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The Signal Molecule for β-Lactamase Induction in *Enterobacter cloacae* Is the Anhydromuramyl-Pentapeptide

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β-Lactamase induction in *Enterobacter cloacae*, which is linked to peptidoglycan recycling, was investigated by high-performance liquid chromatographic analysis of cell wall fragments in genetically defined cells of *Escherichia coli*. After treatment of cells with β-lactams, we detected an increase in aD-tripeptide (disaccharide-tripeptide, *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid), aD-tetrapeptide (disaccharide-tetrapeptide, *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid-D-alanine), and aD-pentapeptide (disaccharide-pentapeptide, *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid-D-alanyl-D-alanine) levels in the periplasms of bacterial cells. Furthermore, only the accumulation of aD-pentapeptide correlates with the β-lactamase-inducing capacity of the β-lactam antibiotic. The transmembrane protein AmpG transports all three aD-peptides into the cytoplasm, where they are degraded into the corresponding monosaccharide peptides. In the absence of AmpD the constitutive overproduction of β-lactamase is accompanied by an accumulation of aM-tripeptide (monosaccharide-tripeptide, anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid) and aM-pentapeptide (L1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid-D-alanyl-D-alanine), but not aM-tetrapeptide (anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid-D-alanine), in the cytoplasm. Only the amount of aM-pentapeptide is increased upon treatment with imipenem. These findings indicate that aD-pentapeptide is the main periplasmic muropeptide, which is converted into the cytoplasmic signal molecule for β-lactamase induction, the aM-pentapeptide.

The emergence of resistance during a course of antimicrobial therapy can be a serious therapeutic problem. In a study with *Enterobacter cloacae*, even under a combination therapy with an aminoglycoside and a broad-spectrum cephalosporin resistance to all β-lactams except the carbapenems developed in nearly 50% of all patients investigated (10). Although it is now generally accepted that the induction itself of the chromosomally mediated cephalosporinase by β-lactam antibiotics (20, 25, 30, 33) is not a real clinical problem, the regulation of β-lactamase production is of great interest because of its high mutation rate of 10^{-5} to 10^{-8} (29) and the fact that nearly all members of the family *Enterobacteriaceae* and *Pseudomonas aeruginosa* strains produce a chromosomal AmpC β-lactamase. Furthermore, this process is linked to the peptidoglycan recycling (14), which is a general feature in eubacteria. Upon interaction with murein synthesis, β-lactams lead to an increased degradation of the murein sacculus. Thus, degradation products accumulate in the periplasm (18). They are transported into the cytoplasm, where they can act as an inducer, converting the transcriptional regulator AmpR into an activator of β-lactamase expression (18, 27).

In our recent study, we demonstrated that AmpD, a cytosolic protein (27), which functions as a negative regulator of AmpC expression (18, 21, 26, 18), is an *N*-acetyl-anhydromuramyl-L-alanine amidase (17–19). Mutations in the *ampD* gene result in constitutive β-lactamase overproduction. Simultaneously, large quantities of aM-tripeptide (anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid) accumulate in the cytoplasm, indicating that this compound is an AmpR-activating ligand (18). Moreover, this molecule is transported into the periplasm (8).

AmpG, a transmembrane protein, is required for β-lactamase induction (23). Jacobs et al. (18) suggest that this protein acts as a permease for the anhydromuropeptide *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid (aD-tripeptide), being a precursor molecule for aM-tripeptide.

In the study described in this paper, we studied the influences of antibiotics with and without the capacity to induce the β-lactamase on the formation of anhydromuropeptides. We analyzed whether AmpG also transports anhydromuropeptides other than D-tripeptide into the cytoplasm, where, as we suggest, these anhydrodisaccharide peptides are cleaved to the corresponding anhydromonosaccharide peptides.

MATERIALS AND METHODS

Bacteria, plasmids, and culture conditions. The *Escherichia coli* K-12 strains used in this study were JRG582 and its derivative JRG58201. The *ampD* and *ampE* genes are totally deleted from strain JRG582 (15). JRG58201 carries an APH cassette inserted in the *ampG* gene (28).

Plasmids pBP21 and pBP22 are derivatives of pUC-19 (pBP19) (3) carrying kanamycin resistance and the *Enterobacter cloacae ampE* gene and the *ampD* and *ampE* genes, respectively. Plasmid pBP21 was constructed as follows. The 1.1-kb *Sph*I-*Xba*I fragment of the wild-type strain *E. cloacae* 14 carrying the *ampE* gene was obtained by PCR. The *Xba*I site of this PCR fragment was blunt ended with DNA polymerase T4, and the resulting fragment was inserted into the unique *Sma*I and *Sph*I sites of pBP19. pBP22 was constructed by subcloning the 600-bp *Bam*HI-*Eco*RI fragment of pBP21 into pBP20 (21).

Recombinant DNA testing techniques and transformation of DNA were performed as described by Maniatis et al. (31). The *E. coli* strains were grown in M9 medium supplemented with glucose (0.2%), Casamino Acids (0.1%), thiamine (1 μg/ml), uracil (50 μg/ml), nicotinamide (5 μg/ml), and $MgSO_4$ (1 mM) at 37°C. When required, sulfamethoxazole (500 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (30 μg/ml) were added.

The various antibiotics, which were tested for their capacities to induce the

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TABLE 1. Amounts of aM-tripeptide, aM-pentapeptide, aD-tripeptide, and aD-tetrapeptide in the hot water extract and the culture medium of JRG58201 (*ampD* and *ampG* negative) and JRG582 (*ampD* negative)

Strain and extract or medium	% Area in the corresponding peak of the HPLC chromatogram[a]			
	aM-tripeptide	aD-tripeptide	aM-pentapeptide	aD-tetrapeptide
JRG58201				
Hot water extract[b]	0.4 (0.3)	0.5 (0.2)	0	2.5 (0.15)
Culture medium[c]	0	0.7	0	3.8
JRG582				
Hot water extract[d]	65	0.3	5.6	0.1
Culture medium[c]	12.4	0.3	0.9	0.3

[a] A value of 0 indicates that the area was less than the limit of detection. Values in parentheses are standard deviations.
[b] Values are means for six extracts.
[c] Values resulting from one analysis.
[d] Values are means for two extracts.

AmpC β-lactamase, were kindly provided by the following companies: cefotaxime by Hoechst AG, Frankfurt, Germany; cefoxitin by Merck Sharp & Dohme GmbH, Munich, Germany; imipenem by Merck Sharp & Dohme, West Point, Pa.; meropenem by Zeneca Pharmaceuticals, Macclesfield, United Kingdom; piperacillin by Cyanamid GmbH, Wolfratshausen, Germany; sulbactam by Pfizer AG, Karlsruhe, Germany; and vancomycin by Eli Lilly & Co., Indianapolis, Ind.

Preparation of muropeptides from different compartments by hot water extracts. (i) Preparation of muropeptides. Muropeptides were prepared by a method based on the one described by Jacobs et al. (18). A 1-liter Erlenmeyer flask containing 200 ml of minimal medium M9 was inoculated with 2 ml of an overnight culture and grown to the mid-logarithmic phase (optical density at 456 nm [OD_{456}] = 0.5), and 15 μCi of [^{3}H]diaminopimelic acid (0.35 Ci/mmol; Amersham, Braunschweig, Germany) was added. The cells were harvested by centrifugation at 4°C and washed once with one-half volume of 10 mM Tris-HCl (pH 8.0). The bacteria were suspended in 20 ml of boiling water and heated at 100°C for 20 min. After removal of particulate matter by centrifugation at 16,000 × *g* for 10 min, the supernatant (hot water extract) was recovered and lyophilized.

(ii) Preparation of periplasmic and cytoplasmic muropeptides. Cells from the *ampG*-positive strain JRG582 and from the *ampG*-negative strain JRG58201 were fractionated into periplasmic and cytoplasmic fractions by treatment with lysozyme and EDTA (37). The formation of spheroplasts could be seen by osmotic sensitivity and conversion into spheres after diluting an aliquot of the suspension 1:5 in distilled water (1). To check if the spheroplasting reaction was completed, we measured the β-lactamase activity in the cytoplasmic and periplasmic fractions. We ran our β-lactamase controls for strain JRG58201 in the same experiment with strain JRG582/pBP131-3.

The cytoplasmic and periplasmic fractions were separated by centrifugation and were suspended in 20 ml of boiling water and heated at 100°C for 20 min. After removal of particulate matter by centrifugation at 16,000 × *g* for 10 min, the supernatant (hot water extract) was recovered and lyophilized.

Methanol precipitation. A rapid method for the removal of proteins is methanol precipitation (8). The lyophilized samples of the hot water extracts and the culture media were resuspended in 400 μl of water and chilled on ice for 15 min. Then, 400 μl of ice-cold methanol was added, and after an additional 15 min on ice the samples were centrifuged at 10,000 × *g* for 10 min to obtain a clear supernatant.

HPLC analyses. After methanol precipitation, the samples were lyophilized and suspended in 100 μl of water. Then, the muropeptides were reduced with sodium borohydride (12). Prior to injection, the samples were centrifuged at 6,000 × *g* for 10 min.

High-performance liquid chromatography (HPLC) was performed with a Pharmacia gradient system consisting of a 2152 LC controller, 2150 pumps, and an 11300 ultragrad mixer driver. A C_{18} Hypersil octyldecylsilane reverse-phase column (250 by 4.6 mm; 3 μm particle size) from Bischoff (Leonberg, Germany), together with a guard column containing μBondapack C_{18} Corasil (Waters), was used to separate the muropeptides. Optimal separation of muropeptide monomers with this HPLC system was reached by a linear gradient from methanol-free 50 mM sodium phosphate (pH 4.31), containing 0.8 mg of sodium azide per liter (buffer A) to 35% 75 mM sodium phosphate (pH 4.95), containing 15% methanol (buffer B) over 100 min at 38.5°C at a flow rate of 0.5 ml/min.

Fractions of the column effluent of 0.25 ml were collected. The radioactivity of each fraction was determined by liquid scintillation counting. Peak areas and area percentage for every peak was calculated with the Microsoft Excel program. The standard anhydromuropeptides aD-tripeptide and aD-tetrapeptide (disaccharide-tetrapeptide, *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid-D-alanine) were kindly provided by J.-V. Höltje.

HPLC analyses of imipenem-treated cells. The prepared anhydromuropeptides of the *ampG*- and *ampD*-negative strain JRG58201 were separated with a gradient composed of two linear gradients. The first part of this gradient is the one described above. The second part is a linear gradient of the same buffers from 36 to 100% buffer B over 25 min.

Determination of β-lactamase activity. We used JRG582 carrying plasmid pBP131 (24), which contains the *E. cloacae* genes (*ampC* and *ampR*) required for the expression of the *E. cloacae* β-lactamase, and plasmid pBP14105-3 (22). pBP14105-3 carries the *ampD* mutant *ampD05*, which leads to a hyperinducible phenotype (21). The cells were grown to an OD_{465} of 0.1, and various antibiotics were added at concentrations that were half the MICs for 2 h. Then, the cells (20 ml) were harvested by centrifugation at 4°C and washed once with 1 volume of 0.05 M potassium phosphate (pH 7.0). The cells were resuspended in 1 ml of the same buffer and frozen overnight. Sonication on ice with a Branson sonifier yielded the cell extract used for β-lactamase determination. The β-lactamase activity was quantified as described by Peter et al. (36), with nitrocefin (50 μM) used as the substrate (34). The protein content of each sample was determined by the method of Bradford (2), with bovine serum albumin used as the standard.

RESULTS

Accumulation of two anhydromuropeptides in *ampD*-negative cells. Hot water extracts of JRG582/pBP21 (*ampD* negative) and JRG582/pBP22 (*ampD* positive) after methanol precipitation were analyzed by HPLC by using the linear gradient described above (Table 1). For the *ampD*-negative strain, compared to the *ampD*-positive strain, two additional peaks with area percentages of 65 (peak 1) and 5.6 (peak 2) were detected (Fig. 1). Peak 1 was identified by mass spectrometry as the aM-tripeptide, which could also be detected in the culture

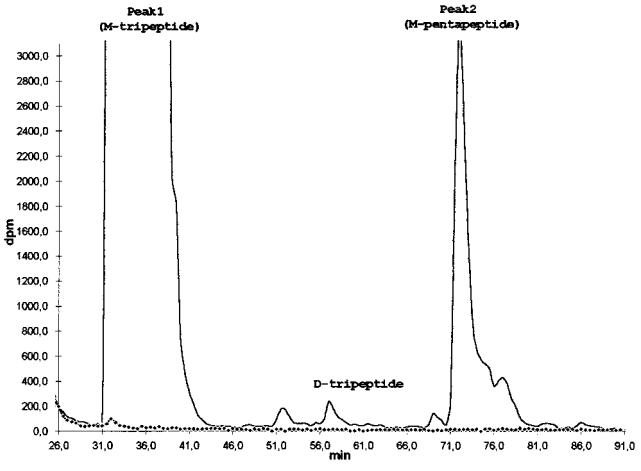

FIG. 1. HPLC analysis of hot water extracts from JRG582/pBP22 (*ampD* positive) and JRG582/pBP21 (*ampD* negative). The chromatogram of the *ampD*-negative strain shows two additional peaks, those for the aM-tripeptide and the aM-pentapeptide. —, *ampD* negative; —◆—, *ampD* positive.

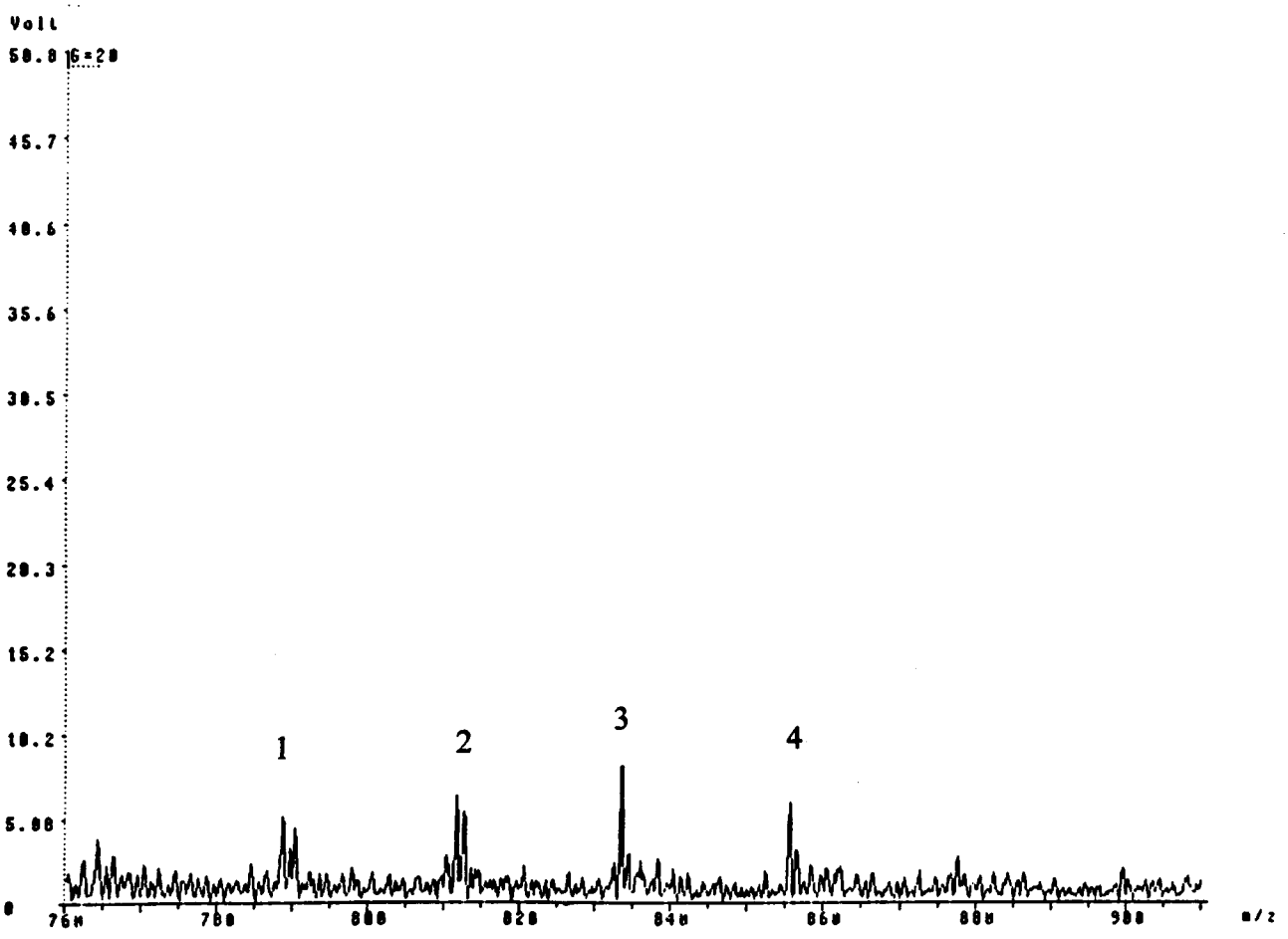

FIG. 2. Mass spectrometry of the compound (Fig. 1, peak 2) which, besides aM-tripeptide, accumulates in *ampD*-negative strains. The mass of the protonated compound (peak 1) corresponds exactly to the mass of aM-pentapeptide (molecular mass, 788 U). Peak 1, aM-pentapeptide + H^+ = 789 U; peak 2, aM-pentapeptide + H^+ + Na^+ = 812 U; peak 3, aM-pentapeptide + $2Na^+$ ($-1H^+$) = 834 U; peak 4, aM-pentapeptide + $3Na^+$ ($-2H^+$) = 856 U.

medium (8). Peak 2 was collected, and the corresponding compound was analyzed by fast-atom bombardment mass spectrometry by using thioglycerol as the matrix. The mass of the protonated compound was 788 (Fig. 2), which exactly corresponds to the mass of 1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid-D-alanyl-D-alanine (aM-pentapeptide).

Furthermore, we could demonstrate that the corresponding compounds are substrates for AmpD. The lyophilized hot water extract of JRG582/pBP21 was dissolved in 1 mM sodium phosphate buffer (pH 6.8), containing 0.02% sodium azide. To one-half of this mixture the MalE-AmpD fusion protein (about 1 μg) (16) was added. Then, we incubated the samples for 12 h at 30°C. The peaks of interest disappeared upon AmpD treatment.

Role of AmpG in peptidoglycan recycling. If the aM-pentapeptide and the aM-tripeptide are formed in the cytoplasm, AmpG would transport the corresponding precursor molecules into the cytoplasm. To investigate this hypothesis, we used an *ampD*- and *ampG*-negative strain (JRG58201). Neither aM-tripeptide nor aM-pentapeptide is generated by this strain (Fig. 3), which supports our hypothesis that in fact AmpG is not only the permease for the precursor molecule of aM-tripeptide (9, 18) but is also the permease for the precursor molecule of aM-pentapeptide.

To investigate this further, we fractionated cells from *ampG*-positive strain JRG582 and from ampG-negative strain JRG58201 into periplasmic and cytoplasmic fractions. Only spheroplasts of *ampG*-positive cells contained aD-tetrapeptide (Fig. 4) as well as aD-tripeptide showing that aD-tetrapeptide can also be transported into the cytoplasm by AmpG. This was already demonstrated by our recent study (9). So AmpG is a specific permease not only for the muropeptide aD-tripeptide but also for aD-tetrapeptide and probably aD-pentapeptide (disaccharide-pentapeptide, *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-*meso*-diaminopimelic acid-D-alanyl-D-alanine), although the latter muropeptide could not be detected in the cytoplasmic fractions of *ampG*-positive cells.

Effect of imipenem on peptidoglycan metabolism. Since there is a direct link between recycling of peptidoglycan fragments and β-lactamase induction (18, 39), it is assumed that imipenem, a strong inducer of β-lactamase production, changes the peptidoglycan metabolism in a specific way.

Treatment of JRG58201 with an imipenem concentration of one-half the MIC for 25 min resulted in a twofold increase of the amount of aD-tetrapeptide (Fig. 5). Furthermore, another anhydromuropeptide appeared only upon imipenem treatment. We analyzed this anhydromuropeptide by fast-atom bombardment mass spectrometry using thioglycerol as the ma-

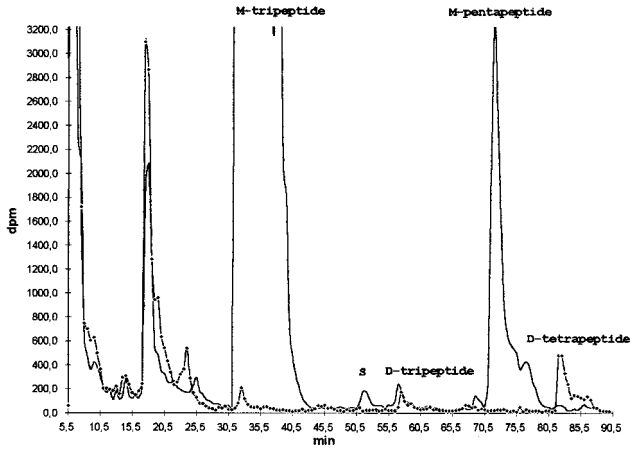

FIG. 3. HPLC analysis of the hot water extracts from JRG582 (*ampD* negative) and JRG58201 (*ampD* and *ampG* negative). S, substrate of AmpD. —, *ampD* negative; —◆—, *ampD* and *ampG* negative.

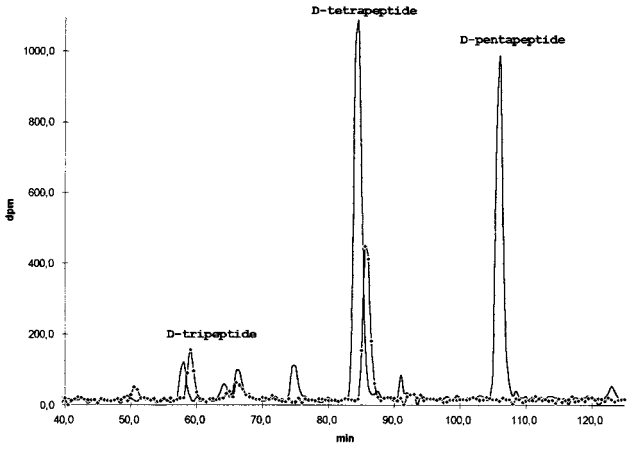

FIG. 5. HPLC analysis of hot water extract from JRG58201 (*ampD* and *ampG* negative). The effect of imipenem (0.25 μg/ml, 25 min) was examined. —, with imipenem; —◆—, without imipenem.

trix. The mass of the protonated compound was 993 (Fig. 6), which exactly corresponds to the mass of aD-pentapeptide.

To investigate if, in addition to aD-tripeptide and aD-tetrapeptide, AmpG also transports aD-pentapeptide into the cytoplasm, we analyzed the cytoplasmic anhydromuropeptides of the *ampG*-positive, *ampD*-negative strain JRG582 after imipenem treatment (0.25 μg/ml, 25 min). The spheroplasts of these cells, in contrast to the spheroplasts of cells not treated with imipenem (Fig. 4), contained aD-pentapeptide (data not shown; area percentage of the corresponding peak, 0.5). Thus, AmpG is also the permease for this anhydromuropeptide. Furthermore, besides aD-pentapeptide only the amount of aM-pentapeptide, not aM-tripeptide, increases on treatment with imipenem, from an area percentage of 2.4 (standard deviation from three experiments, 0.33) to one of 10.9 (standard deviation from three experiments, 0.22).

By using the *ampD*-positive strain JRG582/pBP22, aD-pentapeptide and aM-pentapeptide are only detectable after imipenem treatment (Table 2). The amount of aD-tetrapeptide also increases.

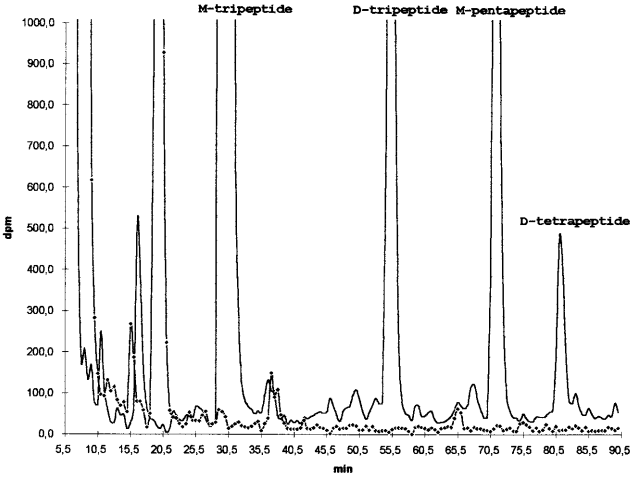

FIG. 4. HPLC analysis of the cytoplasm (spheroblasts) of *ampD*-negative strains JRG582 (*ampG* positive) and JRG58201 (*ampG* negative). —, *ampG* positive; —◆—, *ampG* negative.

Effect of cell wall-active antibiotics. In contrast to β-lactams, other cell wall-active antibiotics like D-cycloserine and vancomycin, as well as the β-lactamase inhibitor sulbactam, do not induce β-lactamase production (data not shown). Therefore, we tried to determine if β-lactams like imipenem, cefoxitin, meropenem, piperacillin, and cefotaxime change peptidoglycan metabolism in a way different from that in which D-cycloserine, vancomycin, and sulbactam change peptidoglycan metabolism.

We treated cells of the *ampD*- and *ampG*-negative strain JRG58201 with antibiotic at a concentration of one-half the MIC to prevent lysis of the cells and separated the prepared anhydromuropeptides with the gradient composed of two linear gradients described above. Since we analyzed an *ampG*-negative strain, aD-tripeptide as well as aD-tetrapeptide and aD-pentapeptide are of periplasmic origin.

As can be seen in Table 3, in contrast to β-lactams, the actions of D-cycloserine, vancomycin, and sulbactam on peptidoglycan synthesis do not alter the release of aD-tripeptide, aD-tetrapeptide, or aD-pentapeptide in the periplasm. All tested β-lactams lead to the same 2- to 4-fold increase in the aD-tripeptide level and 1.5- to 2.5-fold increase in the aD-tetrapeptide level in the periplasm compared to the levels in untreated cells, whereas the release of aD-pentapeptide correlates with the β-lactamase-inducing capacity of the β-lactam antibiotic. For example imipenem, a strong inducer of β-lactamase induction, leads to a 25-fold increase in the aD-pentapeptide level compared to that induced by cefotaxime, a weak inducer.

DISCUSSION

Murein recycling in *E. coli* can use a pathway (18) which involves the products of the genes *ampD* (26) and *ampG* (23). AmpD and AmpG also play important roles in β-lactamase induction. Recently, it could be shown that the cytosolic protein AmpD is an *N*-acetyl-anhydromuramyl-L-alanine amidase (17–19). Furthermore, the *ampD* mutant accumulates aM-tripeptide in the cytoplasm (8, 18). Therefore, it is assumed that AmpD exerts its negative effect on AmpC expression by hydrolyzing this anhydromuropeptide, the signal molecule for β-lactamase induction.

Here we demonstrate that in an *ampD* mutant not only does aM-tripeptide accumulate but to a lesser extent, another an-

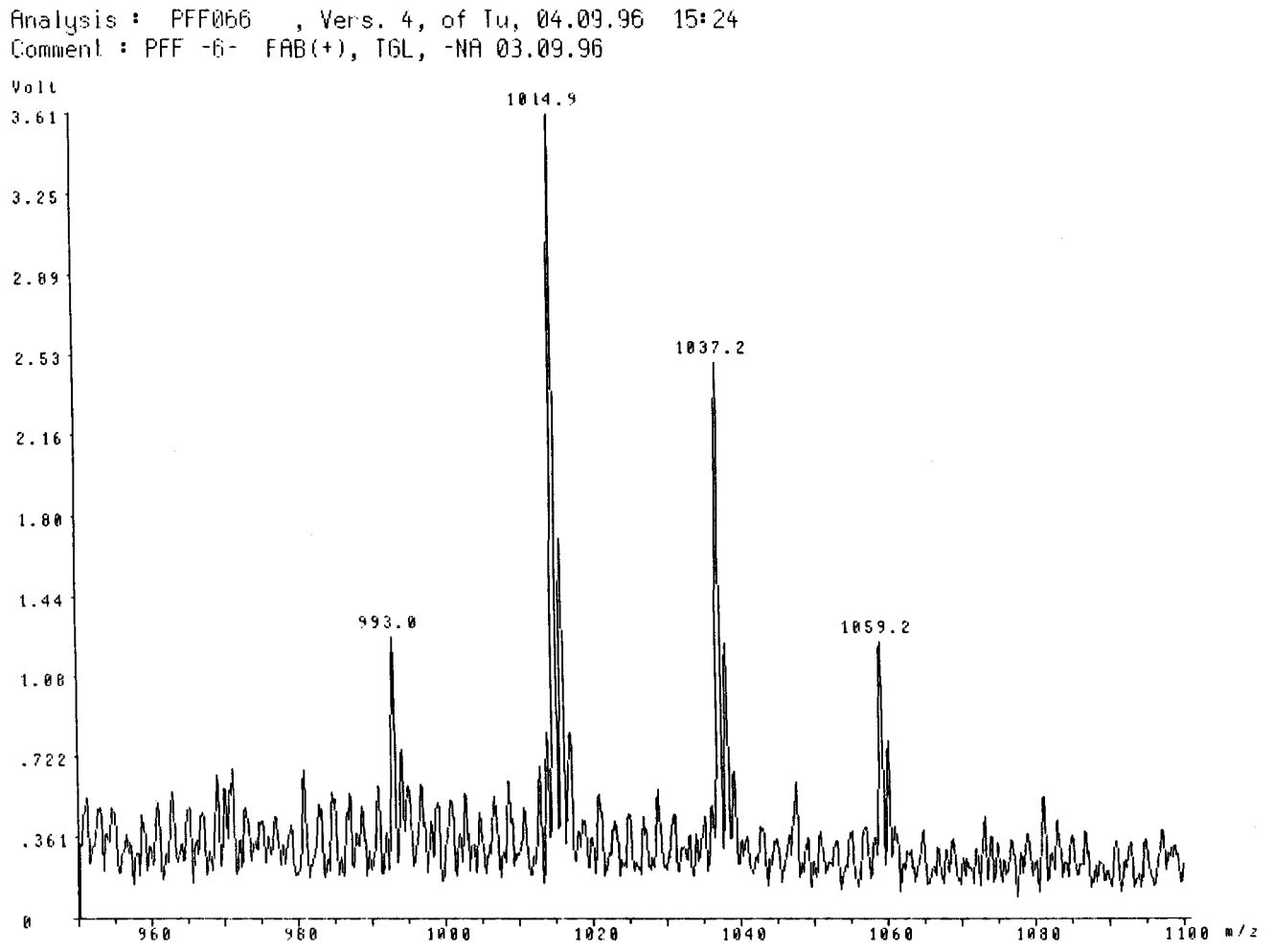

FIG. 6. Mass spectrometry of the compound which appears upon imipenem treatment of JRG58201 (*ampD* and *ampG* negative). The mass of the protonated compound corresponds exactly to the mass of aD-pentapeptide (molecular mass, 992 U). aD-pentapeptide + H^+ = 993 U; aD-pentapeptide + Na^+ = 1,015 U; aD-pentapeptide + $2Na^+$ ($-1H^+$) = 1,037 U; aD-pentapeptide + $3Na^+$ ($-2H^+$) = 1,059 U.

hydromuropeptide, aM-pentapeptide, also accumulates. So far aM-pentapeptide accumulation has not been demonstrated by other workers, and in our recent study it was supposed to be the aM-tetrapeptide (9). Furthermore, we show that both anhydromuropeptides are absent from an *ampD ampG* double mutant. Thus, both anhydromuropeptides are formed only in the cytoplasm, indicating that AmpG is the permease for the precursor molecule not only of aM-tripeptide but also of aM-pentapeptide. Therefore, aD-pentapeptide should be present in the cytoplasm of *ampG*-positive cells rather than *ampG*-negative cells. In both cases, we could not detect any aD-pentapeptide (Fig. 4). It was surprising that in *ampD*-negative cells the aM-pentapeptide accumulates in the cytoplasm (about 3×10^7 molecules per cell).

aD-tripeptide and aD-tetrapeptide could be detected only in the spheroplasts of *ampG*-positive cells (9). Thus, AmpG transports these two anhydromuropeptides into the cytoplasm. Since in exponentially growing cells the cell wall and the culture medium contain four to five times as much aD-tetrapeptide as aD-tripeptide (Table 1), it makes sense that AmpG also transports aD-tetrapeptide into the cell. These findings indicate that AmpG is not specific for one anhydromuropeptide.

TABLE 2. Amounts of aM-tripeptide, aM-pentapeptide, aD-tripeptide, aD-tetrapeptide, and aD-pentapeptide in the hot water extract of JRG582/pBP22 (*ampD* positive)

Imipenem concn (μg/ml)	% Area of corresponding peak in HPLC chromatogram[a]				
	aM-tripeptide	aD-tripeptide	aD-tetrapeptide	aM-pentapeptide	aD-pentapeptide
0	0.22 (0.05)	0.5 (0.2)	0.5 (0.28)	0	0
0.25[b]	0.3 (0.2)	0.6 (0.13)	1.4 (0.1)	0.2 (0.13)	2.2 (0.17)

[a] Results are means for from three extracts. Values in parentheses are standard deviations.
[b] Imipenem was added for 25 min after the cells reached an OD_{465} of 0.5.

TABLE 3. Influence of cell wall-active antibiotics on the peptidoglycan metabolism of JRG58201 (*ampD* and *ampG* negative)[a]

Antibiotic (concn [μg/ml])[a]	Relative value in the indicated expt no.[b]									β-Lactamase (U/mg)[c]
	aD-tripeptide			aD-tetrapeptide			aD-pentapeptide			
	1	2	Mean	1	2	Mean	1	2	Mean	
None	1	1	1	1	1	1	1	1	1	1
D-Cycloserine (8)	1	0.7	0.9	1.1	0.7	0.9	0	1.25	0.7	1.2
Vancomycin (16)	1.2	0.7	1	1.2	0.9	1	1	1.9	1.45	0.7
Sulbactam (8)	1		1	1		1	1		1	0.5
Piperacillin (0.8)	2		2	1.4		1.4	ND[d]		ND	1.8
Cefotaxime (0.25)	3.5	2.5	3	3.4	1.6	2.5	6.3	6.3	6.3	2.5
Meropenem (0.024)	4.3		4.3	2.3		2.3	12		12	8.1
Cefoxitin (2)		3.5	3.5		2.2	2.2		47.5	47.5	41.6
Imipenem (0.25)	1.9	2.2	2	2.3	1.8	2.05	150	170	160	174

[a] The antibiotic was added for 25 min after the cells reached an OD_{465} of 0.5. The concentration of each antibiotic was one-half the MIC.
[b] The values for no antibiotic are linked as 1. The absolute values are as follows: for aD-tripeptide, 0.55 and and 1.1 (mean, 0.83); for aD-tetrapeptide, 2.4 and 7.4 (mean, 4.9); for aD-pentapeptide, 0 and 0.08 (mean, 0.04). The values of the anhydromuropeptides indicate the area of the corresponding peak in the HPLC chromatogram relative to the mean value for untreated cells.
[c] The antibiotic was added for 2 h after the cells reached an OD_{465} of 0.1. The value without antibiotic (0.68) was linked as 1.
[d] ND, not determined.

AmpG is probably also the permease for other anhydromuropeptides released from the cell wall, like aD-pentapeptide. These anhydromuropeptides could then also be recycled. This is in agreement with the fact that AmpD is an amidase for many anhydromuropeptides (17).

Since aM-tetrapeptide (anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid-D-alanine) does not accumulate in the cytoplasm (Fig. 3 and 4), we assume that the terminal amino acid, the D-alanine, is cleaved off in the cytoplasm. A corresponding enzyme has not been found in the cytoplasm thus far. Although aM-tetrapeptide does not accumulate in the cytoplasm of *ampD*-negative cells, it is probably present, since *ampD*-negative cells also contain low levels of other anhydromuropeptides (substrates of AmpD) which have not been identified. One of these anhydromuropeptides, called S (Fig. 3), is detectable only in the presence of AmpG, thus probably being a 1,6 anhydro-monosaccharide peptide. Because of its retention time it might be the aM-tetrapeptide.

We could demonstrate that β-lactams, in contrast to other cell wall-active non-β-lactam-antibiotics like D-cycloserine and vancomycin and in contrast to the β-lactamase inhibitor sulbactam, which do not induce β-lactamase production, lead to increased levels of release of aD-peptides from the peptidoglycan sacculus (Table 3). The addition of all tested β-lactams, the strong inducers of β-lactamase production imipenem and cefoxitin, the moderate inducer meropenem, and the weak inducers cefotaxime and piperacillin resulted in the same increase in aD-tripeptide and aD-tetrapeptide levels in the periplasms of *ampG*-negative cells. Furthermore, another anhydromuropeptide, the aD-pentapeptide, emerges only upon treatment with β-lactams. However, the amount of this anhydromuropeptide correlates with the β-lactamase-inducing capacity of the β-lactam antibiotic (Table 3).

By form spheroplasts of *ampG*-positive (JRG582) and *ampG*-negative (JRG58201) cells after imipenem treatment, we could prove that AmpG transports aD-pentapeptide into the cytoplasm, because only the spheroplasts of *ampG*-positive cells contained aD-pentapeptide. Furthermore, upon imipenem treatment of *ampG*-positive cells, only the amounts of aM-pentapeptide and aD-pentapeptide increased (data not shown). Since in the absence of imipenem these *ampD*-negative cells produce β-lactamase at very high levels and accumulate only the aM-pentapeptide (Table 1), not the aD-pentapeptide (area percentage of the corresponding peak, 0.1), we assume that only or especially the aM-pentapeptide functions as the signal molecule for β-lactamase expression.

The increase in aM-pentapeptide levels must be linked to an increase in the number of pentapeptide side chains in the murein sacculus. The D,D-carboxypeptidases PBPs 4, 5, and 6, which cleave off the terminal D-alanine, reduce the amounts of these pentapeptide side chains very effectively. PBP 5 and PBP 6 account for 80% of the penicillin-binding capacity of cells and are the major D,D-carboxypeptidases (37). PBP 5 seems to be the main carboxypeptidase in exponentially growing cells. PBP 6 is probably important only in cells which are in the stationary growth phase (4). Tuomanen et al. (39) suppose that AmpD might inhibit PBP 5, since in *ampD*-negative cells the amounts of pentapeptide side chains in the peptidoglycan sacculus are decreased. Glauner et al. (12) showed that this difference in the amounts of pentapeptide side chains is significant.

Strong inducers of β-lactamase induction like imipenem and cefoxitin might increase the amount of aM-pentapeptide in the cytoplasm by binding and inhibiting PBPs 4, 5, and 6. Therefore, these D,D-carboxypeptidases are not active enough to remove the terminal D-alanine from pentapeptide side chains of the peptidoglycan sacculus.

The results presented in this paper led to a modified model of the recycling of anhydromuropeptides and their role for the regulation of class I chromosomal β-lactamase (Fig. 7) based on the one by Jacobs et al. (18). All β-lactams bind to at least one of the essential penicillin-binding proteins (PBPs; PBPs 1 to 3), resulting in degradation of the murein sacculus because of the increased amounts of aD-tripeptide and aD-tetrapeptide that are released) and thus in an accumulation of these two anhydromuropeptides in the periplasm. Strong inducers of β-lactamase induction like imipenem and cefoxitin bind to and inactivate not only the essential PBPs but also nonessential PBPs 4, 5, and 6. These D,D-carboxypeptidases cleave off the terminal amino acid from the pentapeptide side chains of the murein sacculus. This step is a prerequisite for the linkage of two peptide side chains to each other to form a bridge between two neighboring sugar strands and thus contributes to the stability of the murein sacculus. Since PBPs 4, 5, and 6 are very effective, pentapeptide side chains are rare and are only a component of newly synthesized murein (13). The concomitant inactivation of an essential PBP and of PBP 4, 5, or 6 leads to an accumulation of aD-tetrapeptide and aD-tripeptide and to

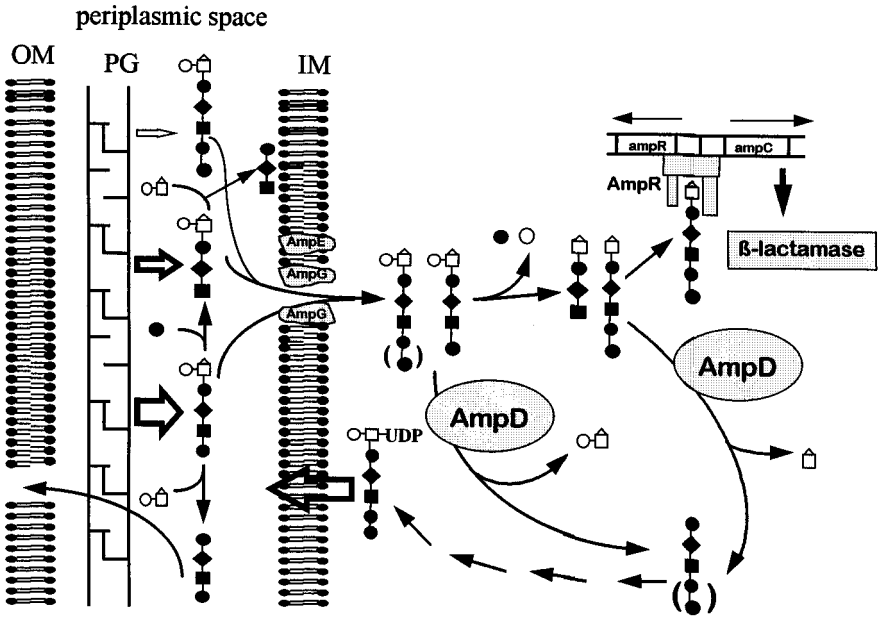

FIG. 7. Model of the recycling of muropeptides and their role in the regulation of class I chromosomal β-lactamase. OM, outer membrane; IM, inner membrane; PG, peptidoglycan; ○-⌂ , disaccharide; ○, GlucNAc; ⌂ , anhMurNAc; □, MurNAc; ●, Ala; ◆, Glu; ■, diaminopimelic acid.

the unusual accumulation of aD-pentapeptide. There is a strict correlation only between the accumulation of the aD-pentapeptide in the periplasm and the strength of an inducer of β-lactamase expression. AmpG transports all three muropeptides into the cytoplasm. There the *N*-acetylglucosamine is cleaved off and the terminal amino acid of the tetrapeptide side chain is also probably cleaved off, although a corresponding enzyme has yet not been found in the cytoplasm. The resulting aM-tripeptide and aM-pentapeptide accumulate in the cytoplasm of *ampD*-negative cells. Since the aM-pentapeptide and not the aD-pentapeptide accumulates in *ampD*-negative cells and these cells simultaneously produce β-lactamase at very high levels, we assume that only or especially the aM-pentapeptide functions as the signal molecule for β-lactamase induction. In the presence of AmpD the peptide side chains are cleaved off the disaccharide or monosaccharide peptides. At least the resulting tripeptide (14, 32), but also probably the pentapeptide, can then be reintroduced into the biosynthetic pathway for murein synthesis. This model also points to an assumption of how substances other than β-lactams can induce β-lactamase production. For *Enterobacter*, *Pseudomonas*, and *Proteus* these substances are, for example, glycine, D-tryptophan, and D-methionine (6, 7, 11, 35). They are probably incorporated into the peptidoglycan sacculus by a penicillin-insensitive L,D-transpeptidase, whereas up to 45% of all peptide side chains can be involved (5, 12). As a result there are increases in the amounts of tetrapeptide and pentapeptide side chains which carry the corresponding D-amino acid or glycine (5). Another effect of these substances is the inhibition of at least one of the essential PBPs, PBPs 1 to 3, leading to an increased degradation of the peptidoglycan sacculus (5, 16, 38). Thus, D-tryptophan, D-methionine, and glycine should, like β-lactams, lead to an accumulation of anhydromuropeptides in the periplasm, including the aD-pentapeptides carrying the unusual D-amino acids or glycine. Thus, we suppose that these unusual aD-pentapeptides or the aM-pentapeptides may also function as signal molecules for β-lactamase induction.

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