How Bacteria Consume Their Own Exoskeletons (Turnover and Recycling of Cell Wall Peptidoglycan)†

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INTRODUCTION

Many studies of bacterial cell walls have been concerned with their structures and mechanisms of synthesis. Much less studied and hidden from view is the story of how gram-negative bacteria such as Escherichia coli break down and reutilize all components of their cell wall peptidoglycan (PG). Our present knowledge of PG recycling is actually quite limited and fragmented. This review brings together these diverse observations and assesses their significance.

Overview of PG Recycling

Nine enzymes, one permease, and one periplasmic binding protein that appear to have the recovery of degradation products from PG as their sole function, thereby making them
available for the cell to resynthesize more PG or to use as an energy source, have been identified in *E. coli*. Since PG represents only 2% of the cell mass, it does not represent a large source of nutrients. However, faced with a sudden loss of carbon, recycling may provide the necessary building blocks to complete a round of cell division and enter stationary phase. Alternatively, in the competition for food in the large intestine, the ability to recycle PG may have survival value. As will be discussed below, gram-negative bacteria living in the colon tend to have a complete repertoire of enzymes for recycling, whereas other organisms have a more limited but nevertheless purposeful arsenal.

What is known is that recycling is not essential under laboratory conditions. *E. coli* cells lacking *ampG* permease (essential for recycling) grow well in minimal media, although cells freshly transduced to *H9004* initially grow poorly for unknown reasons. With one exception, mutations in the genes known to be involved in recycling have no obvious phenotype. Thus, the genes do not appear to be required for viability, normal growth rate, normal morphology, or normal PG content. All of the enzymes are produced and are active during logarithmic growth. All are active on substrates that are present at concentrations in the range of 10^{-5} to 10^{-6} M. A loss of one of the enzymes usually results in the accumulation of millimolar amounts of its substrate. This may be taken to be an indirect indication of their normal activity.

Table 1 lists the proteins associated with PG recycling, their genes, their locations in the cell, and references.

**Biological Effects of PG Recycling Intermediates**

A discussion of the known biological effects of PG recycling intermediates is beyond the scope of this review. Activities such as cytotoxicity, pyrogenicity, arthritogenicity, and sleep promotion are well documented and have been reviewed by Adam and Lederer (1). Recent work has focused on the role of PG recycling in pathogenicity and the role of PG fragments in stimulating the innate immune response. Excellent reviews of these subjects by Boneca (11), Chaput and Boneca (19), and Cloud-Hansen et al. (23) have recently been published.

**PG Structure and Composition**

The term PG was originally defined as a class of polymers composed of approximately equal amounts of amino acids and sugars, and the term murein was reserved for the PG present in the cell envelopes of bacteria. However, as the only PG polymer presently recognized in nature is that found in bacterial cell walls and since the term murein is easily confused with murine, PG has been used increasingly in place of murein as a name for the polymer. The term murein sacculus survives to describe the rigid, shape-forming component of the cell envelope of bacteria that is composed of PG. In this review, PG and murein will be used interchangeably to refer to the polymer, and cell wall and murein sacculus to refer to the rigid structure free of inner and outer membranes, which together with the cell wall make up the cell envelope.

As shown in Fig. 1, PG consists of glycan strands cross-linked by short peptides referred to as stem peptides. The glycan strands consist of alternating units of N-acetylglucosamine (GlcNAc)- and N-acetylmuramic acid (MurNAc)-linked β-1,4. MurNAc is a derivative of GlcNAc with D-lactic acid attached to position 3 by an ether linkage. In gram-negative bacteria, the stem peptide attached to the carboxyl group of each muramic acid usually consists of L-Ala-D-Glu-(L)-meso-diaminopimelic acid (Dap)-D-Ala, although the stem peptides of other bacteria differ in their amino acid composition.

**Table 1. Murein recycling enzymes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Activity</th>
<th>Location</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ampG</em></td>
<td>GlcNAc-anhMurNAc permease</td>
<td>Inner membrane</td>
<td>21, 57</td>
</tr>
<tr>
<td><em>ampD</em></td>
<td>anhMurNAc-l-Ala amidase</td>
<td>Cytoplasm</td>
<td>48, 57</td>
</tr>
<tr>
<td><em>mpl</em> (yjfG)</td>
<td>UDP-MurNAc-L-Ala-γ-D-Glu-Dap ligase</td>
<td>Cytoplasm</td>
<td>46, 83</td>
</tr>
<tr>
<td><em>ldeA</em> (E04 gene)</td>
<td>LD-Carboxypeptidase</td>
<td>Cytoplasm</td>
<td>115, 124</td>
</tr>
<tr>
<td><em>mpaA</em> (ycfI)</td>
<td>γ-D-Glu-Dap amidase</td>
<td>Cytoplasm</td>
<td>120</td>
</tr>
<tr>
<td><em>ycfG</em></td>
<td>l-Ala-l-Ala-Glu epimerase</td>
<td>Cytoplasm</td>
<td>105</td>
</tr>
<tr>
<td><em>nagZ</em> (ycfO)</td>
<td>β-N-Acetylglucosaminidase</td>
<td>Cytoplasm</td>
<td>20</td>
</tr>
<tr>
<td><em>nagA</em></td>
<td>GlcNAc-6-P deacetylase</td>
<td>Cytoplasm</td>
<td>130</td>
</tr>
<tr>
<td><em>annK</em> (ydhH)</td>
<td>anhMurNAc kinase</td>
<td>Cytoplasm</td>
<td>121</td>
</tr>
<tr>
<td><em>murQ</em> (yfeU)</td>
<td>MurNAc-6-P etherase</td>
<td>Cytoplasm</td>
<td>123</td>
</tr>
<tr>
<td><em>amiD</em> (ybR)</td>
<td>anhMurNAc-l-Ala amidase</td>
<td>Outer membrane</td>
<td>118</td>
</tr>
<tr>
<td><em>mppA</em></td>
<td>murein peptide binding protein</td>
<td>Periplasm</td>
<td>94</td>
</tr>
</tbody>
</table>

**FIG. 1.** Basic structure of PG of *E. coli*.  

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TABLE 1. Murein recycling enzymes
peptide often lacks D-Ala or, more rarely, terminates in D-Ala-D-Ala. About one-half of the stem peptides are involved in cross-links between neighboring glycan strands. The glycan strand length varies widely, with an average length of 30 disaccharide-peptide units (47). A comprehensive review of PG composition in a wide variety of bacteria was done by Schleifer and Kandler (104).

Turnover of PG and Discovery of the Recycling of Murein Tripeptide

Turnover refers to the actual loss of PG components from the cell. However, the products of turnover are normally captured and reutilized by the cell by a process termed PG recycling. The PG recycling phenomenon was accidentally discovered by Goodell and Schwarz in 1985 during an investigation of cell wall turnover (40). At the time, there was evidence of substantial loss, i.e., turnover, of the cell wall murein of gram-positive bacilli such as Bacillus subtilis (77) and Lactobacillus acidophilus (12) and of the gram-negative organism Neisseria gonorrhoeae (38, 43). The rate of loss ranged from 25% to 50% per generation.

In marked contrast, turnover of E. coli murein ranged from 0% (77) to perhaps 5% per generation (18). When Goodell and Schwarz reinvestigated this phenomenon (40), they observed a 6 to 8% loss of Dap from the sacculus per generation. The Dap-containing materials released into the medium were identified as being L-Ala-γ-D-Glu-meso-Dap-D-Ala, L-Ala-γ-D-Glu-meso-Dap, and Dap-D-Ala. All of these results were obtained from pulse-chase experiments employing radioactive Dap. The small differences in turnover between these three reports were presumably due to strain variation or cultural conditions. However, as an internal control, no turnover of protein was detected in any of these studies.

Goodell and Schwarz then went on to do a pulse-chase experiment in which the Dap-containing compounds present in the cytoplasm were studied, and this led to the discovery that much of the Dap present in the wall was being recycled (40). An E. coli lys dap mutant was labeled with [3H]Dap for two generations and then chased. The pulse-chase experiment using [3H]Dap described by Goodell and Schwarz is shown in Fig. 2. During the first 2 h of chase, the amount of radioactive Dap in the sacculi actually increased as the cytoplasmic pool of Dap was utilized to synthesize PG. During this initial 2-h period, the free [3H]Dap content of the cytoplasm dropped almost 100-fold. In sharp contrast, the amount of radioactive Dap in the UDP-MurNAc-L-Ala-γ-D-Glu-meso-Dap-D-Ala-D-Ala (UDP-MurNAc-pentapeptide) pool decreased only two-fold, although one would expect it to decrease more rapidly than the Dap pool because of its smaller size. UDP-MurNAc-pentapeptide, a precursor for the synthesis of PG, is present in relatively high concentrations in the cytoplasm (approximately 0.27 mM in rich medium) (79) (see Fig. 3 for a diagram of the PG biosynthetic pathway). Radioactive Dap to replenish the UDP-MurNAc-pentapeptide pool could have come from PG only, and hence, recycling of cell wall material was occurring. From the data in that experiment, Goodell calculated that about 45% of the PG was being recycled per generation (37).

From data indicating that L-Ala-γ-D-Glu-[3H]Dap was taken up and used without degradation and that oligopeptide per-

FIG. 2. Kinetics of labeling of intracellular [3H]Dap-labeled compounds during a chase. (Reprinted from reference 40 with permission.)

FIG. 2. Kinetics of labeling of intracellular [3H]Dap-labeled compounds during a chase. (Reprinted from reference 40 with permission.)

mease (Opp) was required for its uptake, Goodell and colleagues speculated that recycling involved the cleavage of PG to release the tripeptide into the periplasm, followed by uptake via Opp, and the direct utilization of the tripeptide in the biosynthetic pathway (37, 39). As described below, only the last of these three predictions proved correct. Eight years after the discovery of recycling, it was demonstrated that the deletion of genes for Opp permease did not prevent recycling by E. coli (92). Instead of turning over 45% of their cell wall per generation, as predicted if cells required Opp for the uptake of compounds containing Dap, it was demonstrated that several opp-negative strains turned over only 0 to 8% of their Dap per generation, depending on the strain studied (92). Hence, contrary to the original prediction (37), Opp played no detectable role in recycling. Obviously, there must be another permease for the Dap-labeled compound(s). When this result was published, S. Normark (personal communication) suggested that AmpG was a possible permease candidate. AmpG is a membrane protein required for the induction of AmpC β-lactamase in several gram-negative bacteria such as Citrobacter freundii and Enterobacter cloacae, hence the name AmpG (67, 76). It was considered to function either as a sensor or as a permease for the activating molecule. When an ampG mutant was tested for turnover of cell wall Dap, a turnover rate of 40% per generation was observed (57). Thus, AmpG was the permease
required for the recycling of cell wall degradation products containing Dap. Note that *E. coli* does not have a /H9252-lactamase-inducible system, but most studies of /H9252-lactamase induction utilize plasmids in *E. coli* that carry the *ampC* and *ampR* genes from an inducible bacterium. Another protein has been named AmpD because the deletion or mutation of *E. coli ampD* led to the constitutive expression of *ampC* /H9252-lactamase from a plasmid carrying the *C. freundii ampR* and *ampC* genes. AmpD was thus considered to be a negative regulator involved in /H9252-lactamase induction (75). AmpD also proved to be required for recycling. In its absence, the turnover rate was comparable although slightly less than that in the *ampG* mutant (57). As demonstrated by molecular sieve chromatography, the cytoplasm of *ampD* cells labeled with Dap contained huge amounts of a compound or compounds with molecular weights (MWs) 600 to 1,000 that were not present in wild-type cells. The material did not accumulate in *ampG* cells, indicating that the material was derived from PG. An *ampG ampD* double mutant also contained only trace amounts of this material, which indicates that AmpG functions before AmpD.

The compound that accumulated was identified as being 1,6-anhydro-N-acetylmuramyl-L-Ala-D-Glu-Dap (anhMurNAc-tripeptide) (57). This at once suggested that anhMurNAc-tripeptide was the inducer of /H9252-lactamase in *ampD* cells and that AmpD served as a “negative regulator” of /H9252-lactamase synthesis by preventing its accumulation. Since *E. coli* does not have an inducible /H9252-lactamase operon, participation in PG recycling is probably the normal function of AmpG and AmpD. The relationship of recycling to /H9252-lactamase induction will be discussed below.

### Lytic Enzymes of *E. coli* and Their Role in PG Recycling

We shall now turn to the question of what compounds are being imported into the cytoplasm by the AmpG permease. The compounds must be derived from PG since they contain Dap. The PG degradation products released into the periplasm and, hence, available for uptake by AmpG permease were determined by the relative activities of the various lytic enzymes during the growth of *E. coli*. *E. coli* has three classes of enzymes that cleave bonds in PG. Class 1 includes endopeptidases such as penicillin-binding protein 4 (PBP4), PBP7, and MepA that cleave the cross-link between D-Ala of one glycan strand and Dap of a neighboring strand. Obviously, the endopeptidases acting alone would not release PG fragments. Class 2 includes the MurNAc-L-Ala amidases AmiA, AmiB, and AmiC, which are present in the periplasm and have been shown to participate in cell separation, that cleave the bond between MurNAc and the stem peptide of intact PG (44, 99). Another amidase, AmiD, which is present in the outer membrane, was recently identified (118). The amidases thus release murein tri-, tetra-, and pentapeptides into the periplasm from which they diffuse out of the cell, although a small fraction may enter the cytoplasm via the MppA-OppBCDF permease. Class 3 includes the lytic transglycosylases, most of which are exoenzymes, that cleave the glycan strand between carbon 1 of MurNAc and carbon 4 of GlcNAc and simultaneously form the 1,6-anhydro bond of anhMurNAc (49). The principal lytic transglycosylase responsible for the turnover of PG is SltY, a soluble lytic transglycosylase found in the periplasm. Figure 4 shows the release of GlcNAc-anhMurNAc-tetrapeptide from the end of a glycan strand by the exoenzyme SltY. In addition, there are multiple membrane-bound lytic transglycosylases (re-
viewed in reference 47). Some of these membrane-bound enzymes that cleave glycan strands are thought to serve specialized functions, such as providing space for a flagellum (31, 66, 102, 134). Surprisingly, mutants lacking three lytic transglycosylases (SfY, MtA, and MtB) grow normally, although the rate of turnover is reduced (69).

In addition to the lytic enzymes, PBP4, PBP5, and PBP6 have D,D-carboxypeptidase activity that converts stem pentapeptides to stem tetrapeptides (47). Mutations in some of the PG hydrolases affect septum cleavage (45) and also cell shape. DAP carboxypeptidases, for example, have D,D-carboxypeptidase activity that converts stem pen-tapeptides to stem tetrapeptides (47). Mutations in some of these membrane-bound enzymes appear to have evolved to allow cells to recycle the membrane proline (17). Interestingly, two of the three mutations previously reported to result in a loss of function (76) are in the cytoplasmic hydrophobic domains, which suggests a role for these regions in transport (17).

It is now known that AmpG is the permease involved in the transport of anhydromuropeptides (57). Nearly all of the Dap from PG that is turned over and recycled passes through the AmpG permease. The ampG gene and yajG, coded upstream of ampG, form an operon. However, it has been shown that yajG, a putative lipoprotein, was not involved in either β-lactamase induction or the expression of AmpG (76). AmpG, which belongs to the major facilitator superfamily, is an interesting permease in that it transports relatively large molecules (MW 992). Because freeze-thawed E. coli cells were known to be permeable to these large-MW muropeptides, a simplified method for measuring the uptake of radioactive muropeptides using freeze-thawed cells was developed (21).

Although the freeze-thawed cells exhibited high background (TP74 ampG) (Fig. 5), it was possible to demonstrate clearly that AmpG was specific for the free GlcNAc-anhMurNAc disaccharide and the disaccharide carrying stem peptides and that proton motive force was required (Fig. 5) (21). AmpG did not transport anhMurNAc-tripeptide; hence, GlcNAc-anhMurNAc-peptides, the primary products of the action of lytic transglycosylases on PG, are the compounds imported by AmpG.

**DISCOVERY AND CHARACTERIZATION OF ENZYMES INVOLVED IN RECYCLING PG AMINO ACIDS**

In order to study the fate of the PG amino acids, PGs labeled with [3H]Dap and various [3H]Dap-labeled substrates were prepared from the labeled murein or from extracts of mutant cells known to accumulate a given substrate of interest (21, 58, 118, 120). All the strains used in these studies lacked LysA to prevent the conversion of Dap to L-Lys. The following seven enzymes appear to have evolved to allow cells to recycle the amino acids from their murein (Fig. 6).
AmpD: Anhydro-N-AcetylMuramyl-t-Ala Amidase

As noted above, an E. coli strain lacking AmpD contained large amounts of anhMurNAc-tripeptide in its cytoplasm. This suggested that anhMurNAc-tripeptide might be a substrate for AmpD. When AmpD was cloned and a highly purified sample was obtained, it was shown to cleave the anhMurNAc-t-Ala bond. Interestingly, it proved to be highly specific for the anhMurNAc-t-Ala bond (58). The rate of cleavage of MurNAc-peptides and UDP-MurNAc-pentapeptide was at least 10,000 times slower than that of anhMurNAc-tripeptide (58). Thus, AmpD functioning in the cytoplasm rapidly cleaves the anh-MurNAc-peptides without destroying the UDP-MurNAc-pentapeptide needed for the synthesis of PG. Höltje et al. also demonstrated that AmpD was specific for the anhMurNAc-t-Ala bond from the anhMurNAc-peptides at the ends of PG strands but not from the MurNAc-t-Ala bonds in the remainder of the glycan strands (48).

The three-dimensional structure of AmpD from Citrobacter freundii has been solved by nuclear magnetic resonance (72). It contained a zinc ion that was shown to be essential for activity (33, 72). Replacement of one of the presumed zinc triad amino acids (His34, His154, and Asp164) with Ala resulted in a lack of a zinc ion in the active site and loss of amidase activity (33). Tyr63 and Lys162 were proposed to be involved in the binding of the substrate by site-directed mutagenesis (33).

Mpl: UDP-N-Acetylmuramyl-t-Alanyl-γ-D-Glutamyl-meso-Diaminopimelate Ligase

In Goodell’s study of PG recycling, it was demonstrated that the murein tripeptide t-Ala-γ-D-Glu-meso-Dap was reused directly without first being degraded to Dap (37). When L-Ala-γ-D-Glu-[3H]Dap was added to growing E. coli cells, radioactive UDP-MurNAc-pentapeptide and PG were formed even in the presence of a huge excess of nonradioactive Dap (37). One candidate for an enzyme to add murein tripeptide to UDP-MurNAc was MurC, the ligase that adds L-Ala to UDP-MurNAc (Fig. 3). However, purified MurC was shown to totally lack activity for the addition of the tripeptide to UDP-MurNAc (73).

A search of the E. coli chromosome database revealed an open reading frame, yifG, or o457, with 25.8% amino acid sequence identity to MurC (15). Since paralogs often have similar functions, it was speculated that yifG was the gene encoding UDP-MurNAc:t-Ala-γ-D-Glu-meso-Dap ligase (83). This proved to be the case, and ifyG was renamed mpl for murein peptide ligase (83). Strains overexpressing the yifG gene product overproduced Mpl activity, and strains in which the mpl gene was disrupted by the insertion of the 1.28-kb kanamycin resistance cartridge lacked Mpl activity (83). One of the mpl::Km strains was studied further and was shown to have normal morphology and PG content and had a 12-fold-higher pool of murein tripeptide in its cytoplasm than the wild type. This was in contrast to an ampD mutant, which accumulated well over a 100-fold-higher pool of its substrate in the cytoplasm. Interestingly, the cytoplasm was also found to contain an increased level of t-Ala-γ-D-Glu, indicating the presence of an enzyme capable of cleaving Dap from the tripeptide.

The properties and specificity of the Mpl ligase have been studied recently. Mpl was shown to utilize tripeptide, tetrapeptide, and pentapeptide with similar efficiencies (46). Mpl also accepts peptides containing lysine in place of Dap (5). During entry into stationary phase, the expression level of Mpl increases under the control of sigma S (114).

LdcA: L-D Carboxypeptidase

The principal compound to accumulate in an E. coli strain lacking ampD was anhMurNAc-t-Ala-γ-D-Glu-Dap (57). It lacked the GlcNAc and D-Ala normally present in the principal anh-muropeptide imported by AmpG. This indicated that a β-N-acetylglucosaminidase removes GlcNAc and a peptidase that removes D-Ala are likely to be present in the cytoplasm. Indeed, a peptidase named LdcA was found to be present in the cytoplasm (115). The crystal structure of LdcA from Pseudomonas aeruginosa shows that LdcA is a serine protease with a Ser-His-Glu catalytic triad (68). LdcA was shown to cleave D-Ala from t-Ala-γ-D-Glu-meso-Dap (tetrapeptide), disaccharide-tetrapeptide, anh-disaccharide-tetrapeptide, MurNAc-tetrapeptide, and UDP-MurNAc-tetrapeptide at similar rates, although free tetrapeptide was cleaved most efficiently (115). Surprisingly, the cross-linked dimers bis-disaccharide-tetrapeptide and bis-anhydrodisaccharide-tetrapeptide as well as murein sacculi were not substrates. It was shown previously that UDP-MurNAc-pentapeptide was not a substrate (124).

Among the 11 genes involved in recycling, a mutation in the ldcA gene is the only known mutation that produces a phenotype. The ldcA mutant forms thick, oval cells in late log phase, and many lyses (115). Baum et al. identified a dithiazoline inhibitor of LdcA and showed that the drug causes cell lysis in stationary phase, which is similar to the phenotype of an ldcA mutant (6). Those authors’ explanation for this phenotype is that in the absence of LdcA, tetrapeptide accumulates and is used by Mpl to form UDP-MurNAc-tetrapeptide (115). In fact, as noted above, the tetrapeptide is a good substrate for Mpl (46). The MurNAc-tetrapeptide is incorporated into the murein sacculus instead of MurNAc-pentapeptide. Hence, a shortage of pentapeptide, which is required for cross-linking the glycan chains, leads to a deficiency of cross-links and a weakened wall, resulting in bulging and lysis. The fact that the phenotype develops during the onset of stationary phase suggests that recycling may be of critical importance during this period.

MpaA: γ-D-Glutamy-meso-Diaminopimelate Amidase

There were two clues that a γ-D-Glu-Dap amidase may be present in the cytoplasm of E. coli: (i) Dap-per D-Ala was identified in the cytoplasm by Goodell and Schwarz (40), and (ii) t-Ala-γ-D-Glu was identified in the cytoplasm of an mpl mutant (83). These dipeptides could be derived from murein tetrapeptide, and t-Ala-γ-D-Glu could also be produced from murein tripeptide, by cleavage of the bond between D-Glu and Dap.

Bacillus sphaericus contains a sporation-related γ-D-Glu-Dap amidase named ENP1 (2, 32). The catalytic domain of ENP1 contains a zinc binding site, suggesting that a zinc ion is essential for its activity (51). In a search of the E. coli protein
database, regions of the YcjI amino acid sequence displayed significant homology with the catalytic domain and putative zinc-binding triad of ENP1, although the overall identity between the two sequences was only 13% (120). In particular, the putative zinc-binding triad His162-Glu165-His307 of ENP1 corresponds to His69-Glu72-His177 of YcjI in the alignment of the two amino acid sequences (120). YcjI proved to be the gene for an enzyme that cleaved the γ-D-Glu-Dap bond and was named mpaA for muropeptide amidase A. An mpl mpaA (y cjI::Cm) double mutant was constructed and was shown to contain over 10 times as much tripeptide as the mpl mutant and over 100 times as much tripeptide as the wild-type strain in its cytoplasm (120). This demonstrated that the enzyme was active in vivo, as expected. It was difficult to detect activity in vitro. When cloned and overproduced, MpaA formed huge inclusion bodies in the cytoplasm, and purification of MpaA was unsuccessful. However, extracts from cells expressing mpaA from a plasmid were shown to cleave the tripeptide with the release of Dap. More information on the substrate specificity of MpaA and on a possible requirement for zinc awaits overproduction and purification of the enzyme.

YcjG: 1-Alanyl-d/l-Glutamate Epimerase

Schmidt et al. showed that YcjG, divergently coded downstream of mpaA, is l-Ala-d/l-Glu epimerase, which converts l-Ala-d-Glu to l-Ala-l-Glu (105). l-Ala-l-Glu is a substrate for PepD, a dipeptidase of E. coli with a broad specificity (107). The epimerase belongs to the enolase superfamily (42), and the reaction mechanism of the enzyme has been studied (42, 64).

Thus, E. coli can release all of the amino acids from the tripeptide. However, this is a minor pathway because most of the tripeptide is utilized by Mpl to form the PG biosynthesis intermediate UDP-MurNAc-tripeptide.

AmiD: an Outer Membrane Anhydro-N-Acetylmuramyl-l-Ala Amidase

A nagZ ampD mutant accumulated GlcNAc-anhMurNAc-tripeptide in its cytoplasm, as expected (20). However, a significant amount of the disaccharide GlcNAc-anhMurNAc was also present (20). This suggested that in addition to AmpD, E. coli may have a second enzyme with anhMurNAc-l-Ala amidase activity. A search of the genome revealed a paralog, YbjR, with 40% identity to amino acids 65 to 171 of AmpD. The paralog was named AmiD to distinguish it from AmiA, AmiB, and AmiC, the three MurNAc-l-Ala amidases found in the periplasm. Deletion of amiD from the nagZ ampD double mutant produced a strain that totally lacked anhMurNAc-l-Ala amidase activity, demonstrating that AmiD and AmpD were the only enzymes in E. coli able to cleave the anhMurNAc-l-Ala bond (118).

Substrate specificity of AmiD. AmiD was shown to cleave the anhMurNAc-l-Ala bond in GlcNAc-anhMurNAc-tripeptide much more rapidly than that in anhMurNAc-tripeptide. Surprisingly, muropeptides lacking the anhydro ring were good substrates, and, in fact, PG itself was cleaved by the enzyme. Thus, AmiD has a relatively broad specificity. AmiD, like AmpD (33), was shown to require zinc for activity (118).

AmiD is a lipoprotein. The amiD gene product has a short signal sequence and a lipobox motif (L14AGC), followed by Ala, which suggested that AmiD is a lipoprotein destined for the outer membrane (62, 117). Indeed, fractionation of membranes of ampD cells in which AmiD was the only anhMurNAc-l-Ala amidase present demonstrated that the AmiD amidase activity was in the outer membrane (118).

Why GlcNAc-anhMurNAc is present in the cytoplasm of the amiD ampD double mutant even though the strain totally lacks anhMurNAc-l-Ala amidase activity. The amiD ampD nagZ mutant still accumulated disaccharide but only one-fourth as much GlcNAc-anhMurNAc as the ampD nagZ mutant. This indicated that 75% of the GlcNAc-anhMurNAc present in the cytoplasm of the triple mutant was produced by AmiD from anh-muropeptides in the periplasm, followed by the uptake of the disaccharide by AmpG. This demonstrates that significant AmiD activity occurs in growing cells. The activity was somewhat surprising in view of the fact that the amiD gene has tandem rare arginine codons, AGA_AGA, as its second and third codons, suggesting that relatively few AmiD molecules are produced. The remaining 25% of the GlcNAc-anhMurNAc present in the amiD ampD nagZ mutant must have been produced by the action of periplasmic amidases, AmiA, AmiB, and/or AmiC, that cleave the stem peptides from PG, followed by digestion of PG by lytic transglycosylases to release GlcNAc-anhMurNAc and uptake of the disaccharide by AmpG.

Interestingly, an amiD ampG mutant, which one would expect to accumulate GlcNAc-anhMurNAc-peptides in the periplasm, because their MW exceeds 850 and diffusion through the outer membrane would therefore be expected to be slow (85), actually released these large-MW compounds into the medium (118). Not incidentally, the main compound released into the medium, GlcNAc-anhMurNAc-tetrapeptide, is identical to tracheal cytotoxin (TCT) of Bordetella pertussis (101).

MppA, a Periplasmic Murein Peptide-Binding Protein, and OppBCDF and Their Role in Uptake of Murein Tripeptide

When the probable existence of a UDP-MurNAc-l-Ala-γ-D-Glu-Dap-meso-Dap ligase was first predicted (37) (see Fig. 3 for its role in PG synthesis) and the role of AmpG in recycling the tripeptide became known (57), efforts to identify the ligase gene were undertaken. Based on Goodell’s observation that a Dap-requiring strain of E. coli did not form colonies on plates containing low levels of the murein tripeptide L-Ala-γ-D-Glu-meso-Dap for PG synthesis and that the intact tripeptide was apparently used directly (37), mutants that required murein tripeptide as a source of Dap for growth of a Dap auxotroph were sought. E. coli TP981 (opp′ ampG::Km lysI− dapD2) was mutagenized by transposon mutagenesis (94). Mutants of the Dap-requiring strain that did not form colonies on plates containing low levels of the murein tripeptide L-Ala-γ-D-Glu-meso-Dap as the sole source of Dap were selected by the replica plating technique. Four mutants were obtained from about 10 million colonies replicated (94). Two of the mutants carried the transposon in a previously unidentified gene whose product was about 46% identical to the OppA amino acid sequence. This gene product has been named MppA, for murein peptide permease, because of its functional and structural similarity to OppA, the periplasmic binding protein required for the uptake of oligopeptides. MppA was shown to be a periplasmic binding protein.

On April 14, 2021 by guest
http://mmbr.asm.org/
Figure 6 illustrates the pathways for the utilization of cell wall amino acids. The peptides are released from anh-muropeptides by AmpD amidase. LdcA carboxypeptidase cleaves α-Ala from the tetrapeptide. The tripeptide is efficiently used by Mpi ligase to form UDP-MurNAc-tripeptide, which is a normal intermediate in the pathway for PG synthesis (Fig. 3). It is surprising that the cell also maintains a second pathway utilizing MpaA amidase and L-Ala-α,α-D-Glu epimerase (YejG) in its arsenal, which together allow the cell to recover all the individual amino acids from the muropeptides.

**Pathways for Utilization of PG Amino Acids**

Since E. coli can recycle amino acids from the cell wall, it was natural to consider whether the cells can also utilize the amino sugars. In order to study the fate of PG amino sugars, the PG amino sugars were specifically labeled with [3H]GlcN. This was possible by using strains that carried the nagB mutation, which prevented the conversion of GlcN-6-phosphate (GlcN-6-P) to fructose-6-P (Fig. 7). Thus, essentially all of the label was restricted to GlcNAc and MurNAc of PG and the GlcNAc of lipopolysaccharide. As reported below, it was found that both free GlcNAc and anhMurNac derived from PG were converted to GlcNAc-6-P and were thus available for reuse. The following five enzymes were found to be involved in recycling of the PG amino sugars.

**NagA: N-Acetylgloscosamine-6-P Deacetylase**

NagA, an N-acetylgloscosamine-6-P deacetylase (129), is an essential enzyme for the metabolism of amino sugars and for recycling GlcNAc and anhMurNac. As indicated in Fig. 7, conversion of GlcNAc-6-P to UDP-GlcNAC requires that it first be deacetylated by NagA and then converted to GlcN-1-P by GlmM (80), followed by reacetylation and reaction with UTP by GlmU to form UDP-GlcNAc (81, 82). Alternatively, GlcN-6-P can be converted to fructose-6-P by NagB (129). As indicated, the reverse reaction to synthesize Glc-N-6-P is catalyzed by GlmS (reviewed in reference 116).

**NagZ: β-N-Acetylglosaminidase**

Since AmpG permease transported only compounds containing GlcNAc-anhMurNac, whereas anhMurNac-tripeptide rather than GlcNAc-anhMurNac-tripeptide accumulated in ampD cells, it was apparent that GlcNAc must be cleaved from the incoming disaccharide-peptides. A β-N-acetylglosaminidase from E. coli was characterized by Yem and Wu in 1976 (132). It was shown to be present in the cytoplasm and to cleave p-nitrophenyl-β-N-acetyl-d-glucosaminide and muropeptides obtained from PG by digestion with lysozyme. Those authors obtained mutants deficient in enzyme activity but did not map the mutations (131). Hrebenda also reported

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**DISCOVERY AND CHARACTERIZATION OF ENZYMES INVOLVED IN RECYCLING PG AMINO SUGARS**

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the isolation of a mutant deficient in enzyme activity and found that the mutation cotransduced with the tryptophan operon at a low frequency (52). As these mutants were no longer available, Cheng et al. isolated a mutant deficient in β-N-acetylglycerosaminidase from *E. coli* cells treated with nitrosoguanidine (20). Individual colonies were screened for the ability to hydrolyze p-nitrophenyl-β-N-acetyl-d-glucosaminide, and one with reduced activity, TP75, was chosen for further studies. The mutated gene mapped 3.2 min counterclockwise from the tryptophan operon and contained a point mutation. This identified an open reading frame, *ycfO*, which was renamed *nagZ*. A deletion mutant of *nagZ* totally lacked β-N-acetylglycerosaminidase activity and contained large amounts of the disaccharide GlcNAc-anhMurNAc in its cytoplasm (20). Thus, *NagZ* is the only β-N-acetylglycerosaminidase expressed in *E. coli*. As one would expect, a *nagZ* *ampD* double mutant accumulated GlcNAc-anhMurNAc-tripeptide in its cytoplasm (20). The enzyme was active against muropeptides as well as anh-muropeptides. The substrate specificity of *NagZ* was also studied by Vötsch and Templin (128). *NagZ* was inhibited by GlcNAc, the product of the reaction, and, interestingly, by bulgocin, which is known to inhibit the soluble lytic transglycosylase SltY (128). The *NagZ* enzyme has a substrate specificity similar to that of ExoII (22), an unusual β-N-acetylglycerosaminidase from *Vibrio furnissii* whose amino acid sequence is 57% identical to that of *NagZ*. 

It was reported previously that a strain lacking *NagZ* can be induced to synthesize only one-fourth as much β-lactamase as normal from the *ampC-ampR* system of *E. cloacae* (128). Small-molecule inhibitors of *NagZ* from *Vibrio cholerae* were synthesized and were shown to form a complex with *NagZ* by crystallographic methods (112). One of these compounds decreased β-lactamase induction by fourfold (112), which is consistent with the result obtained with a *nagZ* mutant (128).

**NagK: N-Acetylglucosamine Kinase**

The cleavage of anh-muropeptides by the cytoplasmic β-N-acetylglycerosaminidase *NagZ* leads to the release of GlcNAc in the cytoplasm. The free sugar was shown to be recycled into PG and lipopolysaccharide, but exactly how the cell was able to utilize the free GlcNAc was not determined (90). Recycling of this GlcNAc would be readily achieved if it could be converted to the phosphorylated form (Fig. 7). A kinase that phosphorylated GlcNAc was purified from *E. coli*, and its properties were studied in 1966 (3). Building on this purification procedure, a GlcNAc kinase from *E. coli* was purified, and the gene was identified by determining the N-terminal sequence of the purified enzyme (121). The gene, *ycfX*, was named *nagK* for *N*-acetylglycerosaminokinase. A null mutant exhibited very low kinase activity. The *nagK* gene was cloned, and the *NagK* protein was overexpressed and purified. The kinase was shown to be highly specific for GlcNAc, although at extremely high concentrations, glucose was also phosphorylated (121).

A *nagEBACD* mutant fed [3H]GlcNAc was shown to lack radioactive UDP-GlcNAc in its cytoplasm and accumulated large amounts of radioactive GlcNAc-P and GlcNAc because of the absence of NagA deacetylase (90). In contrast, a *nagK* derivative of the *nagEBACD* mutant contained just the opposite, i.e., large amounts of UDP-[3H]GlcNAc but relatively little [3H]GlcNAc and no [3H]GlcNAc-P (121). This suggested the intriguing possibility that a pathway that is able to utilize GlcNAc without first phosphorylating it had been activated (121). This hypothetical pathway remains to be investigated.

As judged by its amino acid sequence (which includes the conserved domain of the ROK family), *NagK* is a member of the ROK family, which includes repressors, open reading frames, and kinases. A crystal structure of Mlc, which is a transcriptional regulator belonging to the ROK family, revealed that the conserved domain of the ROK family coordinates a zinc ion, which is essential for its function (103). However, whether zinc is required for *NagK* activity remains unknown.

**AnnK: Anhydro-N-Acetylmuramic Acid Kinase**

As noted above, preventing the recycling of PG amino sugars by the inactivation of *nagZ* caused an accumulation of GlcNAc-anhMurNAc. Likewise, the deletion of *nagEBACD* resulted in high levels of GlcNAc and GlcNAc-P in the cytoplasm, since NagA is required for the utilization of GlcNAc-P. Surprisingly, the *nag* operon deletion strain completely lacked anhMurNAc. This result strongly suggested that anhMurNAc was metabolized, since during PG recycling, equal amounts of each amino sugar are released into the cytoplasm, and anhMurNAc was not found in the cytoplasm or in the spent medium.

Incubation of [3H]anhMurNAc with soluble cell extract from 1 ml of an *E. coli* culture converted only a trace amount to a compound or compounds that were separable from anhMurNAc by high-performance liquid chromatography or thin-layer chromatography (90). This was surprising, since in the case of the other enzymes involved in recycling, this amount of cell extract was sufficient to convert essentially all of the substrate to product in less than 2 h. However, when ATP and Mg²⁺

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**FIG. 7.** Pathway for recycling PG amino sugars.
were included in the incubation mixture, the reaction proceeded rapidly. With thin-layer chromatography as a guide, the enzymes(s) was partially purified and used to digest anhMurNAc. Analysis of the digest mixture by mass spectrometry unexpectedly identified both GlcNAc-P and MurNAc-P as products (123). Further purification on a MonoQ column separated an activity that converted anhMurNAc to MurNAc-P from an activity converting MurNAc-P to GlcNAc-P (123). Analysis of a tryptic digest of the fraction containing the partially purified anhMurNAc kinase by mass spectrometry identified three known proteins and one unknown open reading frame, ydhH, which proved to be the gene for anhMurNAc kinase, renamed AnmK (123). Thus, the first step in the conversion of anhMurNAc to a metabolizable form was phosphorylation. The reaction is unusual in that it involves the cleavage of the 1,6-anhydro bond in addition to phosphorylation. A comparable reaction converts 1,6-anhydroglucose (levoglucosan) to glucose-6-P (135). An anmK mutant did not accumulate anhMurNAc in its cytoplasm. This is in contrast to other enzymes in the recycling pathway in which the loss of an enzyme resulted in the accumulation of the substrate for said enzyme in the cytoplasm. In the case of AnmK, the substrate anhMurNAc was found in the spent medium, suggesting that an efflux pump for anhMurNAc is present in E. coli that lacks AnmK (123).

**MurQ: N-Acetylmuramic Acid-6-P Etherase**

In 1983, Parquet et al. (95) reported the surprising finding that E. coli cells could grow on MurNAc as the sole source of carbon and energy. Dahl et al. (26) recently demonstrated that a special phototransferase system, MurP (YfeV), was required for growth on MurNAc and that MurNAc was phosphorylated during uptake. It was further demonstrated that cells lacking GlcNAc-6-P deacetylase (ΔnagA) or glucosamine-6-P deaminase (ΔnagB) could not grow on MurNAc. This suggested that GlcNAc-6-P was an intermediate involved in the utilization of MurNAc and that MurNAc-6-P was converted to GlcNAc-6-P. This was followed by the identification of a gene, murQ (yfeU), for the hypothetical “etherase” (59). Jaeger et al. showed that murQ was required for growth on MurNAc; that in the absence of murQ, cells fed MurNAc accumulated MurNAc-P; and that purified MurQ converted MurNAc-6-P to GlcNAc-6-P with the release of d-lactate (59). Interestingly, murQ proved to be in an operon together with murP and a gene for PBP4B (126). MurQ was subsequently shown to be the only MurNAc-P etherase in E. coli, and it was shown that both MurQ and AnmK were required for the utilization of anhMurNAc derived from PG (122). Data indicating that anhMurNAc was taken up by cells provided that MurP was present and that AnmK was required for phosphorylation were also presented (122). Thus, the pathway for recycling anhMurNAc and the pathway that allows the growth of E. coli on MurNAc (and possibly anhMurNAc) merge at MurNAc-6-P. Growth on anhMurNAc was not adequately tested due to the lack of sufficient substrate, although in a preliminary experiment, E. coli cells failed to grow on anhMurNAc as a sole carbon source.

**Pathway for Utilization of PG Amino Sugars**

As shown in Fig. 7, the amino sugars from PG enter the cell via AmpG permease in a bound form as GlcNAc-ahmMurNAc-peptides. The peptides are removed by anhMurNAc-t-Ala-amidase (AmpD), and the disaccharide is cleaved by β-N-acetylglycosaminidase (NagZ), thus freeing GlcNAc and anhMurNAc. NagK, a highly specific GlcNAc kinase, converts the free GlcNAc derived from PG to the phosphorylated form, GlcNAc is normally phosphorylated upon uptake by a phototransferase system, and GlcNAc-P is readily metabolized (Fig. 7). anhMurNAc is also phosphorylated by a specific kinase (AnmK) and then converted to GlcNAc-P by MurNAc-P etherase (MurQ). Hence, both amino sugars are converted to GlcNAc-P and can enter the normal metabolic pathways for the synthesis of UDP-GlcNAc and for glycosylation.

**OCCURRENCE OF ORTHOLOGS OF PG RECYCLING ENZYMES IN OTHER GENERA**

Table 2 lists orthologs of PG recycling enzymes present in selected bacteria. A more extensive list can be found in Table S1 in the supplemental material. With the reservation that the presence of an ortholog does not guarantee the expected activity, one can see that many familiar bacteria have the four genes (ampg, ampD, ldcA, and npl) involved in the recycling of the murein tripeptide. Several bacteria lack LdcA, which may not be needed if the tetrapeptide content of their murein is low or if their murein peptide ligase does not use the tetrapeptide efficiently. It is interesting that many bacteria may possess the outer membrane anhMurNAc-t-Ala amidase (AmiD) even though it is not directly involved in recycling. The degradation of the tripeptide by MpaA and YqG appears to be employed by fewer organisms.

With regard to the proteins involved in the utilization of PG amino sugars (AmpG, NagZ, NagK, AnmK, and MurQ), it appears that many of the listed bacteria may have all five proteins. Remarkably, about one-half of the bacteria listed, as well as *Xanthomonas, Shewanella, Legionella*, and *Ralstonia* (see Table S1 in the supplemental material), have an *anmK* ortholog but lack a *murQ* ortholog. The product of *anmK*, MurNAc-P, presumably cannot be utilized unless converted to GlcNAc-P by MurQ. This suggests that a structurally unrelated protein serves the MurQ etherase function or that AnmK serves a different function.

**Helicobacter pylori** and *Borrelia burgdorferi* lack AmpG and enzymes to cleave PG degradation products. Hence, these compounds, when cleaved from PG by lytic transglycosylases, must be released into their surroundings or injected into the cytoplasm of the host (unless their SltY ortholog is inactive). It has been demonstrated that *H. pylori* injects Dap-containing material into the host cytoplasm via a bacterial type IV secretion system (127).

It is surprising that *Bordetella pertussis* and *Neisseria gonorrhoeae* possess orthologs of AmpG and most of the recycling enzymes because *Bordetella pertussis* secretes huge amounts of the intact TCT (anh-disaccharide-tetrapeptide) (24) and *Neisseria gonorrhoeae* also releases TCT (109). This suggests that AmpG proteins of *B. pertussis* and *N. gonorrhoeae* are either not expressed or not active. A strain of *B. pertussis* which
TABLE 2. Orthologs of proteins involved in PB recycling

| Organism               | Presence of ortholog of: | | | | | |
|------------------------|--------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                        | AmpG | AmpD | Mpl | LdcA | AmiD | NagZ | NagK | AnmK | MurQ | MpaA | YcjG |
| Escherichia coli K-12  | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Escherichia coli CFT073| +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Salmonella enterica   | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Yersinia pestis       | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Shigella flexneri     | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Haemophilus influenzae| +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Pseudomonas aeruginosa| +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Bordetella pertussis  | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Neisseria gonorrhoeae | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Vibrio cholerae       | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Legionella pneumophila| +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Caulobacter crescentus| +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Helicobacter pylori    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Borrelia burgdorferi  | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Bacteroides fragilis  | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |
| Bacillus subtilis      | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |

* This table lists selected organisms listed in Table S1 in the supplemental material. Organisms with two orthologs of a gene are indicated as + +. * indicates orthologs containing a putative signal sequence.

overexpresses AmpG and lacks a gene encoding dermonecrotic toxin is attenuated (84), suggesting that TCT, which is normally released into the medium, is taken up by AmpG permease.

Remarkably, mammals have a membrane protein with significant homology to AmpG (see Table S1 in the supplemental material). If active, the protein may play a role in the importation of PG fragments into the cytoplasm, allowing their detection by Nod1 or Nod2.

From the possession of orthologs, we can speculate further how certain other bacteria may recycle their PG degradation products. Legionella pneumophila possesses an ortholog of AmpD. However, AmpD of L. pneumophila appears to have a signal sequence that would export the protein into the periplasm. Thus, GlcNAc-anhMurNAc-peptides may be cleaved in the periplasm to release GlcNAc-anhMurNAc and peptides. GlcNAc-anhMurNAc would be imported by AmpG into the cytoplasm, which contains orthologs of the enzymes needed for the degradation and reutilization of amino sugars. Free peptides could be taken up by the MppA-OppBCDF system and reused by Mpl.

In E. coli, murQ and murP are in the same operon. Vibrio cholerae is unique in that it possesses two MurQ orthologs, both of which are in chromosome 1. One MurQ is in an operon with murP, and the other MurQ is coded just downstream of anmK. This seems to indicate the horizontal transfer of one of these operons. Interestingly, NagZ of V. cholerae is coded divergently to anmK.

Pseudomonas aeruginosa has three ampD orthologs, one of which is amnD and all of which are involved in β-lactamase induction (61). It may also have two ampG genes.

Although the gram-positive bacterium Bacillus subtilis lacks AmpG permease, it has orthologs of NagZ (YbbD), MurQ (YbbI), MurP (YbbF), LdcA (Ykfa), MpaA (YaggT), and YcjG (Ykfb). B. subtilis cleaves PG primarily by a muramidase and an amidase to produce GlcNAc-MurNAc and peptides. GlcNAc-MurNAc could be cleaved by NagZ, which seems to be exported by a putative signal sequence of the protein. GlcNAc is imported into the cytoplasm by a specific phosphotransferase system, NagP (YffF), and metabolized by NagA and NagB. MurNAc is phosphorylated and imported into the cytoplasm by MurP to produce MurNAc-6-P, which is converted into GlcNAc-6-P by MurQ. Interestingly, NagZ, MurQ, and MurP are encoded in a putative operon that also includes a gene coding for a putative β-lactamase. Free peptides are imported by an unknown permease and degraded by Ykfa, a putative γ-D-Glu-Dap amidase (Ykfc), and YcjG epimerase (Ykfb). Ykfa, Ykfb, and Ykfc are encoded downstream of the dpp operon, which is regulated by the transcriptional regulator CodY and is expressed in the early stationary phase (108, 110). Thus, B. subtilis may have the potential to utilize PG degradation products when carbon and energy sources become scarce.

### RELATED TOPICS

Some conclusions from early work die hard. For instance, although it has been known for many years that the Opp permease plays no detectable role in recycling PG amino acids (92) and that the uptake of murein tripeptide via Opp cannot occur unless MppA is present (94), both of these notions persist. The same might be said for the hypothesis that the cell elongates the sacculus by inserting three glycan strands at a time to replace one existing strand. This idea was a good attempt to explain cell wall turnover, but the concept is incompatible with the fact that single strands are inserted one at a time during elongation (25, 27, 89).

### β-Lactamase Revisited: Relation of Recycling Intermediates to β-Lactamase Induction

In 1994, anhMurNAc-tripeptide accumulation in an ampD mutant of E. coli was shown to correlate with the constitutive expression of C. freundii ampC β-lactamase carried on a plasmid (57). This was followed by the demonstration that AmpR was an activator of ampC expression and that the concentra-
tion of UDP-MurNAc-pentapeptide normally present in *E. coli* acted as a corepressor to repress *ampC* expression (56). The amount of anhMurNAc-tripeptide present in the *ampD* mutant was sufficient to restore expression (56). This explained the constitutive expression of the *C. freundii* *ampC* β-lactamase in the *ampD* mutant but did not explain the induction of *ampC* β-lactamase expression caused by the addition of cefoxitin or imipenem to growing *E. coli* cells. As reported in the original publication, anhMurNAc-tripeptide did not accumulate under these conditions, although a significant amount of a smaller-MW compound did accumulate (57). It was recently shown that the compound that accumulates in this fraction is the pentapeptide t-Ala-γ-D-Glu-Dap-D-Ala-D-Ala and that a smaller amount of anhMurNAc-pentapeptide also accumulates (T. Uehara, K. Suefuji, and J. T. Park, unpublished data). Dietz et al. (30) showed that cefoxitin and imipenem, which are good inducers of β-lactamase, caused cells to accumulate anh-disaccharide-pentapeptide in their periplasm, whereas four other β-lactam antibiotics that caused minimal induction failed to accumulate anh-disaccharide-pentapeptide. Those authors concluded that anhMurNAc-pentapeptide is the active inducer, although the above-described result suggests that free pentapeptide may be a more likely candidate. In vitro studies of *ampC* mRNA synthesis are required to identify the true inducer.

However, other observations suggest that UDP-MurNAc-pentapeptide may not be the corepressor of AmpR. For instance, both a temperature-sensitive *murG* mutant at the restrictive temperature (*MurG* is required for lipid 2 synthesis) (Fig. 3) and *envA* cells carrying *C. freundii* *ampR* *ampC* and treated with ramoplanin (which binds to lipid 2) have been shown to express β-lactamase (113). *envA* cells have a leaky outer membrane, allowing the entry of ramoplanin and other antibiotics (87). In addition to ramoplanin, other PG synthesis inhibitors including vancomycin, moenomycin, fosfomycin, and cycloserine also induce β-lactamase but to a lesser extent (113). All these conditions may result in the reduced availability of lipid 2. It has been shown that a crude preparation of lipid 2 from 1 ml of *E. coli* culture is sufficient to block AmpR-driven *ampC* β-lactamase mRNA synthesis in a cell-free system (K. Suefuji, T. Uehara, and J. T. Park, unpublished data). We are left with several observations that need to be explained before the induction of β-lactamase by β-lactam antibiotics can be fully understood: (i) the fact that AmpG is required for β-lactamase induction by cefoxitin and imipenem implicates PG recycling intermediates other than anhMurNAc-tripeptide in the process; (ii) the fact that cefoxitin and imipenem, which are good inducers, are the only β-lactams tested that caused the accumulation of anhMurNAc-pentapeptide implicates this compound in the induction process (30); (iii) the fact that pentapeptide is the principal compound to accumulate in cells treated with cefoxitin implicates pentapeptide as being the more likely inducer than anhMurNAc-pentapeptide; and (iv) reports that both ramoplanin treatment and a *murG* (Ts) mutant at the restricted temperature cause β-lactamase induction seems to implicate lipid 2 as a corepressor. Unfortunately, a clear connection between lipid 2, recycling intermediates containing pentapeptide, and UDP-MurNAc-pentapeptide remains to be established.

More questions about β-lactamase induction concerning the possible role of AmpE, a membrane protein coded just downstream of *ampD*, have arisen. AmpE is involved in the regulation of β-lactamase expression (50, 75), but the mechanism is unknown. Recently, AmpE in *Pseudomonas aeruginosa* was shown to play an indirect role in resistance to β-lactams (60). While an *ampE* mutation did not change the amounts of PG recycling intermediates in the cytoplasm of *E. coli*, treatment of the *ampE* mutant with cefoxitin increased the amount of free pentapeptide present in the cytoplasm over 100-fold compared to that of the wild type (T. Uehara, unpublished data), indicating that AmpE is involved in PG recycling when the peptide moiety contains γ-Ala-d-Ala. It is very surprising that pentapeptide accumulates in the cytoplasm of the *ampE* mutant since Mpl protein ligates pentapeptide as well as tripeptide to UDP-MurNAc, and this should act against the accumulation of pentapeptide (46).

**Recycling intermediates and gene regulation.** Due to the fact that recycling intermediates triggered the induction of β-lactamase, it was suggested that changes in the cytoplasmic levels of an intermediate might be sensed and cause a response to cell wall damage (57, 93). However, no such relationship has emerged in *E. coli*. On the other hand, in *Pseudomonas aeruginosa*, the AmpR transcriptional regulator regulates pyocyanin production and the expression of LasA protease and LasB elastase in addition to β-lactamase (65). It has also been reported that the treatment of *P. aeruginosa* biofilms with imipenem, a good inducer of β-lactamase, changes global gene expression and alginate production as well as β-lactamase expression (4). Another piece of evidence that supports the linkage of alginate production to PG recycling is that algD transcription is increased by a mutation in *ampD* in *Azotobacter vinelandii* (88). Encystment and the cell shape of *A. vinelandii* are also affected by *ampD* and *ampE* (88). Lastly, in *Acinetobacter baylyi*, mutants in *ampD* and *mpl* are hypersensitive to β-lactam antibiotics (36), whereas *ampD* mutants of *E. cloacae* and *C. freundii* are hyperresistant to β-lactams by overproducing AmpC β-lactamase (74). In *A. baylyi*, PG degradation products might negatively regulate an efflux pump involved in the acquisition of resistance. Thus, recycling intermediates may play a regulatory role in some bacteria.

**Turnover of New PG Formed during Septation**

The recent discovery that the process of cell separation may involve the removal of the equivalent of one or perhaps two layers of murein from between the two newly formed cell poles dramatizes the hidden nature of turnover (119). One of us, T. Uehara, realized that the amount of anhMurNAc-peptides that accumulated in *ampD* mutants could be used as a measure of turnover of the cell wall. This led to a series of experiments measuring the amount of synthesis and degradation occurring during elongation and septation of the cell, with surprising results (119).

When cells were treated with A22, a compound known to inhibit MreB function (34, 63), elongation was inhibited, septation continued, and the cells gradually changed their shape, becoming round (55). Thus, by the exposure of *E. coli* MG1655 *lysA ampD* mutant cells to A22 to prevent the PG synthesis associated with elongation, followed by pulse-labeling of the cells with *[^3H]Dap* (or *lysA ampD nagB nagZ* cells with *[^3H]GlcN*), it was possible to determine the rate of murein
turnover occurring during septation (119). Surprisingly, even though the poles of the cell are known to be stable and do not turn over (16, 28, 29), at least 30% of the murein made during septation was rapidly turned over during a 10-min labeling period (119). This indicates that a large fraction of the PG made during septation is removed as the two new poles are being formed and the daughter cells separate. One interpretation of this result is that at least three layers of wall are laid down simultaneously as the division machinery forms the septum, and most of the middle layer(s) is rapidly removed by lytic transglycosylases. An early indication that material from between the new poles may be removed during septation can be seen from the electron-transparent space between the new poles revealed in thin-section micrographs of septating E. coli B cells shown in Fig. 8 (14). Similar open spaces have been seen in thin-section micrographs of the chain-forming envA (86) and envC (91) mutants. Presumably, the space between the poles contains proteins or other polymers, including partially cross-linked PG, that keep the poles separated. Interestingly, if the loss was only one-third of the new polar material made during septation, it would be consistent with the three-for-one (actually, three formed and one removed) model for septation proposed previously by Höltje (47). However, there is a major caveat. The turnover of murein observed is a minimum value because it does not take into account the unknown amount of PG made and not yet turned over, the amount turned over and not trapped by the ampD cells, or the undercount of label due to a lower specific activity in the anh-muropeptides than in the sacculi. If only 67% of the unstable septal murein was degraded during the 10-min experimental period, it would indicate that four layers of PG are formed during septum formation. Another reason to consider four layers as a possibility rather than three is the report that PBP1B, which is believed to form the glycan chains during septation, is reported to be active when in the form of a dimer (8). If both enzymes of the PBP1B dimer are forming glycan chains, this would produce an even number of glycan layers. The results could not help distinguish between three layers or four layers of PG in the growing septa (119).

Amidase activity during septation. The amidases present in the periplasm, AmiA, AmiB, and AmiC, have been shown to facilitate cell separation (44, 99). Without the amidases, many cells remain attached to each other in chains, indicating that separation is delayed but not completely prevented. However, in terms of the release of Dap from cells during septation, only one-sixth of the septal PG cleaved by the lytic transglycosylases had been cleaved by the amidases, indicating that lytic transglycosylases are primarily responsible for septal PG degradation (119). The murein tri- and tetrapeptides detected in spent media by Goodell and Schwarz (40) are products of amidase activity. Amidase activity seems to be greater during septation than during elongation because it was shown previously that asynchronously growing cells turn over more PG than elongating cells (92). Treatment of septating cells (i.e., A22-treated cells) with furazolocillin, which prevents cross-linking by PBP3 during cell division, resulted in turnover rates of over 75% (our unpublished data). Thus, when cross-linking was blocked, the nascent poles were rapidly degraded, along with the PG between the new poles.

Turnover of New PG Formed during Elongation

When cells overexpress sulA for a short period of time, septation ceases, cell elongation continues, and cells become filamentous (9). It is well established that during elongation, single new glycan strands are inserted between two existing strands (25, 27, 89). Under these elongation conditions, during a 10-min period of labeling of cells, about 7% of their PG turned over, as measured by the entrapment of the anhMurNAc-peptides released by lytic transglycosylases. As noted above, since the PG in the poles of the cell is not turned over during a chase, over 60% of the side wall is presumably turned over each generation for at least the four generations studied (92). The turnover of about 7% of the murein formed during 10 min of elongation by cells with a 50-min generation time is actually less than the rate at which the preexisting side wall turns over (119). Thus, the turnover does not appear to be related to the elongation process.

It is believed that amdinocillin (mecillinam) inhibits the transpeptidation reaction by PBP2 that is required for cross-linking the new glycan strands to old strands during elongation (53, 54, 111). The treatment of elongating cells with amdinocillin resulted in an apparent 78% inhibition of murein synthesis and recovery of the missing material as anhMurNAc-peptides in the cytoplasm of ampD cells (119). Thus, as in the case of furazolocillin-treated septating cells described above, un-cross-linked murein was being made and rapidly degraded by lytic transglycosylases. A very different result was obtained when elongating cells were inhibited by compound A22. At the concentration used (30 μg/ml), cell wall synthesis was inhibited by 60%, but a relatively small amount of anhMurNAc-peptides accumulated, indicating that glycan strands were not being formed (119). One interpretation of these results is that A22 prevented the utilization of the lipid 2 intermediate so that glycan strands could not be formed, whereas in the presence of amdinocillin, un-cross-linked glycan strands were synthesized and rapidly cleaved by lytic transglycosylases.

CONCLUDING REMARKS

Unraveling the process of cell wall recycling has been a pioneering effort. We have reported here how the individual
enzymes were discovered one at a time, how their genes were identified and cloned, and how the proteins were purified and initially characterized. One can see now that *E. coli* and many other colon-dwelling bacteria may have three different recycling pathways. The major one is dependent on Mpl for the direct utilization of murein tripeptide, a second one hydrolyzes the tripeptide to make the individual amino acids available for reuse, and the third, also a major pathway, enables cells to reutilize anhMurNAc and GlcNAc. As speculated in the discussion of orthologs, some bacteria may have only one or two of these pathways.

Lytic transglycosylase and AmpG permease are required for the pathways in *E. coli* and related enteric bacteria. AmpG is a remarkable protein and plays a key role in recycling. Without it, there would be no recycling. AmpG, driven by proton motive force, can transport hydrophilic molecules as large as GlcNAc-anhMurNAc-pentapeptidase MW 992 across the inner membrane. AmpG permease is specific for compounds containing GlcNAc-anhMurNAc. It might be possible to transport biologically active molecules into the cell linked to GlcNAc-anhMurNAc since the disaccharide can carry a peptide of MW 532.

Two other proteins that are required for the recycling of anhMurNAc are no less remarkable. anhMurNAc kinase carries out two reactions: cleavage of the L,α-anhydro bond and phosphorylation of the sugar to yield MurNAc-6-P. MurQ, the enzyme that converts MurNAc-P to GlcNAc-6-P with the release of D-lactic acid, is remarkable for the fact that it cleaves an ether bond. Few such enzymes have been identified in nature.

Interestingly, *E. coli* is also equipped to capture such PG products as murein tripeptide (94), Dap (7), MurNAc (26, 95), and anhMurNAc (122) from the environment.

Although murein turnover and recycling are largely hidden from view, they play a major role in the metabolism of the murein sacculus. Much work remains to be done in the areas of regulation and structural studies. Beyond the question of why turnover and recycling of PG occur, the greatest unsolved mysteries are why over 60% of the side wall is turned over each generation and why the poles of the cell wall are completely resistant to turnover.

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