membrane. Few of them appear to function at the inner side of the cytoplasmic membrane, and it is thus unlikely that any envelope-bound hydrolases have an action on the strictly cytoplasm-located nucleotide precursors, which can nonetheless be used as substrates in *in vitro* assays. The formation of lipid

precursors I and II at the inner side of the cytoplasmic membrane by the transferases MraY and MurG is characterized by their low pool levels and high turnover rates (26, 288). The importance of protecting these lipid intermediates from the potential deleterious effect of hydrolases has been stressed (37). Presumably, the direct channeling of lipid I from MraY to MurG and that of lipid II from MurG to the extracytoplasmic polymerases, perhaps via the hypothetical translocase MurJ (224), avoid their presence as free components in the membrane and their attack by hydrolases.

The way in which the hydrolase activities involved in PG maturation and turnover correlate with the PG-forming HMM PBPs remains largely unknown. Protein-protein interactions with HMM PBPs (293, 297) and subcellular localizations suggest possible associations of hydrolases with the PG-synthesizing machineries responsible for lateral wall and septal PG syntheses. However, clear evidence that PG hydrolases have an essential function in lateral wall PG synthesis is an uncertain matter (104). Although considered a possibility (48), no PG hydrolase has yet been reported to be associated with the elongation complex.

Fifty years of research work have led to the discovery of at least 35 PG hydrolases in *E. coli*, and perhaps more can exist, as judged by the set of PG hydrolases proposed for *B. subtilis* (240). Despite these efforts, our present knowledge of these activities is still fragmentary. Many questions concerning genetic, biochemical, and physiological aspects remain unanswered. It is unknown whether most PG hydrolases are produced constitutively or not. A few examples show that there are variations in hydrolase activity with the growth phase or the cell cycle. This is the case for PBP6 (31, 226), DdpX (144), and LD-carboxypeptidase (13, 14). Although nearly all PG hydrolase genes have been identified, mutational studies have led to disappointingly modest results (49, 92), since many single deficiencies have no effect on growth, morphology, or PG structure.

After purification to homogeneity, the crystal structures and reaction mechanisms of certain *E. coli* hydrolases have been investigated, while the study of the biochemical properties of other ones has not yet been initiated. Moreover, the *in vivo* levels of activity of many hydrolases remain poorly defined. Cell copy numbers are available for some PBPs (60, 243), but we have no idea at what level hydrolases identified only by their gene sequence are present in wild-type cells.

PG hydrolases are involved in various important structural modifications of PG taking place during the growth and stationary phases. The proper control of the PG hydrolases and mostly of those involved in the turnover process is critical for cell integrity and survival. Our present knowledge of the mechanisms regulating the *E. coli* PG hydrolases is yet limited to a few examples: the stringent control modulating Slt70 activity (22); BolA controlling the levels of transcription of PBP5 and PBP6 (226); the activation of AmiA, AmiB, and AmiC amidase activities by EnvC and NlpD (272); and the upregulation of the transcription of *amiA* and *amiC* by the CpxR/CpxA system (302).

A few hydrolases have reasonably well-defined functions. This is true for PBP5 (205), the LD-transpeptidases involved in maturation (159), and the hydrolases of the recycling process (196). The LD-transpeptidases can seemingly function as both

hydrolases and transferases, a possibility not often considered for PG hydrolases (104). LT, endopeptidase, and amidase activities are clearly responsible for turnover and autolysis, but it is difficult to assess whether they fulfill other specific functions.

The exact cellular functions of many PG hydrolases remain elusive, and it is difficult to understand why *E. coli* keeps such a large set of PG lytic enzymes, many of which are seemingly nonessential under laboratory culture conditions. The redundancy of the PG hydrolases and subtle differences in substrate specificities between the enzymes of each family certainly reflect a large variety of as-yet-unidentified specific physiological functions. Their study will be a far more difficult challenge and a longer task than the elucidation of the steps of the PG biosynthesis pathway.

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