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# Growth of *Escherichia coli*: Significance of Peptidoglycan Degradation during Elongation and Septation[▽]

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**We have found a striking difference between the modes of action of amdinocillin (mecillinam) and compound A22, both of which inhibit cell elongation. This was made possible by employment of a new method using an *Escherichia coli* peptidoglycan (PG)-recycling mutant, lacking *ampD*, to analyze PG degradation during cell elongation and septation. Using this method, we have found that A22, which is known to prevent MreB function, strongly inhibited PG synthesis during elongation. In contrast, treatment of elongating cells with amdinocillin, which inhibits penicillin-binding protein 2 (PBP2), allowed PG glycan synthesis to proceed at a nearly normal rate with concomitant rapid degradation of the new glycan strands. By treating cells with A22 to inhibit sidewall synthesis, the method could also be applied to study septum synthesis. To our surprise, over 30% of newly synthesized septal PG was degraded during septation. Thus, excess PG sufficient to form at least one additional pole was being synthesized and rapidly degraded during septation. We propose that during cell division, rapid removal of the excess PG serves to separate the new poles of the daughter cells. We have also employed this new method to demonstrate that PBP2 and RodA are required for the synthesis of glycan strands during elongation and that the periplasmic amidases that aid in cell separation are minor players, cleaving only one-sixth of the PG that is turned over by the lytic transglycosylases.**

The peptidoglycan (PG) layer of the bacterial cell envelope is essential to prevent lysis by the high internal osmotic pressure of the cell (reviewed in reference 65). As the only rigid structure in the cell envelope, it also determines the shape of the cell. Because this unique structure is present only in bacteria, synthesis of PG proved to be the target of many of the early antibiotics, such as penicillins, cephalosporins, vancomycin, D-cycloserine, and bacitracin. Biosynthesis involves construction in the cytoplasm of a precursor molecule, UDP–*N*-acetylmuramic acid–L-Ala–γ-D-Glu–(L)-*meso*-diaminopimelic acid (DAP)–D-Ala–D-Ala (UDP-MurNAc-pentapeptide). Transfer of P-MurNAc-pentapeptide to undecaprenol-P forms lipid 1, followed by transfer of *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to lipid 1 to form lipid 2. This is followed by the formation of glycan strands linked to lipid 2 and cross-linking of the strands to form the final, rigid cell wall known as the murein sacculus (61).

During growth of the rod-shaped bacterium *Escherichia coli*, the PG of the murein sacculus elongates and divides. The division is preceded by formation of a transverse septum at the center of the cell, followed by the splitting of the septum to form the poles of the daughter cells. In the 1980s, three striking observations about the PG enlargement were demonstrated by using autoradiography of *Escherichia coli* cells and by determining the fate of newly synthesized PG (10, 13, 15, 42, 43). First, PG elongates by diffuse intercalation of newly synthesized single glycan strands into the existing sidewall PG. Second, PG synthesis is enhanced at the division site. Third, the PG in the poles of the cell is stable. Most of these observations were later confirmed using a D-cysteine-labeling method (16, 17).

In rod-shaped bacteria, two primary PG synthesis machines exist: one for elongation and one for septation. For elongation, the only essential protein with a known activity is penicillin-binding protein 2 (PBP2), which is a transpeptidase required for formation of cross-linkages in PG during elongation (29, 30, 53). Without PBP2, elongation ceases and cells gradually become spherical. Mutational studies demonstrated that RodA, MreB, MreC, and MreD are also essential for elongation (52, 67, 68). RodA, encoded downstream of the PBP2 gene, is a membrane protein of unknown function that belongs to the SEDS family (for shape, elongation, division, and sporulation) (27). MreB is an actin-like protein which polymerizes in vitro in an ATP-dependent manner (59) and forms a helix in the cytoplasm of *E. coli* (36, 51). MreC and MreD are membrane proteins of unknown function encoded downstream of *mreB* (67). An elongation complex consisting of PBP2, RodA, MreB, MreC, MreD, and some transglycosylases has been proposed to exist in *Caulobacter crescentus*, *Bacillus subtilis*, and *E. coli* based on protein-protein interactions between some of the proteins (19, 20, 36, 60). However, the exact functions of these proteins in sidewall synthesis remain unknown.

The situation is somewhat similar for septation in that the only protein essential for septation that has a known enzymatic activity is PBP3, another transpeptidase (65). Without PBP3, septation ceases and filamentous cells are formed. At least nine genes besides that encoding PBP3 cause a similar phenotype when impaired and are required for cell division (22). Cell division is initiated by formation of an FtsZ ring at midcell, and this FtsZ ring recruits the other division proteins, including PBP3. Enhanced PG synthesis at the division site is dependent on FtsZ polymerization (17). Before septal synthesis begins, preseptal PG at midcell is made mainly of new PG, which is

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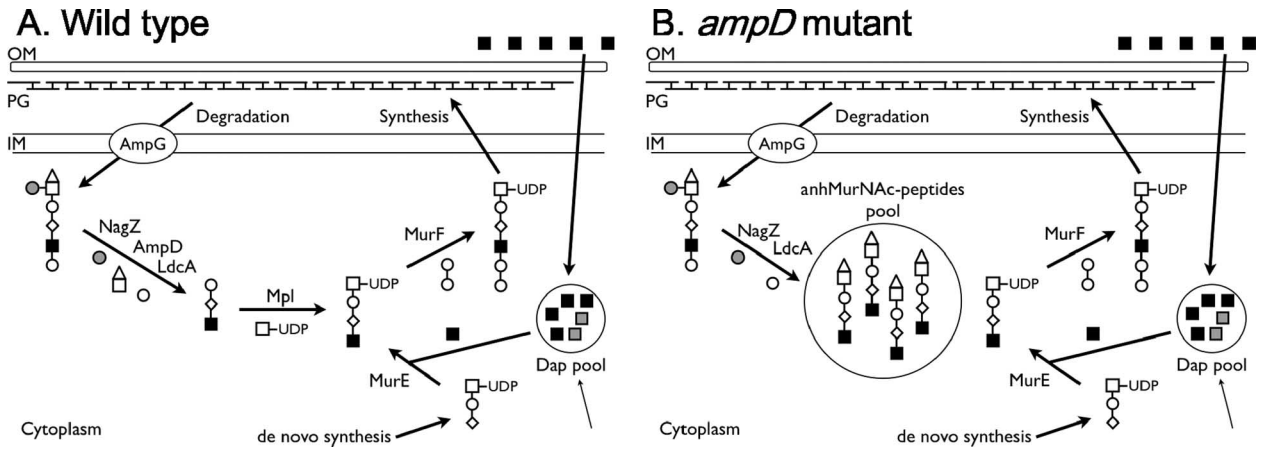



FIG. 1. PG-recycling pathway of the wild type (A) and trapping of anhMurNAc-tripeptide in an *ampD* mutant (B). In the wild type, [$^3$H]DAP from the medium is incorporated into PG via addition to UDP–MurNAc–L-Ala–D-Glu as shown. After degradation of PG, the breakdown products containing GlcNAc-anhMurNAc, e.g., GlcNAc-anhMurNAc-tetrapeptide, are imported by AmpG into the cytoplasm, where they are further degraded by NagZ, AmpD, and LdcA to release the two amino sugars, D-Ala and murein tripeptide. The tripeptide directly reenters the PG synthesis pathway (Fig. 1A) and GlcNAc, anhMurNAc, and D-Ala are also available for reuse. In an *ampD* mutant, PG breakdown products are trapped in the cytoplasm principally as anhMurNAc-tripeptide (Fig. 1B), leaving only GlcNAc and D-Ala available for reuse. OM, outer membrane; IM, inner membrane; black squares, radioactive DAP; gray squares, nonradioactive DAP; gray circles, GlcNAc; white squares, MurNAc; white squares with triangles, anhMurNAc; white circles, D/L-Ala; diamonds, D-Glu.

dependent on FtsZ but not PBP3 (17). The FtsZ-dependent sidewall synthesis at midcell is also observed for *C. crescentus* (1). Furthermore, FtsZ directs PG synthesis into portions of the sidewall (63). It has been proposed that the elongation machinery is directed not only by MreB but also by FtsZ to positions of the lateral wall to carry out elongation (65).

Elongation can be blocked by two small molecules, amdinocillin (FL 1060) (46) and compound A22 (31). Both compounds cause cells to change shape from rods to spheres. Amdinocillin is a β-lactam antibiotic which specifically inhibits PBP2 (29, 30, 53). In *C. crescentus*, MreB was shown to be a direct target of A22 (21). In *E. coli*, A22 disturbs subcellular localization of MreB (34).

The initiation of septation can be inhibited by SulA, a specific inhibitor of FtsZ function (7, 41). Once septation and cell separation are complete, the poles of the cell are stable and do not turn over (i.e., are not degraded each generation and recycled) (10, 16, 17). This is in marked contrast to the sidewalls. About 40% of the existing PG of the cell is turned over (23, 24, 32). Since the poles of the cell are stable (10, 16, 17), it follows that as much as 60% of the old existing sidewall is turned over each generation. The turnover is caused by the combined action of lytic transglycosylases and endopeptidases, and about 95% of the PG degradation products are efficiently imported via AmpG permease into the cytoplasm, where they are cleaved further and reused by a process called PG recycling (Fig. 1) (47).

In this study, by making use of the fact that a PG-recycling mutant, lacking *ampD*, traps PG degradation products in the cytoplasm (see Materials and Methods) (47), we have developed a method to measure the amount of newly synthesized PG being degraded as well as that incorporated into cell wall during cell elongation and division. Using this method, we have demonstrated that amdinocillin and A22 have different modes for preventing elongation. We propose that, during septation, additional PG sufficient to form one or possibly two extra poles is synthesized and rapidly degraded. This may serve to facilitate separation of the daughter cells. We have shown that PBP2 and RodA are required for PG glycan strand synthesis during elongation. In addition, we have shown that PG-lytic transglycosylases play the active role in cell division, while the PG amidases play an apparently lesser or different role in this process.

## MATERIALS AND METHODS

**Strains, plasmids, and growth condition.** *E. coli* K-12 strains lacking AmpD and LysA with or without NagZ were the host strains used in this study. ET505 (W3110 *lysA*::Tn*10*) was obtained from the *E. coli* Genetic Stock Center. The *ampD*::Km (i.e., kanamycin-resistant) mutant was obtained from Nara Institute of Science and Technology (2). Plasmid pAV2 carrying the SulA gene under an arabinose promoter and a chloramphenicol (Cm) resistance gene was kindly provided by Kevin Young (64). All transductions were made by the T4gt7 phage (40). TU73 (MG1655 *lysA*::Tn*10* Δ*ampD*) was constructed by transduction and removal of the Km$^r$ gene (14). TP71PBP2$^{ts}$ *ampD*::Km {*ampD*::Km *pbpA45* of TP71 [F$^-$ *lysA opp araD139 rpsL150 deoC1 ptsF25 ftbB5301 rbsR relA1* Δ(*argF-lac*)]} was constructed from TP71PBP2$^{ts}$ (56). TP71RodA$^{ts}$ *ampD*::Km (*ampD*::Km *rodA52* of TP71) was constructed from SP5211 (52) by the same procedure as used to construct TP71PBP2$^{ts}$ *ampD*::Km. TP78BD (*ampD* Δ*amiD nagB*::Km *nagZ*::Cm of TP71) was constructed previously (55). To construct the strains, LB medium containing 25 μg/ml of kanamycin, 10 μg/ml of tetracycline, or 100 μg/ml of ampicillin was used as required. During labeling with [$^3$H]DAP, a *lysA* mutant strain was used to prevent conversion of DAP to lysine. Likewise, a *nagB* mutant was used when labeling with glucosamine (GlcN) to prevent conversion of GlcN-phosphate to fructose-phosphate; hence, GlcN is found only in compounds derived from UDP-GlcNAc, mainly PG and lipopolysaccharide. The medium used for labeling was M9 minimal medium (40) supplemented with 0.1% Casamino Acids, 1 mM $MgCl_2$, 1 μg/ml thiamine, 100 μg/ml lysine, and, as a carbon source, either 0.2% glucose or 0.6% glycerol as indicated. Cells were grown at 37°C, except where described otherwise. To maintain the pAV2 plasmid in the cells, 20 μg/ml chloramphenicol was added to the medium.

**Strategy to measure the rate of degradation of the newly synthesized PG.** Pulse-labeling the PG with trace amounts of [$^3$H]DAP and trapping the turnover products as 1,6-anhydro-muramic acid (anhMurNAc)-peptides in Δ*ampD* cells is rapid even though it involves at least the following 14 steps. (i) About 10 ng of [$^3$H]DAP (1 μCi) is added to 1 ml of growing culture. This amount is sufficient to label cells for 20 min under our conditions (data not shown). (ii) [$^3$H]DAP is imported into the cell via cystine permease (4). (iii) [$^3$H]DAP enters the cyto-

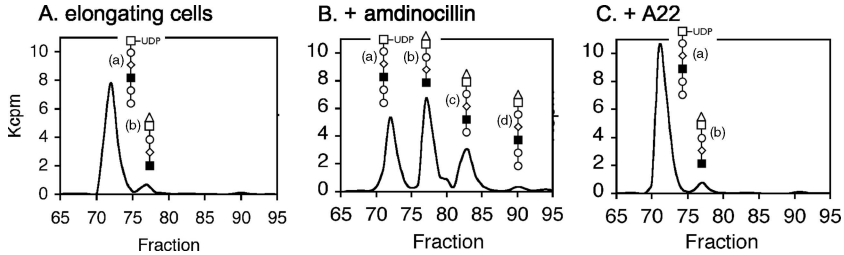



FIG. 2. Contrasting effects of blocking elongation by amdinocillin and A22. TU73 (MG1655 *lysA*::Tn*10* Δ*ampD*)/pAV2 cells in mid-log phase were grown in the M9 glycerol minimal medium containing 0.2% arabinose for 20 min. Control cells (A) and cells treated with 10 μg/ml of amdinocillin (MIC, 0.5 μg/ml) (B) or 30 μg/ml of A22 (MIC, 2 μg/ml) (C) for 5 min were labeled with [$^3$H]DAP for 5 min. The hot-water extracts from cells in 1 ml of culture were analyzed by HPLC, and UDP-MurNAc-pentapeptide (a), anhMurNAc-tripeptide (b), anhMurNAc-tetrapeptide (c), and anhMurNAc-pentapeptide (d) were measured.

plasmic pool of DAP. (iv) DAP is linked to UDP–MurNAc–L-Ala–γ-D-Glu by MurE (61). (v) D-Ala–D-Ala is added by MurF to mix with the UDP-MurNAc-pentapeptide pool, which contains about 200,000 molecules per cell (38). (vi) MraY transfers P-MurNAc-pentapeptide to undecaprenol-P to form lipid 1, with a pool of about 700 molecules (61, 62). (vii) MurG adds GlcNAc from UDP-GlcNAc to form lipid 2 (61) (1,000 to 2,000 molecules in the pool [62]). (viii) Unknown "flippase" moves GlcNAc-MurNAc-pentapeptide to face the periplasm (58, 61). (ix) PBPs polymerize and cross-link the polymer (65). (x) Lytic transglycosylases and endopeptidases cleave PG to form GlcNAc-anhMurNAc-peptide structures (65). (xi) These degradation products (primarily GlcNAc-anhMurNAc-tetrapeptide) are imported via AmpG permease (12, 32). (xii) NagZ removes GlcNAc (11, 66). (xiii) LdcA removes D-Ala from GlcNAc-anhMurNAc-tetrapeptide (54) (xiv) anhMurNAc-tripeptide and other anhMurNAc-peptides accumulate in the cells and serve as a measure of cell wall turnover. In wild-type cells, anhMurNAc-tripeptide is cleaved by AmpD (32, 33), and the product, murein tripeptide, reenters the PG biosynthesis pathway by ligation to UDP-MurNAc (Fig. 1A) (39). In spite of the huge dilution in specific activity upon uptake into the DAP pool in the cytoplasm and passage through a second large pool of UDP-MurNAc-pentapeptide (38), the cells from 1 ml of culture label their murein sacculus with over 20,000 cpm of [$^3$H]DAP in 10 min. By measuring the amount of label incorporated into the murein sacculi (before step x) and the amount of labeled anhMurNAc-peptide accumulating in the cytoplasm of the *ampD* cells (after step xi; Fig. 1B), one can determine the fraction of synthesized PG that has been degraded in any given time period.

In TB78BD (*nagZ nagB ampD*) cells (55), GlcNAc-anhMurNAc-peptide accumulates in the cytoplasm because the strain lacks NagZ β-*N*-acetylglucosaminidase. The amount of PG degradation products trapped compared to the amount being made is an underestimate because 5 to 8% of [$^3$H]DAP-containing peptides are lost to the medium each generation (45). A limitation of this procedure is that the exact amount of each product cannot be determined.

**Procedure for labeling cells and analysis of results.** To study elongation, TU73/pAV2 cells were grown in glycerol minimal medium to mid-log phase (Klett units ≈ 30). One-milliliter cultures were treated with 0.2% arabinose for 20 min to express SulA, block cell division, and cause the cells to filament. The elongating cells were then treated with 30 μg of A22 (the stock solution of A22 contained 10 mg/ml of the compound in dimethyl sulfoxide [DMSO; EMD Chemicals, Inc., San Diego, CA]) or 10 μg of amdinocillin for 5 min followed by incubation with 1 μCi of [$^3$H]DAP (20 Ci/mmol; Moravek Biochemicals, Inc., Brea, CA) for 10 min. In order to block elongation quickly, a concentration of 30 μg/ml of A22, which is 15-fold more than the MIC of A22 for TU73/pAV2, was used. Note that less than 50 μg/ml of A22 does not inhibit processes other than those of MreBCD (34). Arabinose-treated cultures with and without 3 μl of DMSO were used as controls. Three microliters of DMSO did not affect the results (data not shown). Untreated TU73/pAV2 cells, cells expressing SulA for 20 min, and cells treated with 30 μg of A22 for 5 min were also labeled with [$^3$H]DAP for 10 min. Blockage of both elongation and cell division simultaneously did not affect the turbidity or the viability under these condition (data not shown).

TP78BD was grown in the glycerol minimal medium to mid-log phase and treated with A22 for 5 min, followed by labeling for 10 min with 1 μCi/ml of [$^3$H]DAP or 1 μCi/ml of [6-$^3$H]GlcN (21.6 Ci/mol; PerkinElmer, Waltham, MA).

To study temperature-sensitive mutants, TP71 *ampD*::Km, TP71PBP2$^{ts}$ *ampD*::Km, and TP71RodA$^{ts}$ *ampD*::Km were grown at 30°C in the glucose minimal medium to mid-log phase and shifted to 41°C for 15 min, followed by labeling with [$^3$H]DAP for 10 min.

To terminate labeling, cells were chilled by being mixed with cold water on ice, harvested at 4°C, resuspended in 1 ml of cold water, heated for 5 min at 90°C, and centrifuged. The supernatants (hot-water extracts) were collected and lyophilized. The pellets were washed once with 1 ml of water and treated with 400 μg of lysozyme in 1 ml of 10 mM Tris-HCl (pH 8.0) at 37°C overnight. The radioactivity released from the pellet (cell wall) was mixed with Ecoscint H (National Diagnostics, Inc., Atlanta, GA) and counted in a Beckman LS 7500 scintillation counter. The hot-water extracts were analyzed by high-performance liquid chromatography (HPLC) as described below to determine the amounts of radioactivity in UDP-MurNAc-pentapeptide and in the anhMurNAc-peptide structures. It should be noted that all the radioactive anhMurNAc-peptides accumulated in the *ampD*-deficient cells, i.e., anhMurNAc-peptides were not detected in the spent medium (data not shown).

**HPLC analysis.** HPLC was performed with a Waters 1525 binary HPLC pump, a Waters 2487 dual-wavelength absorbance detector, and Breeze software (Waters Co., Milford, MA). Compounds in the hot-water extracts or in the spent media were separated by a Gemini $C_{18}$ column (4.6 by 250 mm, 5-μm particle size; Phenomenex, Inc., Torrance, CA) at a flow rate of 0.5 ml/min by isocratic elution with solvent A (25 mM formate ammonium, pH 3.4) for 10 min, followed by a linear gradient of 0 to 35% solvent B (80% methanol, 20% solvent A) over a period of 35 min.

## RESULTS

**Measurement of the rate of PG degradation during elongation and septation.** Since PG degradation products are trapped in the cytoplasm as anhMurNAc-peptides in an *ampD* mutant (32), we could use the anhMurNAc-peptides as a measure of the amount of PG degraded and determine the fraction of the total PG synthesized that was degraded. With both elongation and division occurring simultaneously in unsynchronized cultures, it is not possible to study either process biochemically in vivo. In this study, we used overexpression of SulA to inhibit cell division in order to selectively study PG synthesis and degradation during elongation. To study septation in relative isolation, we sought a method to specifically block elongation. Both amdinocillin and A22 are known to block elongation; hence, we analyzed their effects on PG synthesis during elongation in cells expressing SulA.

**Modes of action of A22 and amdinocillin on sidewall synthesis.** As shown in Fig. 2B, when elongating cells are treated with amdinocillin, large amounts of anhMurNAc-peptides accumulated, whereas control elongating cells (Fig. 2A) or elongating cells treated with A22 (Fig. 2C) accumulated only trace amounts. Table 1 shows an analysis of the results obtained from the labeling experiments whose results are shown in Fig.

TABLE 1. Degradation of newly synthesized PG in elongating cells and the effects of amdinocillin and A22

| Elongating cells under indicated treatment condition | cpm in TU73/pAV2 cells[a] | | | | % Turnover[e] |
|---|---|---|---|---|---|
| | Cell wall[b] | UDP-MurNAc-pentapeptide | Total anhMurNAc-peptides[c] | Total synthesis[d] | |
| Untreated | | | | | |
| Expt 1 | 9,100 | 7,100 | 900 | 10,000 | 9.0 |
| Expt 2 | 9,900 | 8,200 | 1,000 | 10,900 | 9.2 |
| Amdinocillin | | | | | |
| Expt 1 | 2,200 | 5,600 | 8,200 | 10,400 | 79 |
| Expt 2 | 2,800 | 5,500 | 10,800 | 13,600 | 79 |
| A22 | | | | | |
| Expt 1 | 1,800 | 8,400 | 800 | 2,600 | 31 |
| Expt 2 | 1,900 | 10,700 | 1,000 | 2,900 | 34 |

[a] Growing TU73/pAV2 cells expressing SulA, left untreated or treated for 5 min with 30 μg/ml A22 or 10 μg/ml amdinocillin, were labeled with [$^3$H]DAP for 10 min. All values represent the cpm in cells from 0.5 ml of culture.
[b] The amount of [$^3$H]DAP present in the insoluble fraction after cells were extracted in hot water.
[c] Total anhMurNAc-peptides equals the sum of the anhMurNAc-tripeptide, -tetrapeptide, and -pentapeptide fractions.
[d] Total synthesis is equal to the sum of the cell wall value and total anhMurNAc-peptide value.
[e] Total anhMurNAc-peptide/total synthesis × 100.

2. As shown in this table, 9% of the PG synthesized in untreated cells during the 10-min incubation period was degraded during elongation. This is comparable to the rate of turnover of the existing sidewall calculated from chase experiments (32) and the fact that the poles of the cell are stable once formed (10, 16, 17). Hence, the extensive degradation of sidewall is unlikely to be coupled with insertion of newly synthesized PG into sidewall.

The total amount of PG synthesized in cells treated with amdinocillin was near the normal amount (Table 1). However, 79% of it was degraded and was trapped in the cells as anhMurNAc-peptides. A22, on the other hand, inhibited total PG synthesis drastically, and the amount of degradation was small. These results indicate that amdinocillin did not inhibit the synthesis of glycan strands in elongating cells, whereas A22 did. Moreover, A22 caused accumulation of the major precursor, UDP-MurNAc-pentapeptide, suggesting that A22 inhibits the membrane step of PG synthesis before glycan strands form. Thus, even though both amdinocillin and A22 block cell elongation, their modes of action are strikingly different.

**Septum synthesis.** By using A22 to block elongation, we were able to measure PG synthesis and degradation during cell division in the absence of the PG synthesis required for elongation. Utilizing the PG-recycling mutant as described above, we measured PG synthesis during a 10-min period in control cells, elongating cells, and cells treated with A22. As shown in Table 2, incorporation of [$^3$H]DAP into the cell wall during 10 min was inhibited by 41% when cell division was prevented by SulA and by 74% when A22 was used to inhibit cell elongation. These statistics roughly reflect the fractions of total cell wall synthesis represented by elongation, septation, and concomitant turnover. The large amount of UDP-MurNAc-pentapeptide that accumulated in A22-treated cells represents precursor that would normally have been used to make cell wall. The control cells turned over 11% of newly synthesized PG. Surprisingly, 32% of the newly synthesized PG was turned over in cells treated with A22, compared to only 7% turnover during elongation. This was doubly surprising, since the new poles being formed during septation are stable and do not turn over at all (10, 16, 17).

TABLE 2. Degradation of newly synthesized PG in elongating cells and dividing cells

| Cells | cpm in TU73/pAV2 cells (% inhibition)[a,b] | | | | % Turnover[b] |
|---|---|---|---|---|---|
| | Cell wall | UDP-MurNAc-pentapeptide | Total anhMurNAc-peptides | Total synthesis | |
| Controls | 17,500 (0) | 8,500 | 2,150 | 19,650 | 11 |
| Elongating cells[c] | 10,500 (41) | 6,450 | 800 | 11,300 | 7.1 |
| Dividing cells[d] | 4,600 (74) | 13,700 | 2,200 | 6,800 | 32 |

[a] Growing TU73/pAV2 cells and cells either expressing SulA (elongating cells) or treated with A22 (dividing cells) were labeled with DAP for 10 min. All values represent cpm in cells from 0.5 ml of culture and are the averages of values obtained from two individual experiments. The errors of the paired values obtained were less than 20%.
[b] See the footnotes to Table 1 for definitions of the column headings.
[c] [$^3$H]DAP was added to the culture 20 min after 0.2% arabinose was added to express SulA and arrest septation.
[d] [$^3$H]DAP was added 5 min after 30 μg/ml of A22 was added to arrest elongation.

To examine whether ~32% degradation occurs when sidewall synthesis is blocked by a method other than treatment with A22, temperature-sensitive PBP2 and RodA mutants were used. Cells of both mutant strains grow as rod-shaped cells at 30°C and grow as spheres at 41°C, reflecting inactivation of the elongation pathway in the latter case (52). TP71 *ampD*::Km, TP71PBP2$^{ts}$ *ampD*::Km, and TP71RodA$^{ts}$ *ampD*::Km were grown at 30°C, shifted to 41°C for 15 min, and then labeled with [$^3$H]DAP for 10 min. Note that the mutant cells were still rod shaped after growth at 41°C for 15 min and gradually became spherical over 2 h under the condition used (data not shown). A22 or amdinocillin was added to cultures of TP71 *ampD*::Km 5 min before the addition of [$^3$H]DAP. The results are shown in Table 3. All four conditions inhibited incorporation of DAP into cell wall by about 60%. The rates of degradation were 23% for the temperature-sensitive RodA

TABLE 3. Different effects of blocking elongation by a conditional mutation in PBP2 or RodA or by amdinocillin

| Cell type[a] | cpm in cells[b,c] | | | | % Turnover[c] |
|---|---|---|---|---|---|
| | Cell wall | UDP-MurNAc-pentapeptide | Total anhMurNAc-peptides | Total synthesis | |
| wt | 18,500 | 2,800 | 3,600 | 22,100 | 16 |
| wt treated with amdinocillin | 7,500 | 1,300 | 15,900 | 23,400 | 68 |
| wt treated with A22 | 6,900 | 7,700 | 3,400 | 10,300 | 33 |
| RodA$^{ts}$ | 7,800 | 6,100 | 2,400 | 10,200 | 24 |
| PBP2$^{ts}$ | 7,200 | 9,800 | 3,300 | 10,500 | 31 |

[a] wt, wild type.
[b] TP71 *ampD*::Km, TP71PBP2$^{ts}$ *ampD*::Km, and TP71RodA$^{ts}$ *ampD*::Km were grown at 30°C in M9 glucose minimal medium to mid-log phase and then grown at 41°C for 15 min followed by labeling with [$^3$H]DAP for 10 min. A concentration of 30 μg/ml A22 or 10 μg/ml amdinocillin was added 5 min before labeling of the wild-type cells. All values represent cpm in cells from 0.5 ml of culture and are the averages of values obtained from two individual experiments. The errors of the paired values obtained were less than 15%.
[c] See the footnotes to Table 1 for definitions of the column headings.

mutant and 31% for the temperature-sensitive PBP2 mutant. For the temperature-sensitive RodA mutant, the slightly lower rate of degradation may indicate that the temperature-sensitive mutant RodA protein denatures more slowly than the PBP2 mutant protein and, hence, retains activity longer. The amount of label in UDP-MurNAc-pentapeptide was increased significantly by the mutations and by A22, reflecting the inhibition of PG synthesis. The effects seen with the temperature-sensitive RodA and PBP2 mutants are very similar to the effect of A22 and contrast sharply with those seen with amdinocillin, which lowers the amount of UDP-MurNAc-pentapeptide and greatly increases the amount of degradation.

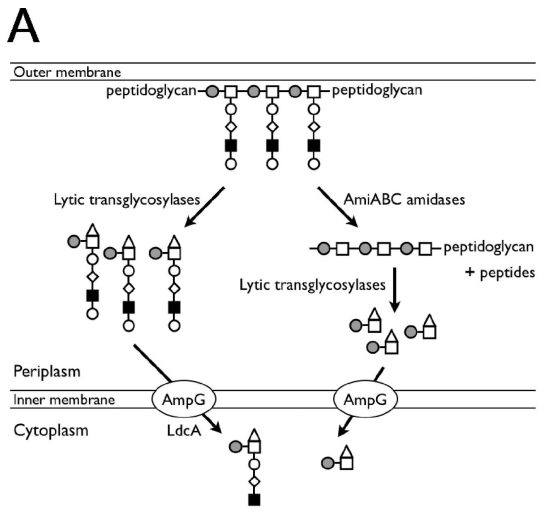
**Activity of periplasmic PG amidases during cell division.** It is known that the three periplasmic PG amidases, AmiA, AmiB, and AmiC, facilitate the separation of the two daughter cells as the last step of cell division (25, 49). We wanted to determine the extent to which the amidases were involved in PG degradation during septum synthesis. However, our system does not detect the degradation products of the amidases, because the radioactive products made by the amidases from [$^3$H]DAP-labeled PG are free peptides. These escape into the medium and cannot be separated from the large excess of [$^3$H]DAP in the medium. To solve this problem, TP78BD, an *E. coli* strain lacking AmpD, AmiD, NagB, and NagZ, was labeled with [$^3$H]GlcN so that glycan strands of PG were labeled. This strain, which lacks the *N*-acetylglucosaminidase that cleaves the GlcNAc-anhMurNAc bond and both amidases that cleave the anhMurNAc–L-Ala bond, has been shown to accumulate GlcNAc-anhMurNAc-tripeptide and the disaccharide GlcNAc-anhMurNAc in the cytoplasm (55). AmiA, AmiB, and AmiC do not cleave the anhMurNAc–L-Ala bond (55) but do cleave the MurNAc–L-Ala bond (25). Since the 1,6-anhydro bond is formed during degradation of PG by lytic transglycosylases (65), any GlcNAc-anhMurNAc recovered in the cytoplasm of TP78BD must have been produced by the action of the amidases on the intact cell wall, followed by the action of lytic transglycosylases (Fig. 3A). Hence, the amount of GlcNAc-anhMurNAc compared to that of GlcNAc-anhMurNAc-peptide reflects the extent of the combined activity of the three PG amidases, AmiA, AmiB, and AmiC, on the PG turned over. TP78BD cells left untreated or treated with A22 for 5 min were labeled with [$^3$H]GlcN or [$^3$H]DAP for 10 min and analyzed as described above. HPLC analyses of the hot-water extracts from cells labeled with [$^3$H]GlcN revealed three new peaks which were not observed in those from cells labeled with [$^3$H]DAP (Fig. 3B). The compound eluted first (corresponding to peak 1) appears to be UDP-MurNAc, and the second new peak (peak 2) was proven to correspond to GlcNAc-anhMurNAc disaccharide by the elution time and by cleavage with NagZ β-*N*-acetylglucosaminidase (data not shown). The third new peak (peak 4) was not identified but was not cleaved by either AmiD or NagZ enzyme (data not shown), indicating that it is not a compound related to PG. As shown in Fig. 3C, when cells were labeled with [$^3$H]DAP, the result was very similar to that obtained with our standard assay strain (TU73/pAV2). A22 inhibited more than half of the incorporation into cell wall, whether labeled with [$^3$H]GlcN or [$^3$H]DAP. The amount of GlcNAc-anhMurNAc was 16% of that of the total degradation products in cells treated with A22, suggesting that one MurNAc–L-Ala bond in six muropeptides of the degraded PG was cleaved by the amidases before the glycan strands were cleaved by lytic transglycosylases. The actual degradation rate measured in [$^3$H]GlcN-labeled cells is slightly higher than that in [$^3$H]DAP-labeled cells because the former group had GlcNAc-anhMurNAc, which was not detected in cells labeled with [$^3$H]DAP. Furthermore, we could detect by digestion with purified hexahistidine SltY followed by HPLC analysis a trace (less than 3%) of PG lacking a peptide in the cell wall of A22-treated cells labeled with [$^3$H]GlcN for 10 min (data not shown). This indicates that glycan strands whose peptides are removed by amidases are rapidly degraded by lytic transglycosylase.

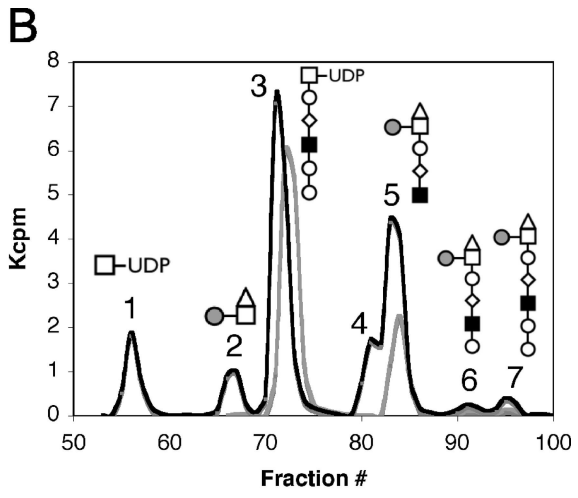
## DISCUSSION

We have developed a new method to determine the rates of PG synthesis and degradation during elongation and septation of *E. coli* cells. Several new insights concerning cell wall metabolism have been obtained by using this method.

**(i) The mode of action of A22 differs from that of amdinocillin even though both are elongation inhibitors.** Amdinocillin and A22 cause cells to grow as spheres (31, 46), suggesting that both drugs inhibit sidewall synthesis. Previous work indicated that amdinocillin inhibited the incorporation of [$^3$H]DAP into the cell wall of normally growing cells by approximately 60% (70) and that it inhibits PBP2 transpeptidase by binding covalently to PBP2 (29, 30, 53). Our result indicates that in the presence of amdinocillin, glycan strands are formed at the normal rate but the nascent glycan strands are rapidly broken down by lytic transglycosylases, resulting in an apparent inhibition of cell wall synthesis. The glycan is degraded presumably because the newly synthesized glycan strands cannot be cross-linked to the existing sidewall when PBP2 transpeptidase is inactive. Since the major lytic transglycosylases in *E. coli* are exoenzymes that cleave the glycan strands from the nonreducing end (3) and the glycan strands elongate at the reducing end (48, 69), one can envision how synthesis and degradation can proceed simultaneously when the transpeptidase activity of PBP2 is blocked by amdinocillin. This is consistent with the model of PG synthesis machinery including PG synthases and hydrolases (28). Surprisingly, very little anhMurNAc-pentapeptide was recovered. This must indicate that the D,D-carboxypeptidases (PBP4, PBP5, and PBP6) are very active. It is also interesting that a large amount of anhMurNAc-tetrapeptide, in addition to the expected anhMurNAc-tripeptide, was detected in cells treated with amdinocillin (Fig. 2B). This demonstrates that the action of LdcA, which removes D-Ala from the stem tetrapeptide (54), is slow compared to the production and import of the degradation products and compared to the action of NagZ (11, 66), which removes GlcNAc from the degradation products.

In contrast to amdinocillin, A22 prevented sidewall synthesis without forming significant amounts of PG degradation products (Fig. 2C and Table 1), indicating that glycan synthesis was indeed inhibited. Inhibition of glycan synthesis was also evidenced by the accumulation of the precursor UDP-MurNAc-pentapeptide. Preliminary results showed that A22 did not affect the levels of lipid 1 and lipid 2 (data not shown), suggesting that A22 inhibits formation of the PG strands from lipid 2. We here conclude that A22 inhibits glycan strand syn-




C

| Material to label cells | peak | [$^3$H]Dap Control | [$^3$H]Dap A22 | [$^3$H]GlcN Control | [$^3$H]GlcN A22 |
|---|---|---|---|---|---|
| Cell wall | | 28400 | 11400 | 62700 | 29800 |
| UDP-MurNAc | 1 | nd | nd | 3400 | 3600 |
| aD | 2 | nd | nd | 2700 | 2400 |
| UDP-MurNAc-pentapeptide | 3 | 6700 | 14100 | 9700 | 15100 |
| Unrelated compound | 4 | nd | nd | 3500 | 3400 |
| aD-tripeptide | 5 | 4200 | 4600 | 9500 | 10500 |
| aD-tetrapeptide | 6 | 170 | 280 | 840 | 770 |
| aD-pentapeptide | 7 | 230 | 330 | 1100 | 970 |
| [1]Total degradation | | 4600 | 5210 | 14140 | 14640 |
| [2]Total synthesis | | 33000 | 16610 | 76840 | 44440 |
| [3]% turnover | | 14% | 31% | 18% | 33% |

FIG. 3. (A) The scheme for estimation of the activity of AmiABC by using TP78BD (*ampD amiD nagB nagZ*). In the TP78BD strain, once PG is cleaved by lytic transglycosylases, the degradation products containing anhMurNAc, which are not substrates of the three amidases, enter the cytoplasm via AmpG permease (55). When PG is first cleaved by the amidases, followed by the action of lytic transglycosylases, GlcNAc-anhMurNAc is produced and imported by AmpG (55). In TP78BD cells, GlcNAc-anhMurNAc and GlcNAc-anhMurNAc-tripeptides imported by AmpG are not cleaved further because AmpD and NagZ are absent; thereby GlcNAc-anhMurNAc and GlcNAc-anhMurNAc-tripeptides accumulate in the cytoplasm (55). Hence, the amount of GlcNAc-anhMurNAc reflects the extent to which AmiABC amidases have cleaved the stem peptides from PG before the action of lytic transglycosylases. See the legend to Fig. 1 for symbol definitions. (B) Combined activity of amidases AmiA, AmiB, and AmiC during cell division. HPLC analysis of the hot-water extracts from TP78BD cells treated with 30 μg/ml of A22 for 5 min followed by labeling with [$^3$H]DAP (gray line) or [$^3$H]GlcN (black line) for 10 min. (C) The table shows the amounts of radioactivity (cpm) in the compounds from 0.5 ml of culture. Total degradation (footnote 1) is the sum of peaks 2, 5, 6, and 7. Total synthesis (footnote 2) is equal to the sum of the cell wall value and the total anhMurNAc-peptide value. Percent turnover (footnote 3) equals total degradation/total synthesis × 100. aD, GlcNAc-anhMurNAc; nd, not detected.

thesis during elongation. Hence as previously suggested by Iwai, the mode of action of A22 is different from that of amdinocillin (31). The direct target of A22 has been shown to be MreB, a bacterial homolog of actin (21, 34). However, it remains unclear whether MreB is required for PG glycan synthesis or whether, in addition, A22 blocks another protein essential for synthesizing glycan strands. The rate of incorporation of DAP into PG was reduced more than 40% when septation was prevented by overexpression of SulA (Table 2). The addition of 30 μg/ml of A22 inhibited about 80% of the residual synthesis occurring in elongating cells (Table 1). There is, however, some 10% of PG synthesis that is not blocked by simultaneous expression of SulA and treatment with A22 to inhibit both cell division and elongation. The residual PG synthesis was not inhibited by amdinocillin (data not shown). We do not know the source of this residual synthesis. It may merely be due to incomplete inhibition by SulA and A22.

**(ii) PBP2 and RodA are required for PG glycan synthesis.** Since A22 blocked synthesis of glycan strands, MreB, a target of A22, is probably required for the reaction. We have also shown that inhibition of PG synthesis and the rate of degradation for the temperature-sensitive mutant cells paralleled those of cells with A22 rather than those of cells treated with amdinocillin (Table 3). This suggests that temperature-sensitive PBP2 and RodA mutants are defective in glycan strand synthesis. It was surprising to observe that denaturation of PBP2 gave results that were completely different from those of

inactivation of the enzyme activity of PBP2 with amdinocillin. Our interpretation of this result is that the native PBP2 protein itself is needed as part of the machinery required for the formation of glycan strands rather than functioning solely to cross-link the new strands between existing strands of the elongating cell wall. The PBP2 protein must be participating in glycan strand synthesis quite apart from its role in cross-linking.

Synthesis of glycan strands from lipid 2 involves two activities: that of a flippase to transfer the hydrophilic GlcNAc-MurNAc-pentapeptide from the inside to the outside of the inner membrane and that of a transglycosylase to form the glycan strands from lipid 2. PBP1A, PBP1B, PBP1C, and MtgA are known transglycosylases (65). Of these, the major enzyme, PBP1B, has in vitro activity that is estimated to be at most only 3% of the in vivo activity (62). A recent report of a study using fluorescent-vancomycin labeling suggested that MreC and RodA are required for PG synthesis activity in *C. crescentus* (18). Without proposing how the proteins essential for cell wall elongation cooperate, we suggest that a putative machine that includes PBP2, RodA, and MreB (and presumably MreC and MreD) together with a transglycosylase is necessary for one or both of these two activities in vivo and that the relevant proteins in combination greatly enhance the activity of the participating transglycosylase.

**(iii) PG degradation during septation is caused by lytic transglycosylases with a minor contribution by PG amidases.** Taking advantage of the fact that an *ampD amiD* double mutant lacks anhMurNAc–L-Ala amidase activity, we have shown that one-sixth of the "extra PG" (i.e., PG that is made and degraded and would normally be recycled during the experimental period) was first cleaved by the periplasmic amidases (AmiA, AmiB, and AmiC), and this was followed by action of lytic transglycosylases. The result confirms that one or more of the amidases are active during cell division, as has been inferred from studies with mutants (25, 50), and that lytic transglycosylases are the major enzymes to degrade the extra PG. There is one caveat. If the amidases created stretches of peptide-free glycan strands that were only slowly digested by MltA and EmtA, the only lytic transglycosylases known to cleave such strands (35, 57), and these strands became detached from the sacculus, our method would not have detected them. Moreover, it remains unknown whether the amidases cleave every sixth bond at random along the glycan strands or whether every sixth glycan strand is degraded as a contiguous whole.

It has been observed that only 30 to 50% of cells lacking six lytic transglycosylases formed short chains consisting of 3 to 8 cells (26), whereas over 90% of cells lacking the three amidases formed longer chains consisting of up to 24 cells (26). This report suggests that the amidases are more important than lytic transglycosylases for cell separation. These conflicting observations might be reconciled if the amidases are required for cleavage of the sidewall at the division site. Imagine a diaphragm forming the septum to consist of multiple layers of PG, and lytic transglycosylases over time remove the hypothetical middle layer(s) from between the two layers that become the new poles. The cells will not separate, because the preexisting sidewall at the division site is still intact. Perhaps the amidases must cleave this region of the sidewall.

**(iv) The significance of the high rate of PG degradation that occurs during septation.** By blocking elongation with A22, we could determine the rate of PG degradation during septation and demonstrated that during septation, a significant amount of PG in addition to the amount required for construction of the stable poles of the daughter cells was synthesized and rapidly degraded (Table 2). The extra PG was over 30% of the total amount synthesized during cell division. A similar figure was also observed when elongation was blocked by the mutation of $PBP2^{ts}$ (Table 3). This is sufficient to form an additional pole. It is reminiscent of the three-for-one model proposed by Höltje (28). In this model of cell division, three layers of PG are synthesized, followed by the removal of the middle layer, resulting in separation of the two layers that become the two daughter poles, each one layer thick. Since the septum is made exclusively of newly synthesized PG (16, 17), 33% degradation of the total PG synthesized is a perfect fit with the model. However, there is a caveat. Our data do not tell us what proportion of the middle layer has been removed during the 10-min experimental period. If only 67% of the middle layer is released during the experimental period, the 33% figure would become 50% and be consistent with a septum in which two layers of PG separate the two poles. One might expect the septum to be made of an even number of PG layers because PBP1B, a major transglycosylase shown to interact with PBP3 (6), functions better as a dimer than a monomer (5). Thin-section electron micrographs of *E. coli* B septa appear to show the poles separated by an electron-transparent gap with patches possibly retaining PG (8). Similar gaps have been observed in the chain-forming *envA* (9) and *envC* (44) mutants. Presumably, the gap exists because lytic enzymes and/or other polymers, including an unknown amount of PG that is sufficient to withstand the osmotic pressure of the daughter cells, are present. Another report that is consistent with our conclusion that the septum may transiently consist of multiple layers is the observation that 20 to 25% of the cell wall is triple layered and the remaining 75 to 80% is single layered (37). An alternative interpretation of the role of lytic transglycosylases, though unlikely in our view, is that only two layers of transverse PG are made and one-third of each is degraded during separation of the poles. Our data indicate only that, in addition to the PG required for formation of two stable poles, extra PG, perhaps sufficient to produce two additional poles, is synthesized and rapidly degraded. Whether the synthesis or degradation of the extra PG is required for cell division remains unknown.

Septation and cell separation is a very intricate and precarious process requiring at least 10 essential genes. It is unlikely that the heretofore undetected activity of the lytic transglycosylases during this process is accidental. To solve these questions, mutants deficient in synthesis or degradation of the extra PG are needed. The greater unresolved questions are why the poles of the cell are stable and resist attack by lytic enzymes and why 60% of the sidewalls of the cell are turned over each generation.

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