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Bacterial cell wall recycling provides cytosolic muropeptides as effectors for β-lactamase induction

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A mechanism for bacteria to monitor the status of their vital cell wall peptidoglycan is suggested by the convergence of two phenomena: peptidoglycan recycling and β-lactamase induction. *ampG* and *ampD*, genes essential for β-lactamase regulation, are here shown to be required for recycling as well. Cells lacking either AmpG or AmpD lose up to 40% of their peptidoglycan per generation, whereas *Escherichia coli* normally suffers minimal losses and instead recycles 40 or 50% of the tripeptide, L-alanyl-D-glutamyl-meso-diaminopimelic acid, from its peptidoglycan each generation. The *ampG* mutant releases peptidoglycan-derived material into the medium. In contrast, the *ampD* mutant accumulates a novel cell wall muropeptide, 1,6-anhydro *N*-acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelic acid (anhMurNAc-tripeptide), in its cytoplasm. This work suggests that AmpG is the permease for a large muropeptide and AmpD is a novel cytosolic *N*-acetylmuramyl-L-alanine amidase that cleaves anhMurNAc-tripeptide to release tripeptide, which is then recycled. These results also suggest that the phenomenon of β-lactamase induction is regulated by the level of muropeptide(s) in the cytoplasm, since an *ampD* mutation that results in β-lactamase expression even in the absence of a β-lactamase inducer coincides with accumulation of anhMurNAc-tripeptide. The transcriptional regulator AmpR is presumably converted into an activator for β-lactamase production by sensing the higher level of muropeptide(s). This may be an example of a general mechanism for signaling the progress of external events such as cell wall maturation, cell division or cell wall damage.

Key words: β-lactamase induction/muropeptides/peptidoglycan recycling

Introduction

Most bacteria have a rigid cell wall that protects the integrity of their cytoplasmic membrane and is therefore essential for survival. The cell wall, termed the murein sacculus, is composed of a heteropolymer known as murein or peptidoglycan. The peptidoglycan consists of glycan chains of alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid which are frequently cross-linked to each other by short peptides. In Gram-negative bacteria, the peptides, usually L-alanyl-D-glutamyl-meso-diaminopimelyl-D-alanine (L-Ala-D-Glu-DAP-D-Ala), are linked to the glycan via an amide bond between the muramic acid carboxyl groups and L-Ala, and to peptides from neighboring glycans by a peptide bond between D-Ala and DAP. Although this highly cross-linked network forms a rigid, insoluble envelope in the shape of the cell, the murein sacculus is, nevertheless, in a dynamic state throughout the life of the cell. Bonds are broken to allow insertion of new glycan chains during enlargement of the cell and to effect separation of daughter cells. In addition, *Escherichia coli,* and presumably most other Gram-negative bacteria, recycles components from 40–50% of its murein sacculus each generation (Goodell, 1985; Goodell and Schwarz, 1985; Park, 1993). The studies by Goodell strongly suggest that as peptidoglycan is degraded in the periplasm, the murein tripeptide, L-Ala-D-Glu-DAP, derived therefrom is transported into the cytoplasm. Here it is incorporated into the precursor, uridine 5′ pyrophosphoryl *N*-acetylmuramyl-L-Ala-D-Glu-DAP-D-Ala-D-Ala (UDP-MurNAc-pentapeptide) and re-utilized for peptidoglycan biosynthesis without breakdown to free DAP. Tripeptide is specifically taken up by the cell via the high-affinity oligopeptide permease transport system (Opp) since *opp* negative strains do not incorporate externally added tripeptide into their murein sacculus (Goodell and Higgins, 1987). Surprisingly, it has recently been demonstrated that *opp* negative strains recycle murein-derived material very efficiently (Park, 1993). It was, therefore, proposed that instead of the Opp permease, a separate low-affinity uptake system for the tripeptide is required for recycling murein tripeptides in *E.coli*. However, as we shall demonstrate, instead of tripeptide a larger muropeptide is transported into the cell, where additional cytoplasmic steps lead to release of tripeptide and its subsequent re-utilization.

A number of Gram-negative species, such as *Citrobacter freundii* and *Enterobacter cloacae,* express a chromosomal β-lactamase (AmpC) that is inducible by β-lactam antibiotics (Lindberg and Normark, 1987). Since β-lactams are not believed to enter the cytoplasm, it has been suggested that β-lactam inhibition of murein biosynthesis releases an endogenous cell wall-derived autoinducer which can enter the cell and bind to the transcriptional

regulator AmpR, converting it into an activator for the *ampC* promoter (Lindquist *et al.*, 1989). Mutants in *ampD*, which in turn encodes a cytosolic protein, are semi-constitutive for β-lactamase production (Honoré *et al.*, 1989; Lindquist *et al.*, 1989). This elevated expression of β-lactamase in the absence of β-lactam inducer suggests that bacterial cells in which AmpD activity is limiting contain a higher concentration of the hypothetical AmpR activating ligand. AmpD would normally maintain the cytosolic concentration of this ligand at a level too low for induction. Tuomanen *et al.* (1991) have shown that *ampD* mutants have an altered composition of peptidoglycan when grown in the presence of a high concentration of DAP, indirectly suggesting a link between peptidoglycan metabolism and β-lactamase induction. The *ampD* gene shares an operon with a second gene, *ampE* (Lindquist *et al.*, 1989). *ampE* was initially thought to be involved in β-lactamase expression, but recent work in which *ampE* was inactivated has shown that *ampE* is not required (Normark *et al.*, 1994). Another gene that is required for induction of β-lactamase is *ampG*, which encodes a transmembrane protein. In the absence of this protein, no induction occurs (Korfmann and Sanders, 1989), nor does constitutive activation of *ampC* take place in *ampG, ampD* double-mutant strains (Lindquist *et al.*, 1993). Its cellular location and the phenotype conferred by the genetic inactivation of *ampG* suggest that AmpG might act as a permease for the hypothetical activating ligand. Interestingly, *E.coli* contains the *ampG* and *ampD* genes, even though this organism lacks *ampR* and the DNA-binding site for AmpR and, hence, its native AmpC β-lactamase is not inducible by β-lactam antibiotics (Korfmann and Sanders, 1989; Lindquist *et al.*, 1989). However, *E.coli* harboring *ampR* and *ampC* from *C.freundii* on a plasmid can be induced to form *C.freundii* AmpC β-lactamase when exposed to β-lactam antibiotics (Lindberg *et al.*, 1985). Furthermore, an *E.coli ampD* mutant overproduces *C.freundii* AmpC β-lactamase in the absence of β-lactams (Lindberg *et al.*, 1987).

These facts suggested to us that AmpG and AmpD might be part of the recycling pathway, and led us into a study of murein turnover in such mutants.

Results

Rate of murein turnover of ampG and ampD negative strains

Since β-lactams interfere with peptidoglycan synthesis and thus may increase the release of murein-derived fragments, and since most recycling of a murein-derived fragment (originally assumed to be the tripeptide) occurs via an unknown uptake pathway clearly distinct from the Opp transport system, we first tested AmpG for involvement in recycling. Strains defective in a protein required for murein recycling should have an increased rate of turnover (i.e. loss of radiolabeled murein components from the cell). As shown in Table I, strains inactivated for *ampG* (TP72, HfrH01 and CH483 *ampG*::kan) lost 20–40% of the [^{14}C]DAP component from their cell wall each generation over several generations, while the control strains lost only ~4–8% per generation. The increase in radioactive murein observed in control cells during the first generation of chase is due to utilization of the [^{14}C]DAP remaining in the cytoplasmic pool.

This result, demonstrating a link between recycling of peptidoglycan and β-lactamase induction, led us to test whether AmpD was involved in a cytoplasmic step of the recycling pathway. As shown in Table I, the strains deleted for *ampD* and *ampE* (JRG582 and TP73) lost label at the same high rate as the *ampG* strains. This high rate of loss always continued for at least four generations in the TP72 and TP73 strains. However, the HfrH01 and CH483 *ampG*::kan strains usually lost label for only two or three generations. We presently have no explanation for this strain difference.

To determine if the turnover rate was dependent on *ampD*, *ampE* or both, we introduced the plasmid pNU404 carrying the *ampD* gene into *E.coli* strain TP73. As shown in Table I, pNU404 complemented the defect and restored normal murein turnover, suggesting that AmpE does not affect turnover. Similarly, introduction of pGKS273-3 carrying *ampG* into the *ampG* negative strain TP72 restored the normal low rate of turnover (Table I).

Dramatic accumulation of a ^{3}H-labeled muropeptide in the ampD negative strain

If AmpD is involved in the recycling pathway, *ampD* mutants should accumulate a murein-derived substrate for AmpD in their cytoplasm. The *ampD* strain was compared with the wild-type and *ampG* strains as follows. The *ampD* and *ampG* single mutants, and the *ampG, ampD* double mutants, were labeled with [^{3}H]DAP for two generations and hot water extracts therefrom were analyzed by molecular sieve chromatography and compared with wild-type for their ^{3}H-labeled components. In the wild-type strain HfrH, only three peaks were observed (Figure 1A). Peak (A) corresponds to the void volume and contains proteins presumably labeled in their lysine residues since the strains used contain DAP decarboxylase (LysA). Indeed, this peak does not appear in the *lysA* strain TP71 which does not convert DAP into lysine (Figure 1F). Peaks (B) and (E) were identified as UDP-MurNAc-pentapeptide and free DAP, respectively, by comparison with known standards (data not shown).

In the *ampG* strain, an additional peak (C), eluting just after the UDP-MurNAc-pentapeptide [peak (B)], was readily detected (Figure 1B). Its position in the chromatogram indicates a mol. wt in the range of 600–1000 rather than the 390 expected for murein tripeptide. Peak (C) contains a mixture of compounds and the muropeptides from the *ampG* strain will be shown to be different from that which accumulates in the *ampD* strain. As can be seen in Figure 1C, the *ampD* strain contained an extremely high level of a muropeptide(s) in this weight range, compared to the content of UDP-MurNAc-pentapeptide which remained relatively constant in all three strains (note the different scale on the *y*-axis of panel C and the reduced amount of cell extract loaded). Expression of the *ampD* gene from pNU404 in the *ampD* mutant, JRG582, restored the normal distribution of the ^{3}H label (Figure 1E). Accumulation of this material in the *ampD* strain thus accounts for its high rate of turnover from the murein sacculus. Gel filtration of the hot water extract of the *ampG, ampD* double mutant JRG58201 (Figure 1D) gave a profile similar to the *ampG* strain HfrH01, i.e. it lacked

Table I. Increased turnover of murein in *ampG* and *ampD* mutants of *E.coli*

Strains	Chromosomal genotype	Plasmid genotype	Loss of remaining [^{14}C]DAP label from the murein per generation (%)			
			1st	2nd	3rd	4th
Derivatives of MC4100[a]	*lysA* , *opp*					
TP71			−9	7	10	12
TP72	*ampG*		24	32	44	43
TP72/pGKS273-3[b]	*ampG*	*ampG* $^{+}$	−9	6	10	10
TP73	*ampDE*		21	34	39	36
TP73/pNU404	*ampDE*	*ampD*$^{+}$	−20	11	6	10
TP74	*ampDE ampG*		22	33	44	45
Derivatives of HfrH[a]						
HfrH			1	10	7	2
HfrH01	*ampG*		16	19	4	2
JRG582	*ampDE*		21	35	31	24
JRG58201	*ampDE ampG*		17	20	2	–
Derivatives of CH483[a]	*opp*					
CH483			−24	3	6	−2
CH483 *ampG::kan*	*ampG*		1	21	27	3

Log-phase cells were labeled with [^{14}C]DAP and chased as described in Materials and methods. Negative values indicate a net gain of ^{14}C label in the murein.
[a]Values are means of three or more experiments with SEs of 5%.
[b]Values are means of duplicate experiments with SEs of 5%.

the huge accumulation of peak (C). Thus, AmpG is involved in a recycling step prior to AmpD, which is consistent with AmpG being the permease required for recycling.

Release of ^{3}H-labeled muropeptides into the culture medium of ampG strains

Since there was little accumulation of ^{3}H-labeled muropeptides in the hot water extracts of the *ampG* strains, HfrH01 and JRG58201, which have high murein turnover rates, the spent medium of these strains, as well as that of HfrH and JRG582, was examined by molecular sieve chromatography to identify the released materials. As can be seen in Figure 2A, except for the enormous amount of free DAP [peak (E)], no other peaks were clearly detectable in the HfrH culture medium. Two components, (C) and (D), of larger molecular weight than DAP were observed in the culture medium of HfrH01 (Figure 2B, zoom). The material in peak (D), from its behavior upon molecular sieve chromatography, could be free tripeptide and/or tetrapeptide. The ^{3}H-labeled materials in the spent medium from cultures of JRG582 and JRG58201 were similar to those of HfrH and HfrH01, respectively (data not shown). Thus, in contrast to the *ampD* deletion strain JRG582, the *ampG* strain HfrH01 and the *ampG*, *ampD* double mutant JRG58201 release murein-derived materials into the medium rather than accumulating them within the cell.

Identification of the compound that accumulates in the ampD strain as a unique muropeptide: 1,6 anhydro N-acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelic acid

The ^{3}H-labeled muropeptide [peak (C), Figure 1C] isolated from extracts of the *ampD* mutant JRG582 by gel filtration were further fractionated by reversed-phase HPLC. Two peaks with a retention time (Rt) of 30 and 32 min, accounting for 5% and 94% of the label, respectively, were obtained with elution method A (see Materials and methods). In this system, tripeptide (Rt = 5.2 min) and tetrapeptide (Rt = 7.2 min) standards were clearly separated from these radioactive peaks (data not shown). Next, the purified ^{3}H-labeled compound was mixed with authentic GlcNAc-anhMurNAc-tripeptide (Rt = 26 min) and GlcNAc-anhMurNAc-tetrapeptide (Rt = 52.5 min), and fractionated by HPLC with elution method B (see Materials and methods). A peak of ^{3}H at 22 min and the expected UV-absorbing peaks at 26 and 52.5 min were obtained. Thus, the ^{3}H-labeled compound that accumulates in the *ampD* deletion strain is clearly neither GlcNAc-anhMurNAc-tripeptide nor GlcNAc-anhMurNAc-tetrapeptide, the usual muropeptides resulting from transglycosylase degradation of murein in *E.coli* (Höltje and Tuomanen, 1991).

We therefore purified a quantity of this new cell wall fragment from *E.coli* JRG582 cells grown in the presence of [^{3}H]DAP as tracer. After gel filtration, the material recovered in peak (C) was further fractionated by HPLC (Figure 3A). The homogeneity of the preparation was confirmed by subsequent HPLC: a single, radioactive UV-absorbing peak was observed (Figure 3B) using method E (see Materials and methods). The purified sample was analyzed for amino acids and amino sugars. For each mol of glutamic acid, 1.00 mol of alanine, 0.92 mol of DAP and 1.12 mol of muramic acid were found, but glucosamine was completely absent. By mass spectrometry, a protonated ion of mass 648 Da was found (Figure 3C). It differs from GlcNAc-anhMurNAc-tripeptide (MH^{+} = 851) by 203 Da, which exactly corresponds to the loss of the *N*-acetylglucosamine. Thus, the *ampD* mutant accumulates a previously undescribed muropeptide: 1,6 anhydro *N*-acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelic acid (anhMurNAc-tripeptide). About 400 nmol of anhMurNAc-tripeptide were obtained from 1 l of culture.

The anhMurNAc-tripeptide is present in the cytoplasm of the ampD strain

The hot water extracts studied thus far contained both periplasmic and cytoplasmic constituents. Since anhMur-

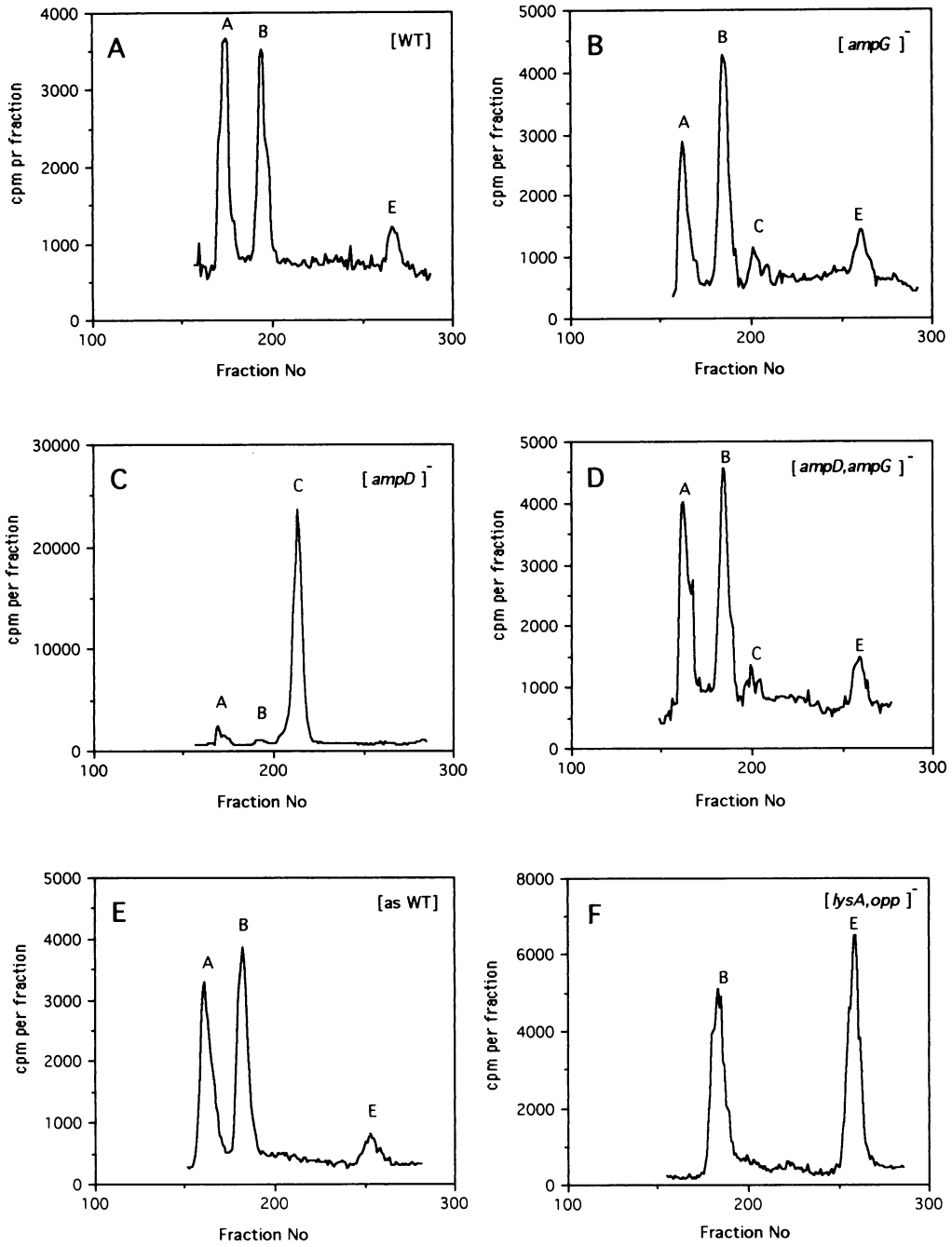

Fig. 1. Molecular sieve chromatography of hot water extracts of *E.coli* strains labeled with [^{3}H]DAP for two generations. The cells were boiled in water and the insoluble particles were removed by centrifugation. The supernatant was then placed on a Toyopearl column and the ^{3}H-labeled compounds were separated according to their size. The peaks, as labeled in all figures (including Figures 2, 4 and 5), represent different size classes: peak (A) = exclusion limit; peak (B) = exclusion limit−1000; peak (C) = 600–1000; peak (D) = 350–500; peak (E) <200. (**A**) Wild-type strain HfrH; 150 000 c.p.m. of the extract were loaded which corresponded to 8 ml culture. (**B**) *ampG* strain HfrH01; 130 000 c.p.m. of the extract were loaded which corresponded to 7.5 ml culture. (**C**) *ampD* strain JRG582; 280 000 c.p.m. of the extract were loaded which corresponded to 2 ml culture. (**D**) *ampD ampG* strain JRG58201; 130 000 c.p.m. of the extract were loaded which corresponded to 7 ml culture. (**E**) *ampD* strain JRG582/pNU404 which carries *E.coli ampD* gene; 100 000 c.p.m of the extract were loaded which corresponded to 8 ml culture. (**F**) *lysA opp* strain TP71; 150 000 c.p.m of the extract were loaded which corresponded to 3 ml culture.

NAc-tripeptide (mol. wt = 647) is large enough to be retained within the periplasmic space, it could have accumulated either in the periplasm or in the cytoplasm of the *ampD* cells. We fractionated [^{3}H]DAP-labeled JRG582/pNU305 cells (expressing the periplasmic β-lactamase of *C.freundii* from the plasmid) into periplasmic and cytoplasmic fractions by treatment with lysozyme and EDTA (Osborn *et al.*, 1972). Essentially all the β-lactamase activity was released from the cells, while the spheroplasts retained 90% of the cellular ATP (data not shown) and, as shown in Figure 4, most of the label was also retained by the spheroplasts and was recovered in peak (C). Thus, anhMurNAc-tripeptide accumulated in the cytoplasm, which is consistent with the fact that AmpD is a cytosolic protein and strongly suggests that this newly found cell wall fragment is a substrate for AmpD.

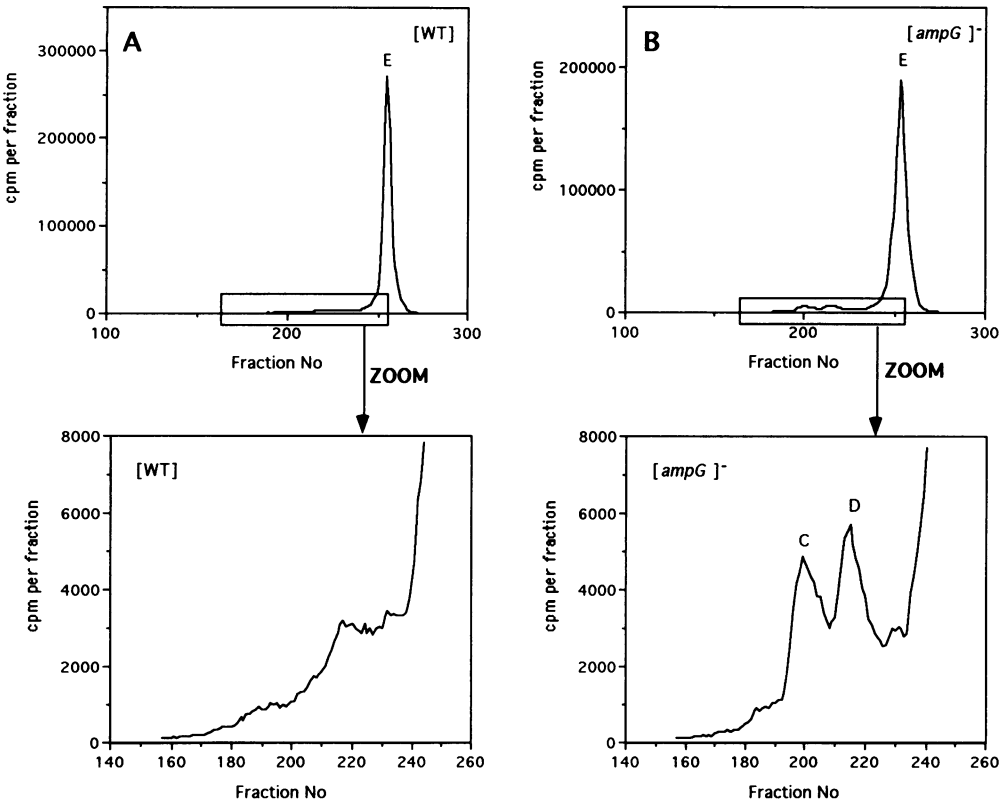

Fig. 2. Molecular sieve chromatography of spent medium of *E.coli* strains labeled with [^{3}H]DAP for two generations. Half of the medium sample (5 ml), after filtration to remove bacteria, was concentrated and chromatographed on the Toyopearl column. Approximately 1 700 000 c.p.m. were loaded. The peaks refer to different size classes defined in the legend of Figure 1. (**A**) Wild-type strain HfrH. (**B**) *ampG* strain HfrH01.

Correlation with β-lactamase induction

We have shown a direct link between recycling of peptidoglycan fragments and β-lactamase induction. Because in the absence of a β-lactam inducer, the *ampD* mutation mimics the induced state, the phenomenon of β-lactamase induction in wild-type cells could be related to an accumulation of anhMurNAc-tripeptide. To test this hypothesis, actively growing cultures of HfrH, HfrH01, JRG582 and JRG58201 each carrying plasmid pNU305, which contains the *C.freundii ampR* and *ampC* genes required for expressing the *C.freundii* β-lactamase, were exposed to the β-lactamase inducer, cefoxitin (10 mg/l), for one generation. Extracts of these cells were made and subjected to molecular sieve chromatography. The wild-type strain HfrH/pNU305 contained a small, but detectable amount of peak (C) and a larger quantity of peak (D) (Figure 5A, compare with Figure 1A). Peak (D) had also been detected in the culture medium of strain HfrH01. Another effect of cefoxitin treatment is that peak (B) (UDP-MurNAc-pentapeptide) was significantly reduced (compare with Figure 1A).

For the *ampG* strain HfrH01/pNU305, exposure to cefoxitin also reduced the pool of UDP-MurNAc-pentapeptide [peak (B)] significantly while peak (C) increased, but no peak (D) was present (Figure 5B). The identical gel-filtration profile was observed for the *ampG, ampD* strain, JRG58201/pNU305 (data not shown). Figure 5C shows that JRG582/pNU305 cultured with cefoxitin is essentially indistinguishable from the untreated culture (Figure 1C).

Thus, peak (D) only accumulates in the wild-type strain and not in the *ampD* or *ampG* strains as a consequence of the presence of cefoxitin. This indicates that the AmpG and AmpD proteins are both required for its accumulation. Because of its apparent molecular weight, peak (D) was provisionally identified as the tripeptide which Goodell and Schwarz (1985) had characterized. Goodell and Higgins (1987) have shown that tripeptide could be efficiently taken up by oligopeptide permease (Opp). To determine if the Opp transport system is involved in the accumulation of peak (D), an extract of the *opp* negative strain TP71/pNU305 grown in the presence of cefoxitin was analyzed by gel filtration and the resulting chromatogram clearly shows the presence of peak (D) (Figure 5D). Thus, Opp is not responsible for the appearance of peak (D) during induction of β-lactamase.

Characterization of the intracellular and extracellular murein-derived material in peaks (C) and (D)

According to our data, AmpG is involved in the recycling of peptidoglycan, most likely by transporting a murein fragment from the periplasmic space to the cytoplasm. The anhMurNAc-tripeptide that accumulates in *ampD* cells is found in the cytoplasm and, therefore, could logically be the component that crosses the cytoplasmic membrane. However, anhMurNAc-tripeptide is presumably formed by removal of *N*-acetylglucosamine from GlcNAc-anhMurNAc-tripeptide and the only β-*N*-acetylglucosaminidase known to be present in *E.coli* is located in the cytoplasm (Yem and Wu, 1976). In an attempt to determine whether GlcNAc-anhMurNAc-tripeptide is

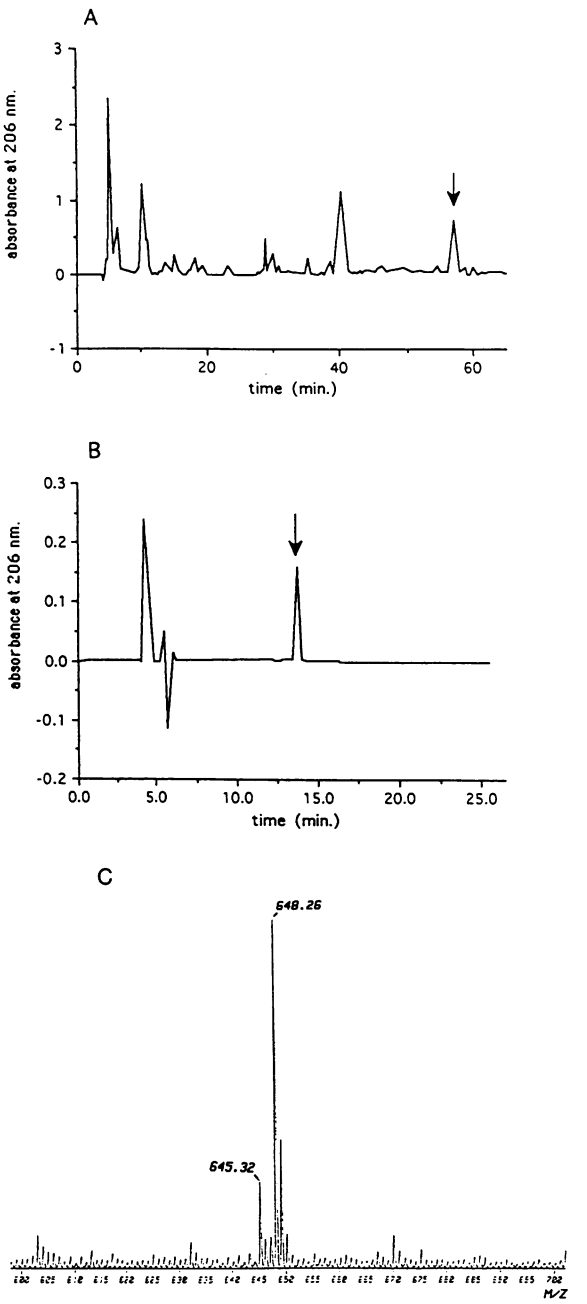

Fig. 3. Purification and characterization of the muropeptide accumulating in *ampD* strain JRG582. The peaks with ^3H labeling are indicated by the arrows. (**A**) C18 reverse-phase HPLC of the material contained in peak (C) of the gel filtration of a soluble extract corresponding to 200 ml culture with a turbidity of 60 Klett units. A linear solvent program from 0 to 20% acetonitrile (containing 0.035% TFA) in water (containing 0.05% TFA) for 90 min at a flow rate of 0.5 ml/min was used. (**B**) C18 reverse-phase HPLC of the ^3H peak from (A) isocratically eluted with 10% acetonitrile (containing 0.05% TFA) at a flow rate of 0.5 ml/min. The single UV-absorbing peak (Rt = 13.7 min) co-eluting with the ^3H label shows the homogeneity of the collected ^3H peak in (A). (**C**) Continuous flow fast atom bombardment mass spectrum of the purified sample: 648.26 = $(M + H)^+$ of sample; 645.32 = $(7 \text{ glycerol} + H)^+$ from matrix.

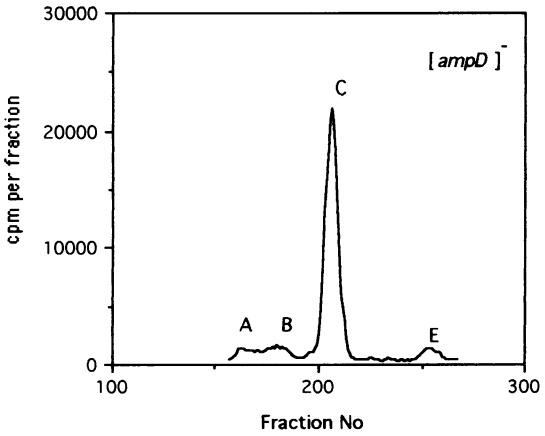

Fig. 4. Molecular sieve chromatography of the cytoplasmic extract of *ampD* strain JRG582/pNU305 labeled with [^3H]DAP. Approximately 230 000 c.p.m. were loaded. The cytoplasmic extract was sonically obtained from spheroplasts prepared by lysozyme + EDTA treatment. The peaks refer to different size classes defined in the legend to Figure 1.

cleaved in the periplasm or in the cytoplasm, we investigated the composition of the material in peak (C) from the culture medium of *ampG* strains, as well as from the hot water extracts of strains HfrH, HfrH01, JRG582 and JRG58201 grown in the presence of cefoxitin for one generation. If anhMurNAc-tripeptide was found in the culture medium or in the hot water extract of *ampG* strains (presumably of periplasmic origin), it would suggest that cleavage of GlcNAc-anhMurNAc-tripeptide occurs in the periplasm. As was done during characterization of the compound that accumulated in the *ampD* mutant, the ^3H-labeled muropeptides in the peak (C) samples were identified by comparison with known standards on HPLC using elution method C (see Materials and methods). The UV-visualized standards were compared with the distribution of ^3H-labeled peaks. Table II gives the percentage of ^3H-labeled anhMurNAc-tripeptide, GlcNAc-anhMurNAc-tripeptide, GlcNAc-anhMurNAc-tetrapeptide and an unidentified compound X (Rt = 78 min) found in the different samples of peak (C). In this system, UDP-MurNAc-pentapeptide eluted after 11 min, demonstrating that compound X was not caused by contamination of peak (C) by peak (B). Since cefoxitin inhibits D,D-carboxypeptidases, muropeptides containing pentapeptide would be present in the murein; hence, compound X is tentatively identified as GlcNAc-anhMurNAc-pentapeptide.

As can be seen from Table II, HfrH01 releases into the medium GlcNAc-anhMurNAc-tetrapeptide and GlcNAc-anhMurNAc-tripeptide, but no anhMurNAc-tripeptide. Because of the size of these muropeptides (mol. wt = 921 and 850), it was unexpected to find them in the medium since only hydrophilic solutes with a mass <600 are thought to readily cross the outer membrane (Decad and Nikaido, 1976). However, the important result is that no anhMurNAc-tripeptide was found either in the culture medium of the *ampG* strain or in the hot water extracts of the *ampG* strain or the wild-type strain, HfrH (Table II). This finding favors the hypothesis that GlcNAc-anhMurNAc-tripeptide, rather than anhMurNAc-tripeptide, is the muropeptide transported across the cytoplasmic membrane. The relative amounts of GlcNAc-anhMurNAc-tripeptide and GlcNAc-anhMurNAc-tetrapeptide found in cefoxitin-induced cells of HfrH (1:4) and HfrH01 (1:2.5) reflect the amounts present in the murein sacculus, whereas

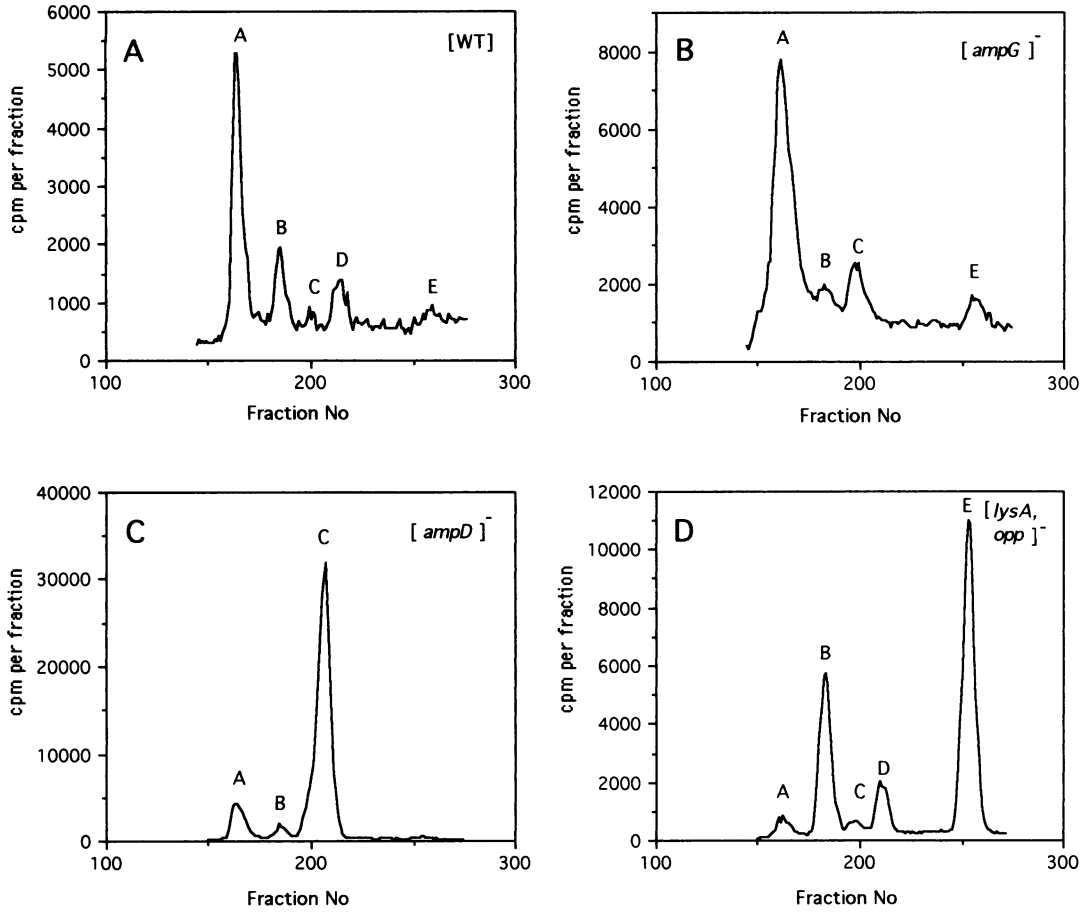

Fig. 5. Molecular sieve chromatography of hot water extracts of *E.coli* strains labeled with [^{3}H]DAP during growth in the presence of cefoxitin (10 mg/l) for 45 min. The peaks refer to different size classes defined in the legend of Figure 1. (**A**) Wild-type strain HfrH/pNU305; 130 000 c.p.m. of the extract were loaded which corresponded to 8 ml culture. (**B**) *ampG* strain HfrH01/pNU305; 130 000 c.p.m. of the extract were loaded which corresponded to 7 ml culture. (**C**) *ampD* strain JRG582/pNU305; 300 000 c.p.m. of the extract were loaded which corresponded to 2 ml culture. (**D**) *lysA opp* strain TP71/pNU305; 140 000 c.p.m. of the extract were loaded which corresponded to 3 ml culture.

the cefoxitin-induced cells of JRG582 contained >4 times more GlcNAc-anhMurNAc-tripeptide than GlcNAc-anhMurNAc-tetrapeptide (Table II). A possible explanation of this result is given in the Discussion.

Using elution method D (see Materials and methods), we confirmed by HPLC that peak (D) from the extract of HfrH grown in the presence of cefoxitin contained ^{3}H-labeled tripeptide exclusively. In marked contrast, the material in peak (D) from the spent medium of a culture of HfrH01 was shown to consist almost entirely of ^{3}H-labeled tetrapeptide.

Discussion

This study strongly suggests that peptidoglycan recycling in *E.coli* involves the product of two genes, *ampD* and *ampG*, previously identified for their role in β-lactamase induction. The following observations lead us to propose a model for recycling muropeptides that differs in important details and in complexity from the one originally proposed by Goodell (1985).

(i) Turnover experiments demonstrated that the *ampG* and *ampD* strains had a considerably reduced ability to recycle muropeptides, as shown by a loss of 20–40% of the [^{14}C]DAP from their murein sacculus each generation compared to an average of 4–8% measured in the isogenic wild-type strains. Thus, the *E.coli* mutants, *ampG* and *ampD*, are not only dramatically affected with respect to β-lactamase production, but have also lost the ability to recycle their murein.

(ii) The high rate of turnover was accompanied by a huge accumulation of a ^{3}H-labeled muropeptide within the cytoplasm of the *ampD* mutant, JRG582. Amino acid and amino sugar analysis, and mass spectrometry, of this material defined its structure as 1,6-anhydro *N*-acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelic acid (mol. wt = 647). This previously undescribed anhMurNAc-tripeptide is a likely substrate for the cytosolic protein AmpD. In fact, we have now demonstrated that purified AmpD rapidly degrades the anhMurNAc-tripeptide into free tripeptide and anhMurNac, and thus acts as a novel *N*-acetylmuramyl-L-alanine amidase (C.Jacobs *et al.*, submitted).

(iii) No such accumulation of muropeptides occurred in the *ampG* strain HfrH01 or in cells of the *ampD ampG* double-mutant strain JRG58201. Thus, the transmembrane protein, AmpG, is involved in the recycling of a peptidoglycan fragment prior to AmpD, most likely serving as the permease. The dominance of the *ampG* mutation over the *ampD* mutation is also seen in β-lactamase induction.

Table II. Distribution of ^{3}H label of peaks (C)

Strains	Per cent of ^{3}H label in peak (C) as			
	AnhMurNAc-tripeptide	GlcNAc-anhMurNAc-tripeptide	GlcNAc-anhMurNAc-tetrapeptide	Unidentified compound X
Soluble extracts of β-lactamase induced culture[b]				
HfrH/pNU305	0[a]	13	57	23
HfrH01/pNU305	0	20	54	23
JRG582/pNU305	75	18	4	1
Spent medium[c]				
HfrH01	0	6	90	0

[a]Under the limit of detection.
[b]The β-lactam inducer was cefoxitin at 10 mg/l which was added to the cultures (at 30 Klett units) for 45 min (until the turbidity of the cultures reached 60 Klett units). Triplicate values within 12% of the mean.
[c]Duplicate values within 5% of the mean.

Turnover was high in *ampG* mutants whether *opp* was present or not, demonstrating that muropeptide uptake via AmpG is the principal route by which tripeptide is released into the cell.

(iv) In *ampG* strains, ^{3}H-labeled cell wall fragments, primarily GlcNAc-anhMurNAc-tetrapeptide and tetrapeptide, were released into the spent medium. This finding is consistent with AmpG being a permease for a specific muropeptide(s).

(v) As demonstrated by the analyses of peak (C) samples summarized in Table II, no anhMurNAc-tripeptide could be detected in the culture medium or hot water extract of the *ampG* strain HfrH01 grown in the presence of the β-lactam inducer cefoxitin. In addition, the only β-*N*-acetylglucosaminidase identified so far in *E.coli* that could form anhMurNAc-tripeptide by removal of *N*-acetylglucosamine from the GlcNAc-anhMurNAc-tripeptide is located in the cytoplasm (Yem and Wu, 1976). This suggests that GlcNAc-anhMurNAc-tripeptide, or possibly the GlcNAc-anhMurNAc-tetrapeptide, rather than anhMurNAc-tripeptide is transported into the cytoplasm via AmpG. The material in peak (C) from the hot water extract of JRG582/pNU305 grown in the presence of cefoxitin contained 4–5 times as much GlcNAc-anhMurNAc-tripeptide as GlcNAc-anhMurNAc-tetrapeptide, which is just the opposite of the normal distribution between these two disaccharide-peptides in the cell wall (1:4), and thus suggests that GlcNAc-anhMurNAc-tripeptide accumulates in the cytoplasm of the *ampD* mutant. Product inhibition of β-*N*-acetylglucosaminidase by the very high concentration of anhMurNAc-tripeptide in JRG582 might cause this accumulation of GlcNAc-anhMurNAc-tripeptide in the cytoplasm.

(vi) Tripeptide accumulated in the wild-type strain HfrH grown in the presence of cefoxitin. In the *ampG* or *ampD* strains, no tripeptide was detected. Thus, its formation requires AmpG and AmpD activities, but not the Opp system since accumulation of tripeptide also occurred in the *opp* strain TP71/pNU305 cultured under the same β-lactamase-inducing conditions.

Based on these observations, we propose the following pathway for peptidoglycan recycling (Figure 6). The first step involves the two lytic transglycosylases degrading the murein into GlcNAc-anhMurNAc-tetrapeptide and GlcNAc-anhMurNAc-tripeptide [for a review of lytic enzymes present in the periplasm of *E.coli*, see Höltje and Tuomanen (1991)]. An L,D-carboxypeptidase removes the terminal D-alanine from the GlcNAc-anhMurNAc-tetrapeptide molecules. The GlcNAc-anhMurNAc-tripeptide formed in the periplasm is efficiently taken up into the cytoplasm by the specific permease AmpG. Once in the cytoplasm, β-*N*-acetylglucosaminidase degrades the GlcNAc-anhMurNAc-tripeptide into anhMurNAc-tripeptide and free *N*-acetylglucosamine. Free tripeptide is released from anhMurNAc-tripeptide by AmpD. Alternatively, AmpD could cleave GlcNAc-anhMurNAc-tripeptide to yield GlcNAc-anhMurNAc and free tripeptide. After formation of the tripeptide, an unknown tripeptide-adding enzyme directly couples the tripeptide to the nucleotide precursor UDP-MurNAc to form UDP-MurNAc-tripeptide in a single step, as was initially proposed by Goodell (1985). This work does not rule out the possibility that GlcNAc-anhMurNAc-tetrapeptide itself is transported into the cytoplasm via AmpG and, in that case, its rapid degradation into GlcNAc-anhMurNAc-tripeptide and free D-alanine would occur as the first cytoplasmic step of recycling since neither [^{3}H]anhMurNAc-tetrapeptide nor free ^{3}H-labeled tetrapeptide was detected inside the cells. However, this alternative seems unlikely since the only known L,D-carboxypeptidase is a periplasmic enzyme (Beck and Park, 1976).

This study also has helped in our understanding of the phenomenon of β-lactamase induction. The presence of cefoxitin in the medium during the growth of the wild-type and *ampG* or *ampD* cultures was accompanied by an increase in GlcNAc-anhMurNAc-tetrapeptide and GlcNAc-anhMurNAc-tripeptide. This result confirms the common hypothesis that the β-lactam inducer, by inhibiting enzymes involved in peptidoglycan synthesis, causes increased breakdown of the peptidoglycan. The increased production of GlcNAc-anhMurNAc-tetrapeptide and GlcNAc-anhMurNAc-tripeptide resulting from cefoxitin action leads to accelerated entry of GlcNAc-anhMurNAc-tripeptide into the cell and accumulation of tripeptide in the cytoplasm. Since derepression of β-lactamase production in the absence of β-lactam inducer occurs as a consequence of an *ampD* mutation, the dramatic accumulation of anhMurNAc-tripeptide in such a mutant strongly suggests that this compound is an activating ligand for the transcriptional regulator AmpR. However, no anhMurNAc-tripeptide could be detected in the wild-type strain HfrH grown in the presence of a β-lactamase inducer.

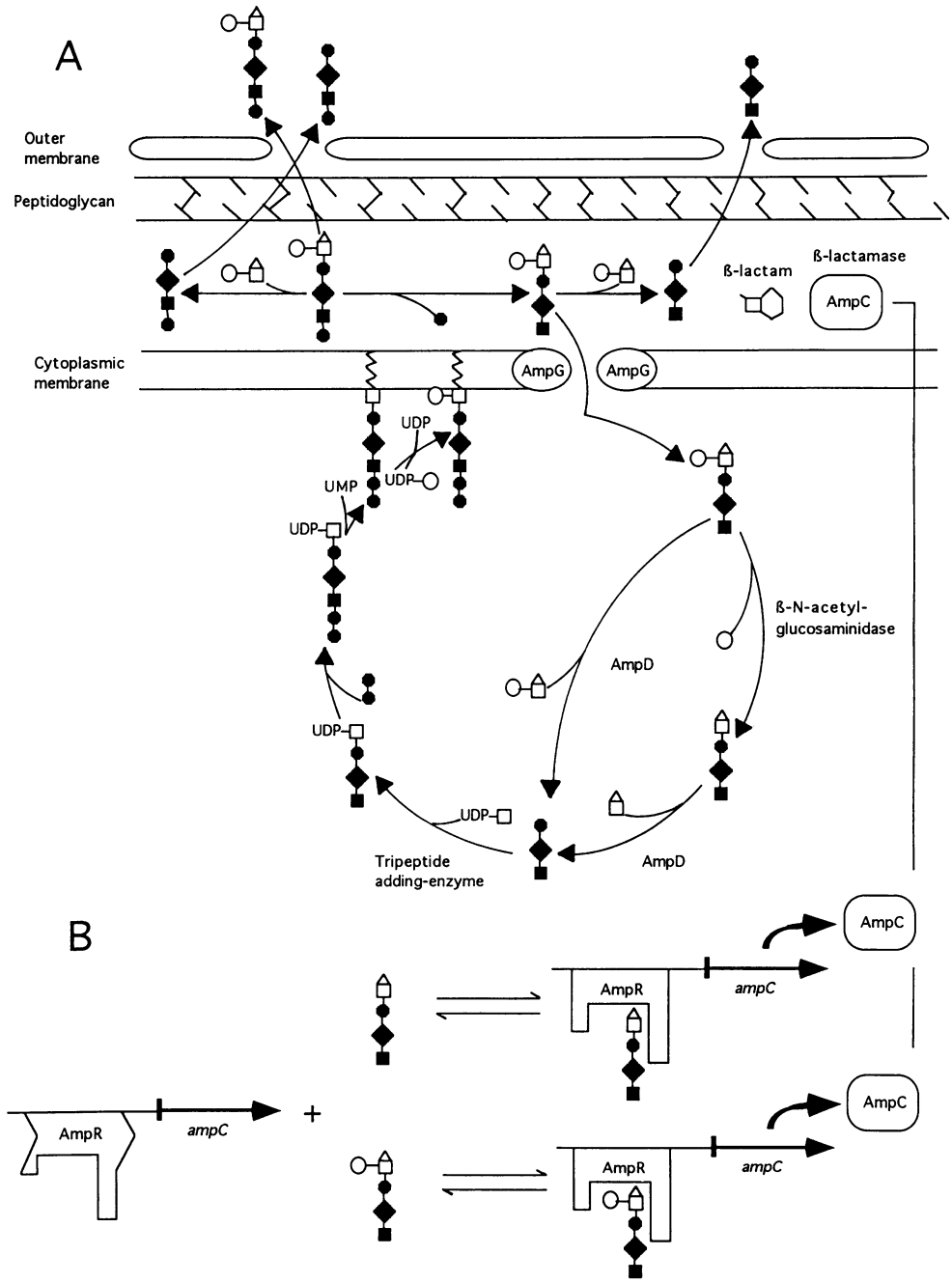

Fig. 6. The proposed interconnected pathway for recycling muropeptides and for their involvement in β-lactamase induction is illustrated. ○, GlcNAc; ⌂, anhMurNAc; □, MurNAc; ●, Ala; ◆, Glu; ■, DAP. (**A**) The recycling pathway. As shown, murein is degraded by known enzymes in the periplasm to the fragments listed. The muropeptide, GlcNAc-anhMurNAc-tripeptide, is transported into the cytoplasm through AmpG. It is then degraded into GlcNAc-anhMurNAc and free tripeptide by AmpD. Alternatively, the β-*N*-acetylglucosaminidase, a cytoplasmic enzyme, removes the *N*-acetylglucosamine of the GlcNAc-anhMurNAc-tripeptide and the resulting anhMurNAc-tripeptide is hydrolysed by AmpD to release tripeptide. Free tripeptide can then be added directly to UDP-*N*-acetylmuramic acid by an as yet unidentified enzyme, thereby reintroducing it into the biosynthetic pathway for murein synthesis. (**B**) Muropeptides as inducers of β-lactamase. Intracellular accumulation of GlcNAc-anhMurNAc-tripeptide as a result of the presence of the β-lactam antibiotics or of anhMurNAc-tripeptide as the result of inactivation of *ampD* triggers production of *C.freundii* AmpC β-lactamase. The muropeptides presumably bind to the transcriptional regulator AmpR and convert it into an activator for *ampC* expression. (*Citrobacter freundii ampR* and *ampC* are expressed from a plasmid.)

Instead, as mentioned above, GlcNAc-anhMurNAc-tetrapeptide and GlcNAc-anhMurNAc-tripeptide and, to a larger extent, tripeptide accumulate as a consequence of the presence of β-lactams in the culture medium. Therefore, AmpR might respond to two or more ligands: the anhMurNAc-tripeptide certainly, the structurally related GlcNAc-anhMurNAc-tripeptide very likely, and the tripeptide much less likely (Figure 6B). The fact that, in the presence of the inducing antibiotic, overexpression of the *ampD* gene from a multicopy plasmid in a wild-type *C.freundii* strain does not allow full induction of β-lactamase expression (Lindberg *et al.*, 1987) suggests that AmpD causes the disappearance of the cytosolic inducer (GlcNAc-anhMurNAc-tripeptide) rather than its formation (tripeptide).

Thus, AmpR can be thought to control β-lactamase production by sensing the cytoplasmic level of muropeptide(s) indicative of the presence or absence of β-lactam antibiotic in the medium, or of other causes of accelerated peptidoglycan turnover.

The existence of such a major degradation–re-utilization pathway is surprising since the *ampG* and *ampD* genes are not essential for viability. Although this study suggests that cell wall recycling has a signaling role in β-lactamase induction, we believe such induction does not represent its primary sensing function because *E.coli* lacks the inducible, AmpR-regulated β-lactamase. It seems quite possible that the dynamic state of the murein sacculus essential for growth and division of the bacterium could require responsive communication with the transcriptional machinery. The level of recycled muropeptides in the cytoplasm may provide this hypothetical communication link. In keeping with this notion, it has recently been reported that in *Bacillus subtilis* transport of wall-derived peptides into the cell may have a signaling role in the initiation of sporulation (Perego *et al.*, 1991).

Materials and methods

Bacteria, plasmids and culture conditions

The *E.coli* K12 strains used in this study were derivatives of HfrH (Low, 1973), CH483 [*thi lac pro galE* Δ(*trp-tonB-oppABCDF*) 467] (Goodell and Higgins, 1987) or MC4100 [F⁻ *araD139* Δ(*argF-lac*) *U169 rpsL150 relA1 flbB5301 ptsF25 deoC1*] (Wientjes *et al.*, 1985). HfrH is the parental strain of JRG582 which is totally deleted for the *ampD* and *ampE* genes (Guest and Stephens, 1980). HfrH01 (derivative of HfrH) and JRG58201 (derivative of JRG582) carry an APH cassette inserted in the *ampG* gene (Lindquist *et al.*, 1993). TP71 is MC4100 *lysA* which was found to also carry *opp* (Park, 1993). TP71 is the parental strain of TP73 in which the operon *ampDE* was deleted by transduction of the deletion from JRG582 with selection for cefotaxime resistance. TP72 and TP74 were derived by P1 transduction of *ampG*::kan from JRG58201 into TP71 and TP73, respectively. Plasmid pNU305 carries the *C.freundii ampR* and *ampC* genes (Lindberg *et al.*, 1985). Plasmid pNU404 is pACYC184 expressing chloramphenicol resistance and the *E.coli ampD* gene (Lindberg *et al.*, 1987). Plasmid pGKS273-3 carries chloramphenicol resistance and the *E.coli ampG* gene (Schmidt, 1991). Transductions (Miller, 1972) and transformations (Sambrook *et al.*, 1989) were carried out as previously described. The *E.coli* strains were grown in minimal medium M9 supplemented with glucose (0.2%), casamino acids (0.1%), thiamine (1 μg/ml), uracil (50 μg/ml), $MgCl_2$ (1 mM), lysine (100 μg/ml), threonine (100 μg/ml) and methionine (100 μg/ml) at 37°C with vigorous agitation. Nicotinamide (5 μg/ml) was added to the cultures of the *ampD*-deleted strains JRG582, JRG58201, TP73 and TP74. Growth of the cultures was assayed by measuring the optical density of the cultures in a Klett–Summerson colorimeter.

Determination of murein turnover

Log-phase cells growing in the above M9 medium were labeled with 0.02 μCi/ml of [^{14}C]DAP (350 mCi/mmol; Commissariat à l'Energie Atomique, Gif-sur-Yvette, France) for 30 min at 37°C. The cells were then chased by diluting the culture with pre-warmed medium containing 100 μg/ml of cold DAP. Aliquots were taken initially and after every generation of growth, as determined by optical density. The aliquots were added to hot SDS to give a final concentration of 4% SDS and incubated at 95–98°C for 30 min. The murein sacculi were collected on 0.22 μm pore size membrane filters (Millipore Corp., Bedford, MA), washed thoroughly with water, and their radioactivity determined by liquid scintillation counting.

Labeling with [^{3}H]DAP

Typically, a 10 ml culture of exponentially growing bacteria (15 Klett units) was labeled with 30 μCi of [3,4,5-^{3}H]DAP (25 Ci/mmol; Commissariat à l'Energie Atomique, Gif-sur-Yvette, France). When the culture reached a turbidity of 60 Klett units, the culture was rapidly chilled to 0°C, filtered on a 0.22 μm pore size membrane filter and washed once with 2 ml of water. The cells collected on the membrane filter were suspended in 10 ml of boiling water, heated at 100°C for an additional 8 min and the resulting solution was centrifuged (12 000 *g*, 10 min, 4°C) to remove particulate matter. The supernatants (hot water extracts) were collected and lyophilized. When required, the spent medium was recovered and lyophilized.

Separation of ^{3}H-labeled components by molecular sieve chromatography

Samples of the hot water extracts and culture media were analyzed by gel filtration on a Toyopearl HW-40S column (1.6×80 cm; TosoHaas, Montgomeryville, PA) equilibrated with 100 mM sodium chloride in 10 mM sodium phosphate (pH 7.5) containing 0.02% sodium azide. The samples were eluted (12 ml/h) with the same buffer; 0.25 ml fractions were collected. Their radioactivity was determined by liquid scintillation counting. When the material in the gel filtration peaks (C) and (D) was required for further studies, only 25 μl of each fraction were analyzed for radioactivity. ^{3}H peak fractions were pooled, lyophilized, desalted on a Toyopearl column (1×30 cm) and lyophilized prior to analysis by HPLC.

HPLC analysis

HPLC was performed with Rainin Rabbit HP pumps and mixer equipment (Rainin Instrument Co., Woburn, MA). Different chromatographic systems using a C18 reverse-phase column maintained at room temperature were employed to characterize the material in peaks (C) and (D).

Method A. Isocratic elution for 10 min with 0.05% trifluoroacetic acid (TFA) in water, followed by a linear gradient of 0–20% of 0.035% TFA in acetonitrile over 30 min; with a flow rate of 0.8 ml/min through a column of LiChrosorb RP-18 (250×4 mm; 5 μm particle size; E.Merck).

Method B. Isocratic elution with 8% acetonitrile in water (containing 0.05% TFA); with a flow rate of 0.5 ml/min through a column of LiChrospher RP-18 (250×4 mm; 3 μm particle size; E.Merck).

Method C. Isocratic elution with 8% acetonitrile in water (containing 0.05% TFA) for 60 min, then a linear gradient from 8 to 20% acetonitrile over 20 min; with a flow rate of 0.5 ml/min through a column of LiChrospher RP-18 (250×4 mm; 3 μm particle size; E.Merck).

Method D. Isocratic elution with 0.05% TFA in water; with a flow rate of 0.5 ml/min through a column of LiChrospher RP-18 (250×4 mm; 3 μm particle size; E.Merck). Tripeptide and tetrapeptide elute at 16.7 and 32.8 min, respectively.

Method E. Isocratic elution with 10% acetonitrile in water (containing 0.05% TFA); with a flow rate of 0.5 ml/min through a column of LiChrospher RP-18 (250×4 mm; 3 μm particle size; E.Merck).

UV-absorbing components of the column effluent were detected at 206 nm by a Spectra-Physics SP8450 UV-detector (San Jose, CA), and peak areas and retention times were recorded with the Rainin Dynamax data-acquisition program.

Purification of anhMurNAc-tripeptide

Two 2 l Erlenmeyer flasks, each containing 500 ml of M9 medium supplemented with glucose (0.2%), casamino acids (0.1%), thiamine (1 μg/ml), uracil (50 μg/ml), $MgCl_2$ (1 mM), lysine (100 μg/ml), threonine (100 μg/ml), methionine (100 μg/ml) and nicotinamide (5 μg/ml), were inoculated with 5 ml of overnight culture of the *ampD* strain JRG582 and grown by incubating on a rotary shaker (180 r.p.m.) at 37°C. When the culture reached a turbidity of 15 Klett units, 60 μCi of [^{3}H]DAP were added to the culture medium. Two generations later, the cells were rapidly chilled to 0°C and harvested by centrifugation in the cold. The bacteria were suspended in 20 ml of boiling water and heated at 100°C for 20 min; following centrifugation at 16 000 *g* for 10 min, the hot water extract was recovered and lyophilized. The sample, dissolved in 1 ml of water, was then loaded on the Toyopearl 40-HWS column as described above. Peak (C) (Figure 1C) was collected and lyophilized. After desalting on a Toyopearl HW-40S column (1×30 cm), the sample was subjected to C18 reverse-phase chromatography (LiChrospher RP-18; 250×4 mm; 3 μm particle size; E.Merck). The solvent program used for the separation was 0–20% acetonitrile (containing 0.035% TFA) in water (containing 0.05% TFA) over a period of 90 min at a flow rate of 0.5 ml/min. The radiolabeled peak fractions were pooled, adjusted to pH 7 with ammonia, and lyophilized following concentration by rotary evaporation to remove acetonitrile.

Determination of ATP content and β-lactamase activity

To determine the ATP content in periplasmic and cytoplasmic extracts, we used the Sigma kit (stock no. FL-ASC) and followed the protocol

recommended by the manufacturer. The technique utilized the property of luciferase to emit light proportionally to the ATP present in an extract. The light was measured with a ILA911 luminometer (Tropix, Inc., Bedford, MA).

β-Lactamase activity in the extracts was determined spectrophotometrically at 482 nm with 150 μM nitrocefin as substrate in 50 mM potassium phosphate (pH 7.0) at 30°C under conditions in which <10% of the substrate is hydrolyzed. The extracts analyzed were prepared from *E.coli* strain JRG582/pNU305 cells harvested by centrifugation at a turbidity of 60 Klett units. The bacterial pellet was washed with 0.9% NaCl and resuspended in 500 μl of the same saline solution. The method used to separate periplasmic molecules from cytoplasmic contents (lysozyme + EDTA treatment) was as described previously (Osborn *et al.*, 1972). The cytoplasmic material was then liberated from the spheroplasts by sonic oscillations at 4°C in 10 s bursts with a Branson cell disrupter 200 (Branson Sonic Power Co., Danbury, CT).

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