Role of Penicillin-Binding Proteins in the Initiation of the AmpC β -Lactamase Expression in *Enterobacter cloacae*

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Penicillin-binding proteins (PBPs) are involved in the regulation of β -lactamase expression by determining the level of anhydromuramylpeptides in the periplasmic space. It was hypothesized that one or more PBPs act as a sensor in the β -lactamase induction pathway. We have performed induction studies with *Escherichia coli* mutants lacking one to four PBPs with DD-carboxypeptidase activity. Therefore, we conclude that a strong β -lactamase inducer must inhibit all DD-carboxypeptidases as well as the essential PBPs 1a, 1b, and/or 2.

The production of β -lactamase is the major mechanism of bacterial resistance to β -lactam antibiotics. These enzymes hydrolyze the β -lactam ring and hence inactivate the antibiotics before they reach their target, the penicillin-binding proteins (PBPs) (8). In members of the family *Enterobacteriaceae* the inducible production of the chromosomal AmpC β -lactamase is mediated by the genes *ampC*, *ampR*, *ampD*, and *ampG* (18–23) and is closely linked with the recycling of the peptidoglycan (5, 11, 14–17, 40; D. Pfeifle, H. Dietz, E. Janas, I. Wiegand, and B. B. Wiedemann, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C-003, 1998). β -Lactam antibiotics differ markedly in their induction potentials. Imipenem and cefoxitin are strong inducers, while aztreonam and ceftazidime are not (5). As these two groups of β -lactam antibiotics differ in their affinities for the PBPs, it was hypothesized that one or more PBPs act as a sensor in the β -lactamase induction pathway (5, 27, 30, 31, 35, 40; Pfeifle, 38th ICAAC).

After addition of a strong inducer like imipenem NAcGlc-anhMurNAc-tripeptide (*N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic-acid), NAcGlc-anhMurNAc-tetrapeptide (*N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic-acid-D-alanine), and especially, NAcGlc-anhMurNAc-pentapeptide (*N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic-acid-D-alanyl-D-alanine) accumulate in the periplasmic space (5, 14). Jacobs et al. (16) demonstrated that anhydromuramyl (aM)-tripeptide and aM-tetrapeptide convert the transcriptional factor AmpR into an activator of β -lactamase expression. We believe that the aM-pentapeptide plays an important role in the induction process, because we could demonstrate a clear correlation between the amount of the aM-pentapeptide, the amount of the β -lactamase, and the induction capacity (5).

The PBPs are cytoplasmic membrane enzymes involved in peptidoglycan biosynthesis (8, 36, 37). Up to 12 PBPs have been identified in *Escherichia coli* (1, 8, 10). They are able to bind to β -lactam antibiotics covalently at a conserved active serine residue because of their structural homology with the natural substrate D-alanine-D-alanine for transpeptidation. High-molecular-weight PBPs 1a, 1b, 2, and 3 are essential for growth and survival of the bacterial cell. PBPs 1a and 1b are

believed to be dual transpeptidases-transglycosylases which catalyze glycan chain elongation and peptidoglycan cross-links, while PBP 2 and PBP 3 act only as transpeptidases. PBP 3 is essential for the formation of the septum during cell division (8, 36, 37). PBP 2, encoded by the gene *pbpA*, is required for lateral cell wall elongation and the maintenance of the rod shape (37). The activity of PBP 2 accounts for about 70% of peptidoglycan synthesis during elongation, indicating that PBP 2 is a major factor in net synthesis (32). Inhibition of PBP 2 with mecillinam leads to spherical cells and causes cell lysis after some generations (28). Mutations in the *pbpA* gene abolish β -lactamase induction (30).

Low-molecular-weight PBPs 4, 5, 6a, 6b, and 7 are dispensable, as their inactivation by mutation does not affect the vitality of the cells (1, 7). Most of the nonessential PBPs function as DD-carboxypeptidases. The DD-carboxypeptidases PBPs 4, 5, and 6 account for about 50% of the penicillin-binding capacity of bacterial cells (6). These enzymes are responsible for the degradation of the pentapeptide side chains to tetrapeptide in the peptidoglycan (1, 4, 36). Only newly inserted murein components carry pentapeptide side chains, which are rapidly degraded by transpeptidases and carboxypeptidases (4, 9). The inhibition of DD-carboxypeptidase leads to an increased level of pentapeptide side chains in the murein sacculus (1, 4, 7).

On the basis of our experiments we postulate that the 1,6-anhydromuramyl-pentapeptide is the main signal molecule for β -lactamase induction (5), for which it sends a signal by converting AmpR from a repressor into an activator (16). Strong inducers of β -lactamase like imipenem and cefoxitin bind to the DD-carboxypeptidases besides the essential PBPs and lead to conservation of pentapeptide side chains in the murein (5).

Here we describe induction studies performed with *E. coli* mutants lacking PBPs with carboxypeptidase activity (Table 1)

TABLE 1. Bacterial strains used in the study

Strain	Genotype and/or phenotype	PBP deletion	Reference
MC4100	F [−] Δ (argF-lac)U169 araD139 deoC1 flbB5301 ptsF25 relA1 thiA rpsL150		1
UGM599	MC4100 Δ dacA::Km	5	1
UGM600	MC4100 <i>dacD1</i>	6b	1
UGM601	UGM599 <i>dacD1</i>	5, 6b	1
JBS200	<i>his supF srl::Tn10 recA56</i>		3
JBS1002	JBS200 Δ dacC1 Δ dacA::Km	5, 6a	1
UGM602	JBS1002 <i>dacD1</i>	5, 6a, 6b	1
UGM603	UGM602 <i>dacB::spc</i>	4, 5, 6a, 6b	1

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TABLE 2. MIC of β -lactams for *E. coli* strains lacking DD-carboxypeptidases^a

Antibiotic	MIC (μ g/ml)							
	MC4100	UGM599 ($\Delta 5$) ^b	UGM600 ($\Delta 6b$)	UGM601 ($\Delta 5$, $\Delta 6b$)	JBS200	JBS1002 ($\Delta 5$, $\Delta 6a$)	UGM602 ($\Delta 5$, $\Delta 6a$, $\Delta 6b$)	UGM603 ($\Delta 4$, $\Delta 5$, $\Delta 6a$, $\Delta 6b$)
Amoxicillin	4	4	4	4	4	4	16	64
Cefaclor	2	2	4	2	1	2	16	64
Cefazolin	2	0.5	4	0.5	1	1	4	256
Cefuroxime	1	0.5	2	0.5	2	1	4	16
Cefotaxime	0.0625	<0.0625	0.125	<0.0312	<0.0312	<0.0312	0.5	0.5
Cefsulodin	32	16	32	32	32	32	32	64
Mecillinam	0.075	0.075	0.075	0.0362	0.075	0.0362	0.0362	0.075
Aztreonam	<0.0312	<0.0312	0.0625	<0.0312	<0.0312	<0.0312	0.0625	1
Imipenem	0.125	0.0625	0.125	0.0625	0.0625	0.125	0.0625	0.0625

^a All strains carry plasmid pBP131, which contains *E. cloacae* genes *ampC* and *ampR*.^b Designations in parentheses indicate the PBPs that have been deleted.

which were transformed with the *Enterobacter cloacae ampR-ampC* operon.

Culture conditions. The *E. coli* strains were grown in M9 medium supplemented with glucose (0.2%), Casamino Acids (0.1%), thiamine (1 μ g/ml), uracil (50 μ g/ml), nicotinamide (5 μ g/ml), and MgSO₄ (1 mM) at 37°C. When required, sulfamethoxazole (1,000 μ g/ml), neomycin (50 μ g/ml), and tetracycline (50 μ g/ml) were added.

The various antibiotics, which were tested for their capacity to induce the AmpC β -lactamase, were kindly provided by the following companies: cefotaxime by HMR Hoechst, Frankfurt, Germany; imipenem by Merck Sharp & Dohme, West Point, Pa.; mecillinam by Leo Pharmaceutical Products, Ballerup, Denmark; and aztreonam and cefsulodin by Grünenthal, Aachen, Germany.

Antibiotic susceptibility testing. Antibiotic susceptibility was tested by a microdilution procedure in Iso-Sensitest broth (Oxoid). MICs were determined with a photometer for microtiter plates (LabSystems Multiscan Multisoft) after inoculation of antibiotic-containing microtiter plates (Merlin-Diagnostika, Bornheim, Germany) with 100 μ l of an appropriate bacterial suspension ($\approx 10^5$ CFU/ml) and incubation for 24 h at 36 $\pm$ 1°C.

Determination of β -lactamase activity. We performed induction studies with *E. coli* PBP deletion mutants transformed with plasmid pBP131 containing the *E. cloacae* genes (*ampC* and *ampR*) required for the expression of *E. cloacae* β -lactamase (19). The cells were grown to an optical density at 546 nm (OD₅₄₆) of 0.5, and various antibiotics were added at concen-

trations that were half the MIC for 40 min. As a positive control imipenem was added at 1 μ g/ml. Then, the cells (10 ml) were harvested by centrifugation at 4°C. The cells were resuspended in 1 ml of 0.05 M potassium phosphate buffer (pH 7.0) and were 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. (33), with nitrocefin (50 μ M) used as the substrate (29). The protein content of each sample was determined by the method of Lowry et al. (26), with bovine serum albumin used as the standard.

Role of PBPs for initiation of β -lactamase induction. The MICs of most of the β -lactamase-sensitive antibiotics were increased only for UGM602 (PBP 5, 6a, and 6b negative) and the quadruple PBP deletion mutant UGM603 (PBP 4, 5, 6a, and 6b negative) (1) (Table 2). These results were a hint that the basal β -lactamase activity had changed.

The inactivation of one or two DD-carboxypeptidases has no influence on the basal β -lactamase level. However, the basal β -lactamase level is increased in the triple mutant UGM602 and is especially increased in the quadruple mutant UGM603. The basal β -lactamase activity is elevated 2- to 3-fold in the triple mutant UGM602 compared to that in the wild type or the mutants with only one or two deletions of DD-carboxypeptidases and is elevated 10-fold in the quadruple mutant (Table 3).

The simultaneous inhibition of the essential PBPs 1a and 1b by cefsulodin and PBP 2 by mecillinam (24) led to a strong β -lactamase induction only in UGM603. The use of higher concentrations of mecillinam and cefsulodin results in the same

TABLE 3. Influence of β -lactam antibiotics on β -lactamase induction in mutants of *E. coli* lacking DD-carboxypeptidases^a

Antibiotics ^b	β -Lactamase activity (μ mol/mg/min) ^c							
	MC4100	UGM599 ($\Delta 5$) ^d	UGM600 ($\Delta 6b$)	UGM601 ($\Delta 5$, $\Delta 6b$)	JBS200	JBS1002 ($\Delta 5$, $\Delta 6a$)	UGM602 ($\Delta 5$, $\Delta 6a$, $\Delta 6b$)	UGM603 ($\Delta 4$, $\Delta 5$, $\Delta 6a$, $\Delta 6b$)
None	0.45 (0.18) ^e	0.4 (0.06) ^e	0.48	0.36 (0.06) ^e	0.46 (0.05)	0.67 (0.22) ^e	1.65 (0.25) ^e	5.40 (1.5) ^e
Cefsulodin	0.43 (0.17) ^e	0.22	0.60	0.34	0.56 (0.05)	0.61 (0.24) ^e	2.66 (0.25) ^e	9.10 (1.5) ^e
Mecillinam	0.44 (0.08) ^e	0.21	0.44	0.28	0.44 (0.17) ^e	0.66 (0.21) ^e	1.68 (1) ^e	14.00 (5.5) ^e
Mecillinam-cefsulodin	0.49 (0.21) ^e	0.57	1.23 (0.55) ^e	0.48 (0.12) ^e	0.52 (0.05)	0.60 (0.2) ^e	1.76 (0.5) ^e	18.86 (7.3) ^e
Imipenem	0.50 (0.19) ^e	0.58	0.53	2.20	0.53 (0.04)	0.67 (0.18) ^e	2.96 (0.37) ^e	8.50 (1.6) ^e
Cefotaxime	0.75 (0.38) ^e	0.23	0.42	0.21	0.75 (0.38) ^e	0.76 (0.43) ^e	2.07 (0.35) ^e	5.75 (1.7) ^e
Aztreonam	0.15 (0.09) ^e	0.21	0.35	0.20	0.15 (0.09) ^e	0.42 (0.27) ^e	1.00 (0.77) ^e	5.50 (2.0) ^e
Imipenem (1 μ g/ml)	12.31 (1.33)	26.59 (5.26)	16.14 (3.29)	33.52 (5.57)	11.08 (1.27)	18.06 (9.75)	12.91 (2.18)	24.9 (5.78)

^a All strains are carrying the plasmid pBP131, which contains the *E. cloacae* genes *ampC* and *ampR*.^b The antibiotic was added for 40 min after the cells reached an OD₅₄₆ of 0.5. The concentration of each antibiotic was half the MIC.^c Data are means from two or three experiments.^d See footnote b of Table 2.^e Values in parentheses in the table body are standard deviations for three experiments.

effect (data not shown). The inhibition of PBP 3 by aztreonam (24) had no consequence for the β -lactamase induction (Table 3). Also, we have tested a mutant that lacks PBPs 4, 5, and 6a, the effect for that mutant was the same as that for UGM603 (data not shown).

These results suggest that only the deletion of three or four DD-carboxypeptidases and the concomitant inhibition of the essential PBP 1a, 1b, and/or 2 results in an increased level of β -lactamase induction. Thus, a strong inducer must inhibit all DD-carboxypeptidases as well as essential PBPs 1a, 1b, and/or 2. In contrast, Sanders et al. (35) suggested a major role for PBP 4 in the induction of AmpC, as in PBP 4-negative and in PBP 4- and PBP 7-negative mutants the inducibility decreases.

On the basis of these results we favor a modified model of the induction of β -lactamase that emphasizes the involvement of PBPs in this process (5, 17). In the periplasmic space the β -lactam antibiotics bind to PBPs, causing an imbalance in the equilibrium of peptidoglycan synthesis and hydrolysis reactions (12). Four different lytic transglycosylases in *E. coli* are known to be associated with PBPs that bind to multienzyme complexes. These lytic transglycosylases hydrolyze glycosidic bonds to allow murein expansion, releasing mucopeptides containing anhydromuramic acid. All four enzymes function as exomuraminidases which cleave the glycan strands in a processive manner starting at the nonreducing glucosamine end. Three enzymes (MltA, MltB, and MltC) are lipoproteins bound to the outer membrane, while Slt70 is a soluble protein (12, 13, 25, 34, 39). Slt70 interacts with the high-molecular-weight PBPs 1a, 1b, and 2 (39). The three-dimensional structure of Slt70 revealed by X-ray crystallography is doughnutlike, with the active site facing the inside (38). It is suggested that peptide cross-links prevent access of Slt70 to the glycan chains and therefore slow down the activity of this enzyme (2). Inhibition of Slt70 leads to a decrease in the level of β -lactamase induction (Pfeifle et al., 38th ICAAC; D. Pfeifle, I. Wiegand, and B. Wiedemann, Program Abstr. 9th Eur. Congr. Clin. Microbiol. Infect. Dis., abstr. 01174, 1999).

Inactivation of DD-carboxypeptidases PBP 4, 5, 6a, and 6b leads to an increase in the level of pentapeptide side chains in the peptidoglycan. Inhibition of PBP 1a, 1b, and/or 2 leads to inactivation of the transpeptidase function, causing a decrease in the level of cross-links in the murein. The reduced amount of cross-links allows further degradation of the murein by the lytic transglycosylases especially by Slt70 (39, 40). Therefore, degradation of the murein sacculus is followed by an accumulation of aM peptides in the periplasm. The most important degradation product, aM-pentapeptide, is transported into the cytoplasm (5), where it probably converts AmpR from a repressor into an activator of *ampC* expression by displacing the UDP-NAcMur-pentapeptide (uridine diphosphate-N-acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelic acid-D-alanyl-D-alanine) from AmpR (16).

Although the process of β -lactamase induction is much better understood in the context of peptidoglycan degradation and recycling, we still lack knowledge about some essential steps of β -lactamase initiation. Studies will be needed to assess the links between inhibition of PBPs, lytic transglycosylases, and β -lactamase induction. Inhibition of the lytic transglycosylases, especially Slt70, could be a possible means of disturbing β -lactamase induction (Pfeifle et al., 9th ECCMID).

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