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Location of *N*-Acetylmuramyl-L-Alanyl-D-Glutamylmesodiaminopimelic Acid, Presumed Signal Molecule for β-Lactamase Induction, in the Bacterial Cell

HELGARD DIETZ, DIETER PFEIFLE, AND BERND WIEDEMANN*

Institut für Medizinische Mikrobiologie und Immunologie, University of Bonn, 53115 Bonn, Germany

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Using a chromatographic method for the isolation and detection of periplasmic and cytoplasmic muropeptides avoiding radioactive labeling, we found that in the *ampD*-negative strain JRG582 the anhydromuropeptide *N*-acetylmuramyl-L-alanyl-D-glutamylmesodiaminopimelic acid (anhMurNAc tripeptide) accumulates not only in the cytoplasm but also in the periplasm. Simultaneously JRG582 carrying the *Enterobacter cloacae* genes *ampC* and *ampR*, which are necessary for the induction of β-lactamase expression, overproduces β-lactamase. We confirmed that the transmembrane protein AmpG transports a precursor muropeptide into the cytoplasm and that the formation of the anhMurNAc tripeptide takes place in the cytoplasm. anhMurNAc tripeptide can then be secreted into the periplasm. Therefore, the amount of anhMurNAc tripeptide in the cytoplasm is reduced not only by AmpD but also by transport out of the cell.

In many enterobacteria such as *Enterobacter cloacae* and some *Citrobacter* species, β-lactam antibiotics can induce the chromosomal AmpC β-lactamase (16, 20, 21). Upon interaction with murein synthesis, β-lactams lead to an increased degradation of the murein sacculus. Thus, degradation products accumulate in the periplasm (9). 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).

AmpG, a transmembrane protein, is required for high-level expression of β-lactamase, whether this arises from induction or loss of AmpD (12). Jacobs et al. (9) suggest that this protein acts as a permease for the muropeptide *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamylmesodiaminopimelic acid (GlucNAc-anhMurNAc tripeptide).

In our recent study, we demonstrated that AmpD, a cytosolic protein (11), functions as a negative regulator of AmpC expression (11, 15, 17), being a *N*-acetyl-anhydromuramyl-L-alanine amidase (8). Mutations in the *ampD* gene result in constitutive β-lactamase overproduction. Simultaneously, large quantities of anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamylmesodiaminopimelic acid (anhMurNAc tripeptide) accumulate in the cytoplasm, indicating that this compound is the AmpR-activating ligand (8, 9). Hence, AmpG transports a precursor of the likely inducer molecule into the cytoplasm.

In this paper, we studied the peptidoglycan metabolism in *ampD*- and *ampG*-positive and -negative *Escherichia coli* strains with a new method for the isolation of periplasmic and cytoplasmic muropeptides without radioactive labeling.

MATERIALS AND METHODS

Bacteria, plasmids, and culture conditions. The *E. coli* K-12 strains used in this study were JRG582 and its derivative JRG58201. The *ampD* and *ampE* genes are deleted in JRG582 (6). JRG58201 carries an APH cassette inserted in the *ampG* gene (19).

Plasmids pBP21 and pBP22 are derivatives of pUC-19 (2) (pBP19) (11) carrying kanamycin resistance and the *E. cloacae ampE* gene (pBP21) or the *ampD* and *ampE* genes (pBP22). Plasmid pBP21 was constructed as follows. A 1.1-kb *Sph*I-*Xba*I fragment from the chromosome of the *E. cloacae* 14 wild type carrying the *ampE* gene was obtained by PCR. The *Xba*I site of this PCR fragment was blunt ended by the 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 (11), which carries the *ampD* gene of *E. cloacae.*

Recombinant DNA techniques and transformation of DNA were performed as described by Sambrook et al. (24). The *E. coli* strains were grown in complete medium standard NI broth (Merck AG, Darmstadt, Germany) at 37°C.

Preparation of hot-water extracts. Hot-water extracts were prepared on the basis of the method described by Jacobs et al. (9). Instead of minimal M9 medium, complete medium was used, and no radioactive diaminopimelic acid was added.

A 1-liter Erlenmeyer flask containing 200 ml of complete medium was inoculated with 2 ml of overnight culture and grown to mid-log phase (optical density at 456 nm = 0.5). Cells were harvested by centrifugation at 4°C and washed once with 1 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.

Preparation of periplasmic and cytoplasmic muropeptides. In order to fractionate the cells into periplasmic and cytoplasmic fractions, we developed this method knowing that even a larger muropeptide like GlucNAc-anhMurNAc tripeptide can pass the outer membrane (9). The washed cells were suspended in 20 ml of 10 mM Tris-HCl (pH 8.0). After incubation of this suspension at room temperature for 40 min, we harvested the cells by centrifugation at 4°C. The supernatant containing the periplasmic muropeptides was collected and lyophilized. The cytoplasmic fraction was obtained by following the method already described for the hot-water extract.

Methanol precipitation. The lyophilized samples of the hot-water extracts or cellular fractions were resuspended in 400 μl of water and chilled on ice for 15 min. Then 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.

High-performance liquid chromatography (HPLC) analyses. After methanol precipitation, the samples were lyophylized and suspended in 100 μl of water. Then, the muropeptides were reduced with sodium borohydride (4). Prior to chromatography, the samples were centrifuged for 10 min at 6,000 × *g.*

HPLC was performed with a Pharmacia gradient system consisting of a model 2152 LC controller, model 2150 pumps, and a model 11300 ultragrade mixer driver. A C_{18} Hypersil reverse-phase column (250 by 4.6 mm; 3-μm particle size) from Bischoff (Leonberg, Germany) together with a guard column containing μBondapak C_{18} Corasil (Waters) was used to separate the muropeptides. UV-absorbing components of the column effluent were detected at 205 nm by a model 2151 UV detector (Pharmacia), and peak areas and retention times were recorded with the maximum data acquisition program from Waters (Erkrath). The signal of the UV detector was integrated, and the area percentage for every peak was calculated.

Optimal separation of muropeptide monomers with this HPLC system was reached by a linear gradient from methanol-free 50 mM sodium phosphate, pH

* Corresponding author. Mailing address: Institut für Medizinische Mikrobiologie und Immunologie, University of Bonn, Meckenheimer Allee 168, 53115 Bonn, Germany. Phone: (0228) 735272. Fax: (0228) 735267.

TABLE 1. Amount of isolated anhMurNAc tripeptide after gel filtration or methanol precipitation

Sample[a]	Peak area (μV/s) of tripeptide in HPLC chromatogram by:	
	Gel filtration	Methanol precipitation
1	18,214,228	17,918,142
2	11,036,691	11,183,195
3	8,135,131	9,449,385

[a] Three samples of extracted JRG582 (*ampD* negative) were halved, with one half treated by gel filtration and the other half treated by methanol precipitation.

4.31, containing 0.8 mg of sodium azide per liter to 28% 75 mM sodium phosphate, pH 4.95, containing 15% methanol over 80 min at 38.5°C with a flow rate of 0.5 ml/min.

Determination of β-lactamase activity. Cells were fractionated as described below, and the β-lactamase activity of the supernatant as well as of the bacterial pellet was determined. The bacterial pellet was resuspended in 1 ml of 0.05 M potassium phosphate (pH 7.0) and frozen overnight. Sonication on ice with a Branson sonifier yielded the cell extract for β-lactamase determination. The β-lactamase activity was quantified as described by Peter et al. (23) with nitrocefin (50 μM) as the substrate (22). The protein content of each sample was determined by the method of Bradford (1) with bovine serum albumin as the standard.

RESULTS

Methanol precipitation versus gel filtration for the removal of proteins. The hot-water extracts as well as the periplasmic and cytoplasmic fractions contain proteins, which must be removed to avoid rapid clogging of the HPLC columns. Three samples of extracted JRG582 were divided in half; one half was treated by gel filtration through a Sephadex G-50 column (30 by 2.5 cm; Pharmacia), and the other half was treated by methanol precipitation. By HPLC analysis comparable results were observed (Table 1).

Accumulation of anhMurNAc tripeptide in *ampD*-negative strains. Hot-water extracts of JRG582/pBP21 (*ampD* negative) and JRG582/pBP22 (*ampD* positive) after methanol precipitation were analyzed by HPLC using the described linear gradient. As expected, in the *ampD*-negative strain an additional peak was detected (Fig. 1) with an area percentage (related to the total area of the chromatogram) of 6.47 (standard deviation = 1.08; $n = 16$); the corresponding value for the *ampD*-positive strain was below the limit of detection, 0.05 ($n = 13$). This peak could also be detected in the supernatant of washed cells. Furthermore, we could demonstrate that the corresponding compound is a substrate 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 hot-water extract the MalE-AmpD fusion protein (about 1 μg) (8) was added. Then, we incubated both halves for 12 h at 30°C. The peak of interest disappeared only upon AmpD treatment.

We collected the peak (retention time = 37.63 min) (Fig. 1b) and analyzed the corresponding compound by mass spectrometry. Mass spectrometry was performed by fast atom bombardment-ionization using thioglycerol as matrix. The mass of the protonated compound was 647.7 (Fig. 2), which exactly corresponds to the mass of anhMurNAc tripeptide.

Presence of anhMurNAc tripeptide in the cytoplasm and in the periplasm. Cells of JRG582/pBP21 were fractionated into periplasmic and cytoplasmic fractions by osmotic shock (7). Surprisingly, the anhMurNAc tripeptide, which was shown to be formed in the cytoplasm (9), was present in the periplasmic fraction but not in the cytoplasmic fraction under these conditions. This suggests that the method damaged the cytoplasmic membrane, facilitating the release of anhMurNAc tripeptide. For this reason, we used the gentler method for the preparation of periplasmic and cytoplasmic muropeptides as described in Materials and Methods. To demonstrate the gentleness of this method, we showed that proteins like β-lactamase can hardly escape through the outer membrane under these conditions. Preparations of JRG582 carrying plasmid pBP131 (13), which contains the *E. cloacae* genes (*ampC* and *ampR*) required for the expression of the *E. cloacae* β-lactamase, revealed that most of the β-lactamase activity was in the bacterial pellet (29 U/ml) and not in the supernatant (3 U/ml). In contrast, fractionating the cells by osmotic shock led to an almost complete release of β-lactamase (35 U/ml in the supernatant and 0.3 U/ml in the bacterial pellet).

Both fractions of JRG582/pBP21 (*ampD* negative) contained the anhMurNAc tripeptide at a high level: the mean area percentages (related to the total area of the chromatogram) of the peptide were 7.28 and 12.49 in the periplasm and cytoplasm, respectively (standard deviations = 0.057 and 1.81, respectively [$n = 3$]). To prove that the muropeptide released from the periplasm is anhMurNAc tripeptide, we performed mass spectrometry on the released material. The mass of this compound was 647.7, corresponding to the mass of anhMur NAc tripeptide.

Exclusive formation of anhMurNAc tripeptide in the cytoplasm. Since anhMurNAc tripeptide was found in the periplasm as well as in the cytoplasm, two questions arose. First, is anhMurNAc tripeptide formed in the periplasm and then transported via AmpG into the cytoplasm? Second, is the anhMurNAc tripeptide formed in the cytoplasm, as suggested by Jacobs et al. (9), and then exported through the inner membrane? In the latter case, AmpG would transport a precursor molecule into the cytoplasm. To investigate this, we used an *ampD*- and *ampG*-negative strain (JRG58201). If AmpG transports anhMurNAc tripeptide being formed in the periplasm into the cytoplasm, we should find this compound in the periplasm. Since anhMurNAc tripeptide was present neither in the periplasm nor in the cytoplasm, we proved that in fact anhMurNAc tripeptide is formed in the cytoplasm and then transported into the periplasm. AmpG being the permease for a precursor molecule of anhMurNAc tripeptide is necessary for its formation in the cytoplasm.

DISCUSSION

Murein recycling in *E. coli* can use a pathway (5, 9) which involves the products of the genes *ampD* (15) and *ampG* (12). AmpD and AmpG play also an important role in β-lactamase induction. Recently, it was shown that the cytosolic protein AmpD is a *N*-acetylanhydromuramyl-L-alanine amidase (8, 10). Since the *ampD* (26) mutant accumulates anhMurNAc tripeptide in the cytoplasm (9), it is assumed that AmpD exerts its negative effect on AmpC expression by hydrolyzing this anhydromuropeptide, the positive signal molecule for β-lactamase induction.

In this study we established a new method for the isolation of periplasmic and cytoplasmic muropeptides without radioactive labeling. Using this method, we could demonstrate that anhMurNAc tripeptide accumulates not only in the cytoplasm but also in the periplasm of *ampD* mutants. Furthermore, we showed that in an *ampD ampG* double mutant anhMurNAc tripeptide is absent in both the periplasm and the cytoplasm. This finding indicates that AmpG transports a precursor molecule into the cytoplasm. Thus, anhMurNAc tripeptide is formed in the cytoplasm and then transported out of the cell.

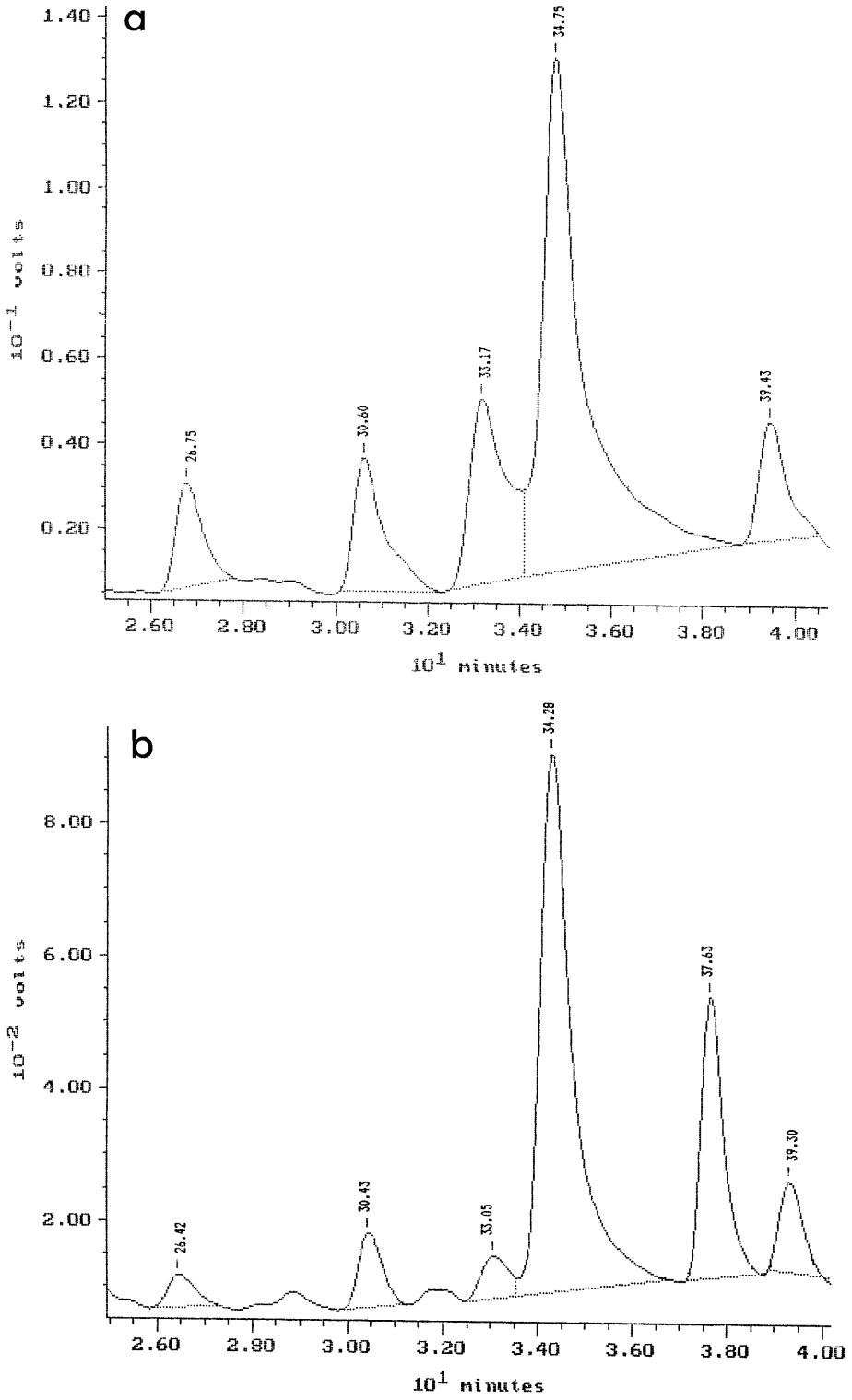

FIG. 1. HPLC analysis of hot-water extracts from JRG582/pBP22 (*ampD* positive) (a) and JRG582/pBP21 (*ampD* negative) (b). The chromatogram of the *ampD*-negative strain shows an additional peak (retention time = 37.63 min).

It will be interesting to find which permease is responsible for the transport and if it also exports other anhydromuropeptides.

This study revealed that the amount of anhMurNAc tripeptide in the cytoplasm is reduced not only by AmpD but also by transport out of the cell. The question of why the bacterial cell established different ways to reduce the amount of this muropeptide in the cytoplasm arises. It is possible that the level of this muropeptide resulting from murein degradation signals

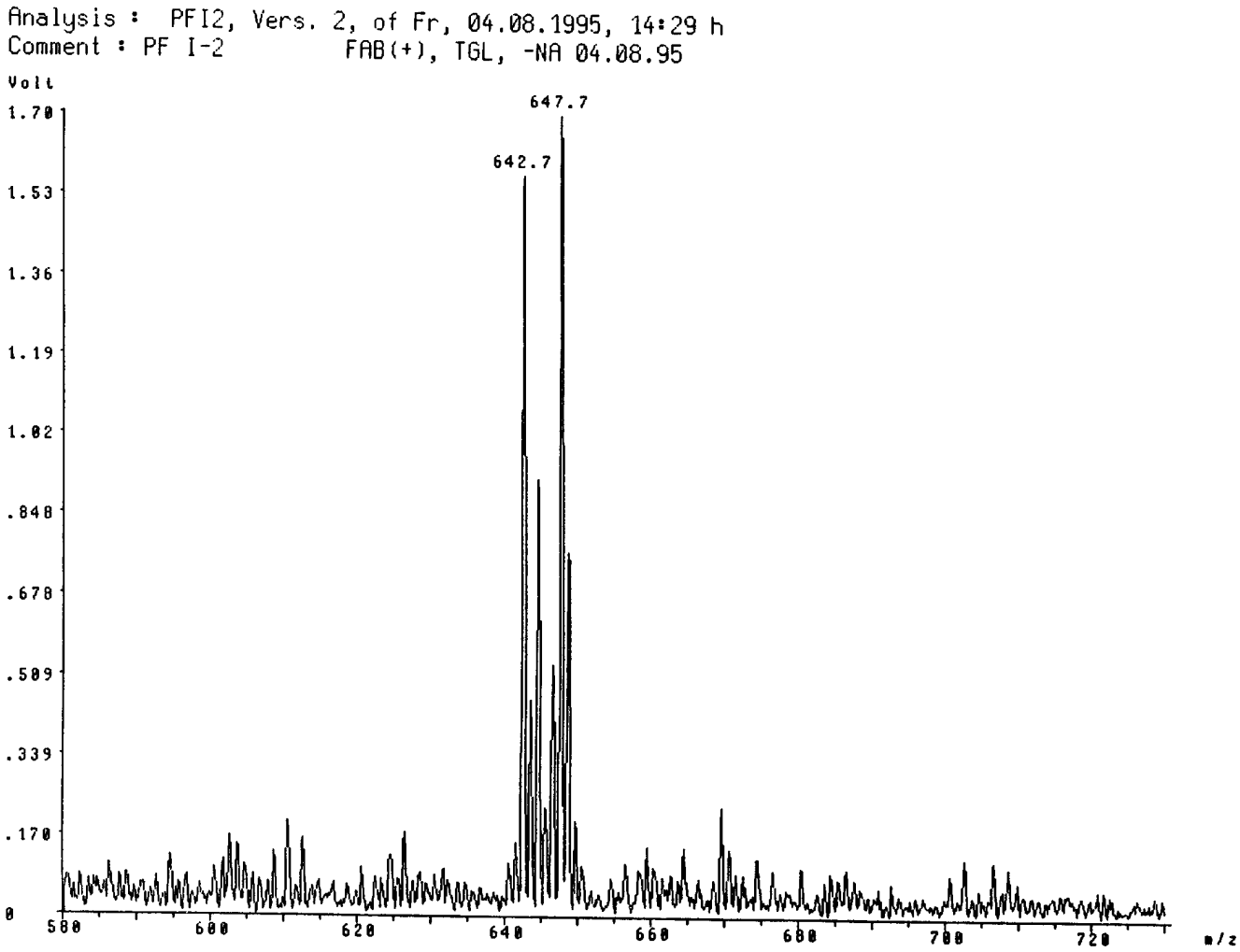

FIG. 2. Mass spectrometry of the compound which accumulates in *ampD*-negative strains. The mass of the protonated compound corresponds exactly to the mass of anhMurNAc tripeptide (molecular weight = 647).

the dynamic state of the murein sacculus. Thus, anhMurNAc tripeptide could be the link between murein metabolism and the transcriptional machinery (9). It cannot be excluded that also other muropeptides can function as a link and simultaneously, as Jacobs et al. speculate (9), act as signal molecules for β-lactamase expression.

Since the dynamic state of the murein sacculus is essential for bacterial growth and cell division, the murein metabolism must be strictly regulated. The accumulation of anhMurNAc tripeptide in an *ampD* mutant impairs the peptidoglycan recycling (9). Simultaneously the cells start producing β-lactamase at a high level. The question of why the cells react to an increased level of anhMurNAc tripeptide by overexpressing β-lactamase arises.

The transport of anhMurNAc tripeptide out of the cell could also serve a completely different task, namely, functioning as a virulence factor. It could be demonstrated that anhydromuropeptides can induce arthritis (3) and meningitis (25) as well as sleep (14). Thus, *ampD* mutants that are more resistant to β-lactam antibiotics might be more virulent to human beings than the wild type.

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