Dependence of induction of enterobacterial AmpC β -lactamase on cell-wall peptidoglycan, as demonstrated in *Proteus mirabilis* and its wall-less protoplast L-form

MICHAEL TÖLG, HERBERT SCHMIDT, RALPH SCHIERL, MARTINA DATZ and HANS H. MARTIN*

Institut für Mikrobiologie, Technische Hochschule Darmstadt, Schnittspahnstrasse 9, D-64287 Darmstadt, FRG

(Received 5 February 1993; revised 17 May 1993; accepted 18 June 1993)

The mobilizable plasmid pMD101 (*ampR*, *ampC*) was constructed by inserting cloned *ampC*, the structural gene for the chromosomal AmpC β -lactamase of *Citrobacter freundii*, and the closely linked *ampR* encoding the transcriptional regulator essential for enzyme induction, into the broad host-range plasmid pKT231. Plasmid pMD101 was transconjugated into *Proteus mirabilis* VI and its isogenic, cell-wall-less protoplast L-form LVI. AmpC β -lactamase was expressed constitutively from cloned *ampR* and *ampC* in bacteria and in some L-form protoplasts. However, induction of the enzyme by β -lactam antibiotics occurred only in bacterial cells and not in the cell-wall- and peptidoglycan-deficient L-form. In agreement with current models, induction of AmpC β -lactamase is thought to be initiated by an induction signal arising from the metabolic disturbance of cell-wall peptidoglycan.

Introduction

Chromosomally encoded class 1 β -lactamase (Sykes & Smith, 1979; molecular class C, Jaurin & Grundström, 1981) occurs in different Gram-negative bacteria. The enzyme is normally synthesized constitutively at low levels but can be induced to much higher concentrations by β -lactam antibiotics with a corresponding increase of bacterial resistance to these antibiotics. In *Citrobacter freundii* and *Enterobacter cloacae*, induced expression of the β -lactamase from the chromosomal gene *ampC* has been shown to be under the control of several regulatory genes. These include *ampR*, closely linked to *ampC*, encoding a transcriptional regulator (Lindberg *et al.*, 1985; Honoré *et al.*, 1986; Lindquist *et al.*, 1989a), and the *ampD*–*ampE* operon, responsible for negative modulation of β -lactamase synthesis (Lindberg *et al.*, 1987; Honoré *et al.*, 1989; Lindquist *et al.*, 1989b). The more recently discovered gene *ampG* (Korfmann & Sanders, 1989) is thought to encode a membrane-bound transducer of an external induction signal (Lindquist *et al.*, 1993). As suggested by these authors, the transducer may modify the external signal and relay the product as activator to the transcriptional regulator AmpR. In the

absence of induction, function of *ampD*, *E* diverts much of the moderate signal inflow from AmpG, either by direct signal inactivation or by inhibition of AmpR function, thus allowing only low-level constitutive β -lactamase synthesis. Induction by β -lactam antibiotics presumably increases signal production above the inactivating capacity of *ampD*, *E* and causes large-scale activation of AmpR and expression of AmpC β -lactamase. The negative control function of *ampD* has been demonstrated in mutants where defects in *ampD* result either in high (semi-)constitutive or in hyper-inducible β -lactamase synthesis (Lindberg *et al.*, 1987; Lindquist *et al.*, 1989b; Honoré *et al.*, 1989). Genes *ampD*, *E* (Lindberg *et al.*, 1987; Honoré *et al.*, 1989; Lindquist *et al.*, 1989b) and *ampG* (G. Korfmann, personal communication; Lindquist *et al.*, 1993) are also present in *Escherichia coli*. Here, AmpC β -lactamase is normally non-inducible because of deletion of *ampR*, but becomes inducible upon introduction of cloned *ampC* and *ampR* from *C. freundii*.

The nature of the signal interacting with *ampG* or its product and the way in which it originates from the action of the inducing β -lactam are so far unknown. The bacterial cytoplasmic membrane is thought to be impermeable to β -lactam antibiotics, and it has been shown that penetration of the antibiotics into the cytoplasm is not required for induction (Everett *et al.*, 1990). In a

* Author for correspondence. Tel. +49 6151 63391; fax +49 6151 164808.

model proposed by Lindberg *et al.* (1985, 1988), the initiation of β -lactamase induction is viewed as an additional effect of the interaction of β -lactams with one or several of the penicillin-binding proteins on the outer surface of the cytoplasmic membrane. The ensuing inactivation of the function of penicillin-binding proteins and disorganization of bacterial peptidoglycan synthesis may then cause the formation of one or more peptidoglycan catabolites with the function of indirect induction signals ('autoinducer'). Evidence for a link between the regulation of AmpC β -lactamase and the biosynthesis of peptidoglycan has also been provided by Tuomanen *et al.* (1991). Induction of β -lactamase would thus depend on the presence of a cell wall and peptidoglycan. In agreement with this hypothesis, we show here that AmpC β -lactamase is expressed constitutively and inducibly from cloned *ampR* and *ampC* of *C. freundii* in normal bacteria of *Proteus mirabilis*, but only constitutively in the isogenic protoplast L-form derived from this bacterium. The L-form organisms have been shown previously to be viable, wall-less protoplasts devoid of a peptidoglycan layer and outer membrane (Martin, 1964; Hofschneider & Martin, 1968; Kroll *et al.*, 1980).

Methods

Bacteria and plasmids. *P. mirabilis* VI and the protoplast L-form LVI were described by Taubeneck (1962*a, b*), Martin (1964) and Hofschneider & Martin (1968). *E. coli* HfrH (Low, 1973) and SN0301 (*ampC8*, β -lactamase-deficient; *ampD1*, Lindquist *et al.*, 1989*b*) were reference strains for inducible and hyperinducible expression of β -lactamase, respectively, from cloned *ampR* and *ampC*. *E. coli* S17-1 (*pro thi*; Simon, 1989) was used as auxotrophic donor for the trans-conjugation of mobilizable plasmids. *E. coli* FL01 (Lindberg & Normark, 1987) served as restriction-deficient host for plasmid construction.

The adjacent genes *ampR* and *ampC* on a 7.5 kb *EcoRI* fragment from *C. freundii* were obtained from the original cloning product, plasmid pNU302 (Lindberg *et al.*, 1985). Subcloned *ampR* and *ampC* on pNU305 (vector pBR322, Tc^R; Lindberg *et al.*, 1985) and pNU344 (vector pACYC184, Cm^R; Lindberg *et al.*, 1987) were used for transformation experiments into *P. mirabilis* and as references for β -lactamase expression in *E. coli*. The mobilizable plasmid pKT231 (Km^R Sm^R, Bagdasarian *et al.*, 1981) was the vector for the construction of plasmid pMD101 (*ampR ampC* Km^R). Its size was determined as 13.3 kb by restriction analysis. Plasmid pGKS273-3 (vector pACYC184, *ampG*, Cm^R) with cloned *ampG* from *E. coli* was kindly made available by Dr Gisela Korfmann (Bayer A. G., Leverkusen, Germany).

Growth media. LB broth and agar medium for *E. coli* was supplemented with 2 g glucose l⁻¹ and 1 mg thiamin l⁻¹ (Lindberg *et al.*, 1987). Minimal media were M9CA medium (Lindberg *et al.*, 1987) supplemented with 2 g Casamino acids (Difco) l⁻¹, 50 mg uracil l⁻¹, 50 mg L-tryptophan l⁻¹ and 1 mg thiamin l⁻¹ for *E. coli*, and the medium of Grabow & Smit (1967) for *P. mirabilis*. Growth of individual, non-swarming colonies of *P. mirabilis* was obtained on the NaCl-deficient, high-glucose agar medium of Naylor (1964). This was

modified to contain 10 g tryptone (Oxoid) l⁻¹, 5 g yeast extract (Oxoid) l⁻¹, 10 g glucose l⁻¹ and 20 g agar l⁻¹.

Bacteria and L-form protoplasts of *P. mirabilis* were grown in the modified L-form medium of Martin *et al.* (1975) containing (l⁻¹): 15 g beef extract Lab Lemco (Oxoid), 2 g glucose, 5 g NaCl and 4.5 g Na₂HPO₄ · 2H₂O. Agar medium for the L-form was supplemented with 100 ml horse serum (Oxoid) l⁻¹. L-form protoplasts for polyethylene glycol transformation were grown in modified LFS medium (Klessen *et al.*, 1989), containing (l⁻¹): 3 g beef extract Lab Lemco (Oxoid), 10 g Bacto Peptone (Difco), 5 g yeast extract (Oxoid), 5 g NaCl and 20 g sucrose.

The following β -lactam compounds were used: cephalothin and cephalixin (Eli Lilly), cefotaxime (Hoechst), aztreonam (Squibb), cefoxitin and imipenem (Merck, Sharp & Dohme), 6-amino penicillanic acid (Bayer) and nitrocefin (Glaxo).

DNA techniques. For the isolation of plasmid DNA, digestion with restriction endonucleases, separation of DNA fragments by agarose gel electrophoresis, and ligation of DNA fragments, standard procedures (Sambrook *et al.*, 1989) were used. Purification of plasmid DNA on a preparative scale was carried out with the Qiagen Plasmid Midi Kit (Qiagen) according to the manufacturer's instructions.

Construction of plasmid pMD101 (*ampR ampC* Km^R) from pKT231 (Km^R Sm^R). Cloning of *ampR* and *ampC* into the streptomycin marker of pKT231 and preservation of kanamycin resistance was necessary to allow the introduction of pMD101 and assay of *ampR* and *ampC* function in the streptomycin-resistant *E. coli* reference strains. From pNU302 (Lindberg *et al.*, 1985), the *C. freundii*-derived 7.5 kb fragment containing cloned *ampR* and *ampC* was excised by *EcoRI* endonuclease and purified. Vector pKT231 (13.3 kb) was cut by *EcoRI* at the unique *EcoRI* site in the promoter region essential for transcription of the streptomycin resistance gene (Bagdasarian *et al.*, 1981). The 7.5 kb *ampR* and *ampC*-containing fragment was ligated into the linearized vector. The ligation product was transformed into *E. coli* FL01, and colonies of transformants were grown on LB-agar medium containing 100 µg kanamycin ml⁻¹. Colonies containing the desired hybrid plasmid pMD101 (*ampR ampC* Km^R Sm^R) were identified by their inability to grow on replica plates of the original transformant colonies on LB-agar medium containing 100 µg streptomycin ml⁻¹. Plasmid pMD101 was isolated from hybrid-plasmid-containing bacteria and its size was determined as 20.8 kb by restriction analysis.

Plasmid transfer procedures. All plasmids were transformed into *E. coli* strains by the CaCl₂ technique (Mandel & Higa, 1970) or by the procedure of Hanahan (1983). Plasmids pKT231 (Km^R Sm^R) and pMD101 (*ampR ampC* Km^R) were transconjugated into cells and L-form protoplasts of *P. mirabilis* from the auxotrophic donor *E. coli* S17-1(pKT231) and S17-1(pMD101). For liquid mating, *P. mirabilis* VI and the donor *E. coli* strains were grown at 37 °C in shake cultures of 30 ml L-form medium to an OD₅₇₈ of 0.8. After 10-fold dilution of the donor *E. coli* cultures with sterile L-form medium, 2 ml of *P. mirabilis* VI culture and of dilute donor cultures were mixed and incubated at 37 °C for 2 h with slow shaking. Then, cells from 1.5 ml mating mixture were sedimented by centrifugation and freed from the L-form medium by two consecutive washings in 0.05 M-phosphate buffer, pH 7, and sedimentation. For the selection of transconjugants of *P. mirabilis* VI and elimination of auxotrophic *E. coli*, different dilutions of the resuspended sediment in phosphate buffer were plated on Grabow-Smit minimal agar medium containing 200 µg kanamycin ml⁻¹ and incubated for 2 d at 37 °C. Cells from the colonies obtained were restreaked twice on the same selective agar medium to isolate individual clones of transconjugants. The presence of the respective plasmids in the transconjugants was verified by standard plasmid analysis. No growth appeared on control platings of *E. coli* donor bacteria and of *P. mirabilis* VI on the kanamycin-minimal agar medium.

Transconjugation of pMD101 into LVI protoplasts was carried out by filter mating of donor *E. coli* S17-1(pMD101) and L-form protoplasts grown as described for liquid mating. A mixture of 0.25 ml donor and 1 ml protoplast cultures was partially sedimented. After removal of the clear supernatant the concentrated mating suspension was spread on a prewashed, sterilized and dried Millipore filter (50 mm diam., 0.22 μ m pore size). The filter was placed on the surface of an L-form agar plate and incubated for 4 h at 37 °C. L-form transconjugants were selected by transferring the mating filter into liquid L-form medium containing 100 μ g kanamycin ml⁻¹ and 200 μ g cycloserine ml⁻¹ for the elimination of the *E. coli* donor by inhibition of peptidoglycan synthesis. After incubation for 24 h at 30 °C, turbidity of the medium and microscopic observation of a protoplast population indicated L-form growth. Subsequent subculture in L-form medium with kanamycin and cycloserine, and then with 100 μ g kanamycin ml⁻¹ alone yielded cultures of uniform L-form protoplasts. Individual clones of LVI(pMD101) were then obtained by plating growing L-form protoplasts on kanamycin-L-form agar medium supplemented with horse serum. Single protoplast colonies were isolated after 2–5 d incubation at 30 °C, and pMD101 was reisolated from the L-form transconjugants.

Polyethylene glycol (PEG) transformation of plasmid pGKS273-3 (*ampG*, Cm^R) into L-form strain LVI(pMD101) was carried out according to Klessen *et al.* (1989), with modification. Transformants were selected by transfer of the PEG-transformation mixture to 30 ml L-form medium containing 20 μ g chloramphenicol ml⁻¹ and incubation at 37 °C with slow shaking. Growing L-form protoplasts appearing after 2 d were spread on L-form agar plates containing 20 μ g chloramphenicol ml⁻¹ and 100 μ g kanamycin ml⁻¹. Colonies of transformants LVI(pMD101 + pGKS273-3) were isolated after 5 d incubation at 30 °C.

β -Lactamase techniques. Induction of β -lactamase by β -lactam antibiotics, preparation of crude extracts of β -lactamase from sonicated bacterial cells, and spectrophotometric assays of β -lactamase activity were performed according to Lindberg *et al.* (1985, 1987). Hydrolysis of the substrates cephalaxin (200 μ M) by enzyme extracts from cells, and nitrocefin (100 μ M) by excreted β -lactamase in culture medium, was measured at wavelengths of 260 and 482 nm, respectively. One unit (U) of β -lactamase hydrolyses 1 μ mol of the respective substrate min⁻¹ in 50 mM-sodium phosphate buffer, pH 7.0, at 30 °C. β -Lactamase excreted from L-form protoplasts grown in 30 ml shake cultures to an OD₅₇₈ of 0.4, was measured in samples of clear growth medium after removal of the protoplasts by centrifugation for 15 min at 6000 g. β -Lactamase activities in U (ml medium)⁻¹ were compared with those in equal volumes of cultures of *P. mirabilis* cells adjusted to the same OD, after liberation of the enzyme by sonication of the cells.

Results and Discussion

Transconjugation of ampR and ampC from C. freundii on plasmid pMD101 into normal cells and L-form protoplasts of P. mirabilis

Initial attempts to introduce cloned *ampR* and *ampC* from *C. freundii* on available *E. coli* host-specific plasmids pNU305 and pNU344 (Lindberg *et al.*, 1985, 1987), into cells of *P. mirabilis* by conventional transformation or electroporation were unsuccessful. This finding agrees with previous reports on the failure or very low efficiency of plasmid transformation into *P. mirabilis*

by different techniques (Merrick *et al.*, 1987; Wirth *et al.*, 1989).

However, transconjugation of plasmid pKT231 (Km^R Sm^R) with extended host range for Gram-negative bacteria (Bagdasarian *et al.*, 1981) from the donor *E. coli* S17-1 containing mobilizing function (Simon, 1989) was routinely achieved by liquid mating into whole cells and by filter mating into L-form protoplasts of *P. mirabilis*. Therefore, *ampR* and *ampC* on pNU302 (Lindberg *et al.*, 1985) was re-cloned into pKT231 as described in Methods, to obtain plasmid pMD101 (*ampR ampC* Km^R 20.8 kb, Fig. 1).

Bacterial transconjugants of *P. mirabilis* VI(pMD101) were selected on kanamycin-containing minimal medium from mating mixtures with the auxotrophic donor *E. coli* S17-1(pMD101). L-form transconjugants could not be isolated in the same way because of their inability to grow on minimal medium. Instead, transconjugants of L-form protoplasts, LVI(pMD101), were obtained as sole survivors of filter mating mixtures in complex medium containing kanamycin plus cycloserine for the selective elimination of the *E. coli* S17-1(pMD101) donor.

Expression of ampC β -lactamase from pMD101 in bacterial and L-form transconjugants of P. mirabilis

The presence of an *ampC*-type gene has been demonstrated in *P. mirabilis* (Bergström *et al.*, 1983). However, weak and non-inducible expression of the chromosomal

Fig. 1. Mobilizable plasmid pMD101 (*ampR ampC* Km^R), containing cloned genes *ampR* and *ampC* on a 7.5 kb *EcoRI* chromosomal fragment from *C. freundii* (stippled segment), inserted into the unique *EcoRI* site of broad-host-range plasmid pKT231 (13.3 kb, Bagdasarian *et al.*, 1981; thin black segment). From the donor *E. coli* S17-1 (pMD101), plasmid pMD101 was transconjugated into bacteria and L-form protoplasts of *P. mirabilis* VI for assays of β -lactamase expression in these hosts.

β -lactamase has been reported in this organism (Smith, 1969). In agreement with this observation we found that neither cells of *P. mirabilis* VI nor protoplasts of the isogenic L-form strain LVI contained measurable β -lactamase activity, and induction of the enzyme to higher concentration also did not occur. Thus, expression of any heterologous ampC β -lactamase from cloned *ampR* and *ampC* of *C. freundii* was expected to be readily detectable here. Specific activities of constitutive and induced AmpC β -lactamase isolated from bacterial transconjugants of *P. mirabilis* and of transformants of *E. coli* reference strains are shown in Table 1. The regular function of *ampR* and *ampC* in pMD101 was demonstrated by the comparable constitutive and hyperinduced expression of β -lactamase from these genes in pMD101 and pNU305 in the ampD1 mutant *E. coli* SN0301 (Lindberg *et al.*, 1987). Also, typically moderate constitutive and 16-fold induced synthesis of β -lactamase occurred in the *ampD* wild-type strain *E. coli* HfrH (pMD101).

In the transconjugant *P. mirabilis* VI(pMD101) the enzyme was synthesized constitutively at low level, and was induced 105-fold by β -lactam antibiotic. Also, with the introduction of pMD101, the inhibitory concentrations of β -lactamase-labile cephalothin and of β -lactamase-stable cefotaxime increased from 2 and $< 2 \mu\text{g ml}^{-1}$ to > 64 and $16 \mu\text{g ml}^{-1}$, respectively, in *E. coli* SN0301, and from 16 and $< 0.25 \mu\text{g ml}^{-1}$ to > 128 and $1 \mu\text{g ml}^{-1}$, respectively, in *P. mirabilis* VI. Obviously, in *P. mirabilis* VI an induction sensor and equivalents of genes for other required functions previously identified in *E. coli*, *Ent. cloacae* and *C. freundii*, such as *ampG* and *ampD*, must be present and capable of interacting with *ampR* and *ampC* of *C. freundii*.

In transconjugants of L-form protoplasts LVI (pMD101), in the absence of an outer membrane and periplasmic space, the β -lactamase was excreted into the growth medium. Only negligible amounts of enzyme were found in the cytoplasm and membranes of the protoplasts (data not shown). Therefore, expression of β -lactamase from pMD101 in protoplasts and whole cells was compared by measuring enzyme activities in the growth medium produced by comparable amounts of bacteria and protoplasts (Table 2). In the L-form LVI(pMD101), AmpC β -lactamase was expressed constitutively, but there was no enzyme induction under conditions where the β -lactamase in the isogenic bacteria was induced 87-fold. The much lower enzyme activity in L-form cultures in the presence of inducer resulted from inactivation of the constitutive enzyme by the added cefoxitin, presumably by stable acylation. This was suggested by the immediate lowering of the activity of the constitutive β -lactamase when cefoxitin at the concentration used for induction was added to the L-

form medium after the protoplasts had been removed by sedimentation.

Non-inducibility of β -lactamase in the L-form is not due to defective ampG or the loss of penicillin-binding proteins

The non-inducibility of β -lactamase in the L-form could possibly have been caused by the spontaneous loss of an additional induction factor, such as the *ampG*-mediated signal transducer, or of a penicillin-binding protein serving as induction sensor. Therefore, cloned *ampG* from *E. coli* on plasmid pGKS273-3 (G. Korfmann, personal communication) which complements the non-inducibility of *ampG*-defective *E. coli* mutants (Lindquist *et al.*, 1993), was introduced into protoplasts of LVI(pMD101) by PEG transformation. However, these *ampG* transformants remained non-inducible (Table 2). Thus, there was no evidence of an *ampG* defect in L-form strain LVI. It is, of course, possible that *ampG* from *E. coli* is unable to complement in *P. mirabilis*.

Previous studies have also shown that all essential penicillin-binding proteins for peptidoglycan synthesis and most or all non-essential ones are present in different *Proteus* L-form strains (Martin *et al.*, 1980; Rousset *et al.*, 1982). If an induction signal interacting with *ampG* or its product could arise by direct, cell-wall-independent emission from an inducer-sensing penicillin-binding protein after the binding of inducing β -lactam, this should also be possible in the L-form.

Loss of constitutive overproduction of β -lactamase in the protoplast L-form

Because of the generally weak expression of AmpC β -lactamase in *P. mirabilis* a low level of enzyme induction in the L-form could possibly be overlooked. Therefore we attempted to detect inducibility with higher sensitivity in a protoplast L-form derived from β -lactamase-overproducing mutants of *P. mirabilis* VI, analogous to the *ampD*-defective mutants of *C. freundii* and *E. coli* (Lindberg *et al.*, 1987; Lindquist *et al.*, 1989b) and *Ent. cloacae* (Honoré *et al.*, 1989) with high semi-constitutive or hyperinducible β -lactamase.

Selection for resistance to cefotaxime ($2\text{--}16 \mu\text{g ml}^{-1}$) in the presence of kanamycin ($100 \mu\text{g ml}^{-1}$) was used to isolate β -lactamase-overproducing mutants of *P. mirabilis*(pMD101) (Lindberg *et al.*, 1987). Platings of 3×10^9 cells were made on high-glucose and NaCl-deficient agar medium with antibiotics on which the swarming of *P. mirabilis* was suppressed and separate colonies were formed. Among eight resistant isolates tested, two strains, *P. mirabilis* VI-R1(pMD101) and VI-R2-

Table 1. Constitutive and induced expression of AmpC β -lactamase from cloned *ampR* and *ampC* of *C. freundii* in *E. coli* and *P. mirabilis*

β -Lactam antibiotics with known inducing potency, 6-aminopenicillanic acid (Lindberg & Normark, 1987), and cefoxitin and imipenem (Martin *et al.*, 1988), were tested at different concentrations. Highest induction without simultaneous cell wall damage was obtained by 6-aminopenicillanic acid in *E. coli* and by cefoxitin in *P. mirabilis*. Induction in this experiment was with 250 μ g 6-aminopenicillanic acid ml^{-1} in *E. coli* and with 10 μ g cefoxitin ml^{-1} in *P. mirabilis*. Results of triplicate measurements with the substrate cephalixin are shown $\pm$ SD. n.m.a., No measurable activity.

Bacteria	Plasmid-borne <i>ampR</i> and <i>ampC</i>	β -Lactamase, sp. act. U (mg protein) $^{-1}$	
		Constitutive	Induced
<i>E. coli</i>			
SN0301 (<i>ampD1</i>)	—	n.m.a.	n.m.a.
SN0301(pNU305)	+	1.1 $\pm$ 0.1	14.8 $\pm$ 1.1
SN0301(pMD101)	+	2.0 $\pm$ 0.3	12.9 $\pm$ 1.4
HfrH	—	n.m.a.	n.m.a.
HfrH(pMD101)	+	0.16 $\pm$ 0.02	2.5 $\pm$ 0.2
<i>P. mirabilis</i>			
VI	—	n.m.a.	n.m.a.
VI(pKT231)	—	n.m.a.	n.m.a.
VI(pMD101)	+	0.02 $\pm$ 0.01	2.1 $\pm$ 0.1
VI-R1(pMD101)	+	8.8 $\pm$ 1.8	11.4 $\pm$ 1.2
VI-R2(pMD101)	+	6.9 $\pm$ 1.1	18.5 $\pm$ 1.5

Table 2. Difference in inducibility of AmpC β -lactamase from cloned *ampR* and *ampC* on pMD101 in normal bacteria and in L-form protoplasts of *P. mirabilis* VI

Induction was with 10 μ g cefoxitin ml^{-1} . Activity of β -lactamase excreted from the L-form protoplasts was determined in the growth medium. The activity of solubilized enzyme from a comparable quantity of bacteria was measured after sonication of the cells. Values of triplicate measurements with the substrate nitrocefin are shown $\pm$ SD.

Bacterium	$10^{-3} \times \beta$ -Lactamase activity (U ml^{-1})	
	Constitutive	Induced
<i>Bacterium</i>		
VI(pMD101)	5.1 $\pm$ 0.5	44.2 $\pm$ 3.5
<i>L-form</i>		
LVI(pMD101)	12.9 $\pm$ 1.8	2.4 $\pm$ 0.8
LVI(pMD101 + pGKS 273-3) (<i>ampG</i>)	13.4 $\pm$ 2	4 $\pm$ 1

(pMD101), surviving on 2 and 8 μ g cefotaxime ml^{-1} , respectively, showed highly elevated constitutive β -lactamase activity and limited further inducibility (Table 1). The minimal inhibitory concentration of cefotaxime for these strains was $> 32 \mu\text{g ml}^{-1}$, in contrast to < 0.25

and $1 \mu\text{g ml}^{-1}$ for *P. mirabilis* VI and VI(pMD101), respectively. Thus, *P. mirabilis* VI-R1(pMD101) and VI-R2(pMD101) resembled *E. coli* mutant SN0302 (*ampD2*) with high semi-constitutive β -lactamase expression from cloned *ampR* and *ampC* (Lindberg *et al.*, 1987; Lindquist *et al.*, 1989b). Therefore, the β -lactamase-overproducing strains of *P. mirabilis* VI were tentatively assumed to be defective in *ampD*. However, this remains to be proven by complementation when a wild-type *ampD*-bearing plasmid becomes available which can be introduced into *P. mirabilis* and is compatible with pMD101.

Conversion of *P. mirabilis* VI-R1(pMD101) and VI-R2(pMD101) to the irreversible, wall-less protoplast L-form was carried out in two steps of progressive disorganization of the cell wall, different from the traditional technique of long-term exposure to high concentrations of benzyl penicillin (Taubeneck, 1962a, b; Martin, 1983). First, the bacteria were converted to the reversible spheroplast L-form with residual but defective cell walls during growth on L-form agar medium containing the β -lactamase-stable monobactam antibiotic aztreonam at a concentration of $100 \mu\text{g ml}^{-1}$. The spheroplasts were then adapted to growth in the same liquid medium (Martin & Gmeiner, 1979; Martin, 1983; Huber & Martin, 1986). For the maintenance of plasmid pMD101 in the spheroplasts, $100 \mu\text{g}$ kanamycin ml^{-1} was present in all media.

In a second step, viable wall-less protoplasts were isolated from the spheroplast culture by complete inactivation of all essential penicillin-binding proteins and inhibition of peptidoglycan synthesis. This was achieved by growth in the presence of aztreonam with preferred binding to penicillin-binding protein 3 and the carbapenem antibiotic imipenem at a concentration of $125 \mu\text{g ml}^{-1}$, which simultaneously inactivates penicillin-binding proteins 1A, 1B and 2 (Huber, 1985; Huber & Martin, 1986).

After initial incubation for 5 d in liquid medium with both β -lactams and kanamycin, growth of L-form protoplasts was obtained which were subcultured repeatedly in the same medium. Upon transfer to β -lactam-free medium and subsequent subculture, growth in the L-form continued in all isolates, and no reversion to the bacterial form took place. This suggested that the stable protoplast L-form strains LVI-R1(pMD101) and LVI-R2(pMD101) had been obtained. Surprisingly, no constitutive or inducible β -lactamase activity could be detected in the growth medium or in the protoplasts of such L-form cultures derived from the β -lactamase-overproducing *P. mirabilis* mutants. Loss of plasmid pMD101 from the L-form was excluded as the cause of the absence of the enzyme. Plasmid DNA was isolated from protoplasts of LVI-R1(pMD101) and LVI-R2(pMD101) and transformed into *E. coli* SN0301 (*ampD1*) with

selection for kanamycin resistance. In the transformants β -lactamase was expressed in the typical hyperinducible fashion.

The total absence of β -lactamase in L-forms LVI-R1(pMD101) and LVI-R2(pMD101) remains unexplained. One may speculate that even constitutive expression of AmpC β -lactamase requires a signal at low level, and that its formation also involves low-molecular-mass components of the cytoplasmic or membrane-bound stages of the peptidoglycan biosynthetic pathway which persist in the cell-wall-less L-form. Conversion of *P. mirabilis* VI-R1 and VI-R2 into the L-form by total inhibition of the essential penicillin-binding proteins may have eliminated such required metabolites which have remained functional in the original, benzyl-penicillin-induced L-form strain LVI. As yet, no information on such differences in *P. mirabilis* L-forms is available. They may resemble the differences in the state of peptidoglycan precursor molecules and biosynthetic enzymes that have been characterized in different L-form strains of *Bacillus subtilis* and *Bacillus licheniformis* (Ward, 1975).

Assuming that β -lactamase overproduction in *P. mirabilis* mutants VI-R1 and VI-R2 are due to defective *ampD*, the observed loss of β -lactamase expression and overproduction in the protoplast L-form descendants of VI-R1 and VI-R2 also agrees with the concept of a 'late' function of *ampD* depending on the arrival of a peptidoglycan-derived induction signal, as suggested in the model of Lindberg *et al.* (1988).

Proposal of a general, peptidoglycan-related mechanism of β -lactamase induction

The reported results support the notion that induction in Gram-negative bacteria is an indirect process dependant on the metabolism of the cell wall. From the inhibition of synthesis of cell-wall peptidoglycan by β -lactam antibiotics, one or several derouted and possibly degraded precursors or breakdown products may arise and these may function as secondary signal (autoinducer, Lindberg *et al.*, 1988) for β -lactamase induction. Obviously, this possibility does not exist in the wall-less L-form protoplasts. It remains possible, of course, that non-inducibility in the L-form is connected with the absence of a still unknown induction sensor or other essential factor that has been lost together with the outer membrane and periplasm.

However, the concept that induction of β -lactamase is initiated by disturbance of peptidoglycan synthesis is also supported by recent observations of 'unspecific' β -lactamase induction by glycine and D-amino acids in *Ent. cloacae* (Gatus *et al.*, 1986; Ottolenghi, 1993) and in hyperinducible *E. coli* SN0301 (*ampDI*)(pNU305) carrying cloned *ampR* and *ampC* from *C. freundii* (Martin &

Schmidt, 1993). Most importantly, it has been shown that β -lactamase was equally non-inducible by β -lactam and by glycine or D-methionine in the *ampG*-defective mutant *E. coli* SN0301-1(pNU305) (Schmidt, 1991; Martin & Schmidt, 1993). Thus, induction by both types of inhibitors of peptidoglycan synthesis depends on the function of the *ampG*-encoded signal transducer.

Glycine and D-amino acids have been extensively characterized as inhibitors of peptidoglycan synthesis (Hammes *et al.*, 1973; Trippen *et al.*, 1976). The mechanism of this inhibition, viz. competitive interference with the construction of peptide side chains of the peptidoglycan, differs drastically from the action of β -lactam antibiotics. Moreover, no mechanism is known by which glycine and D-amino acids or a product of their inhibitory action could interact with an induction-sensing penicillin-binding protein.

The available data make it attractive to assume a general pathway of β -lactamase induction as a response to altered conditions of peptidoglycan synthesis in Gram-negative bacteria. Presumably, in spite of their different mechanisms, the different inhibitors with the simultaneous function as β -lactamase inducers cause the formation of certain peptidoglycan catabolites of sufficiently similar structure and potential for interaction with *ampG* or its product. A requirement for such specifically structured induction signals may also explain the high inducing capacity of certain β -lactams, such as cefoxitin and other cephamycins, and imipenem, in contrast to low induction by other, structurally different β -lactams (Gootz & Sanders, 1983; Martin *et al.*, 1988). The selective affinities of the effectively inducing β -lactams for penicillin-binding proteins and the resulting specific peptidoglycan damage may lead to the most efficient production of an induction signal.

This work was supported by the Deutsche Forschungsgemeinschaft.

References

- BAGDASARIAN, M., LURZ, R., RÜCKERT, B., FRANKLIN, F. C. H., BAGDASARIAN, M. M., FREY, J. & TIMMIS, K. N. (1981). Specific purpose plasmid cloning vectors II. Broad host range, high copy number, RSF1010-derived vectors, and a host vector system for gene cloning in *Pseudomonas*. *Gene* **16**, 237-247.
- BERGSTRÖM, S., LINDBERG, F., OLSON, O. & NORMARK, S. (1983). Comparison of the overlapping *frd* and *ampC* operons of *Escherichia coli* with the corresponding DNA sequences in other Gram-negative bacteria. *Journal of Bacteriology* **155**, 1297-1305.
- EVERETT, M. J., CHOPRA, I. & BENNETT, P. M. (1990). Induction of the *Citrobacter freundii* Group I β -lactamase in *Escherichia coli* is not dependent on entry of β -lactam into the cytoplasm. *Antimicrobial Agents and Chemotherapy* **34**, 2429-2430.
- GATUS, B. J., BELL, S. M. & JIMENEZ, A. S. (1986). Comparison of glycine enhancement with cefoxitin induction of β -lactamase production in *Enterobacter cloacae* ATCC 13047. *Journal of Antimicrobial Chemotherapy* **21**, 163-170.
- GOOTZ, T. D. & SANDERS, C. C. (1983). Characterization of β -lactamase

- induction in *Enterobacter cloacae*. *Antimicrobial Agents and Chemotherapy* **23**, 91–97.
- GRABOW, W. O. K. & SMIT, J. A. (1967). Methionine synthesis in *Proteus mirabilis*. *Journal of General Microbiology* **46**, 47–57.
- HAMMES, W., SCHLEIFER, K. H. & KANDLER, O. (1973). Mode of action of glycine on the biosynthesis of peptidoglycan. *Journal of Bacteriology* **116**, 1029–1053.
- HANAHAN, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* **166**, 557–580.
- HOFSCHNEIDER, P. H. & MARTIN, H. H. (1968). Diversity of surface layers in L-forms of *Proteus mirabilis*. *Journal of General Microbiology* **51**, 23–33.
- HONORÉ, N., NICOLAS, M. H. & COLE, S. T. (1986). Inducible cephalosporinase production in clinical isolates of *Enterobacter cloacae* is controlled by a regulatory gene that has been deleted from *Escherichia coli*. *EMBO Journal* **5**, 3709–3714.
- HONORÉ, N., NICOLAS, M. H. & COLE, S. T. (1989). Regulation of enterobacterial cephalosporinase production: the role of a membrane-bound sensory transducer. *Molecular Microbiology* **3**, 1121–1130.
- HUBER, K. (1985). *Quantitative Bewertung der Funktion von Penicillinbindeproteinen an der Sphäroplasten-L-Form von Proteus mirabilis nach Einwirkung verschiedener Klassen von β -Laktam-Antibiotika*. PhD thesis, Technische Hochschule Darmstadt, Germany.
- HUBER, K. & MARTIN, H. H. (1986). State of peptidoglycan in spheroplasts of *Proteus mirabilis* grown in the presence of different β -lactam antibiotics. In *Biological Properties of Peptidoglycan*, pp. 187–190. Edited by P. H. Seidl & K. H. Schleifer. Berlin, New York: Walter de Gruyter.
- JAURIN, B. & GRUNDSTRÖM, T. (1981). ampC cephalosporinase of *Escherichia coli* K-12 has a different evolutionary origin from that of β -lactamases of the penicillinase type. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 4897–4901.
- KLESSEN, C., SCHMIDT, K. H., GUMPERT, J., GROSSE, H. H. & MALKE, H. (1989). Complete secretion of activable bovine prochymosin by genetically engineered L-forms of *Proteus mirabilis*. *Applied and Environmental Microbiology* **55**, 1009–1015.
- KORFMANN, G. & SANDERS, C. C. (1989). ampG is essential for high level expression of AmpC β -lactamase in *Enterobacter cloacae*. *Antimicrobial Agents and Chemotherapy* **33**, 1946–1951.
- KROLL, H. P., GMEINER, J. & MARTIN, H. H. (1980). Membranes of the protoplast L-form of *Proteus mirabilis*. *Archives of Microbiology* **127**, 223–229.
- LINDBERG, F. & NORMARK, S. (1987). Common mechanism of ampC β -lactamase induction in enterobacteria: regulation of the cloned *Enterobacter cloacae* P99 β -lactamase gene. *Journal of Bacteriology* **169**, 758–763.
- LINDBERG, F., WESTMAN, L. & NORMARK, S. (1985). Regulatory components in *Citrobacter freundii* ampC β -lactamase induction. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 4620–4624.
- LINDBERG, F., LINDQUIST, S. & NORMARK, S. (1987). Inactivation of the ampD gene causes semiconstitutive overproduction of the inducible *Citrobacter freundii* β -lactamase. *Journal of Bacteriology* **169**, 1923–1928.
- LINDBERG, F., LINDQUIST, S. & NORMARK, S. (1988). Genetic basis of induction and overproduction of chromosomal class I β -lactamase in non-fastidious Gram-negative bacilli. *Reviews of Infectious Diseases* **10**, 782–785.
- LINDQUIST, S., LINDBERG, F. & NORMARK, S. (1980a). Binding of the *Citrobacter freundii* AmpR regulator to a single DNA site provides both autoregulation and activation of the inducible ampC β -lactamase gene. *Journal of Bacteriology* **171**, 3746–3753.
- LINDQUIST, S., GALLANI, M., LINDBERG, F. & NORMARK, S. (1989b). Signalling proteins in enterobacterial AmpC β -lactamase regulation. *Molecular Microbiology* **3**, 1091–1102.
- LINDQUIST, S., WESTON-HAFER, K., SCHMIDT, H., PIEL, C., KORFMANN, G., ERICKSON, J., SANDERS, C., MARTIN, H. H. & NORMARK, S. (1993). AmpG, a signal transducer in chromosomal β -lactamase induction. *Molecular Microbiology* **9**, 703–715.
- LOW, B. K. (1973). Rapid mapping of conditional and auxotrophic mutants in *Escherichia coli* K12. *Journal of Bacteriology* **113**, 798–812.
- MANDEL, M. & HIGA, A. (1970). Calcium-dependent bacteriophage DNA infection. *Journal of Molecular Biology* **53**, 159–162.
- MARTIN, H. H. (1964). Composition of the mucopolymers in the cell walls of the unstable and stable L-forms of *Proteus mirabilis*. *Journal of General Microbiology* **36**, 441–450.
- MARTIN, H. H. (1983). Protoplasts and spheroplasts of Gram-negative bacteria, with special emphasis on *Proteus mirabilis*. In *Protoplasts 1983, Lecture Proceedings*, Experientia Supplementum, vol. 46, pp. 213–225. Edited by I. Potrykus, C. T. Harms, A. Hinnen, R. Hütter, P. J. King & R. D. Shillito. Basel: Birkhäuser Verlag.
- MARTIN, H. H. & GMEINER, J. (1979). Modification of peptidoglycan structure by penicillin action in cell walls of *Proteus mirabilis*. *European Journal of Biochemistry* **95**, 487–495.
- MARTIN, H. H. & SCHMIDT, H. (1993). Disturbance of peptidoglycan synthesis by glycine and D-methionine creates a signal for the ampG-mediated induction of AmpC- β -lactamase in *Escherichia coli*. In *Bacterial Growth and Lysis – Metabolism and Structure of the Bacterial Sacculus*, pp. 341–346. Edited by M. A. de Pedro, J. V. Hölte & W. Löffelhardt. New York: Plenum Publishing Co.
- MARTIN, H. H., MASKOS, C. & BURGER, R. (1975). D-Alanyl-D-alanine carboxypeptidase in the bacterial form and L-form of *Proteus mirabilis*. *European Journal of Biochemistry* **55**, 465–473.
- MARTIN, H. H., SCHILF, W. & SCHIEFER, H. G. (1980). Differentiation of Mycoplasma-like forms from bacterial protoplast L-forms by assay for penicillin-binding proteins. *Archives of Microbiology* **127**, 297–299.
- MARTIN, H. H., SCHMIDT, B., BRÄUTIGAM, S., NOGUCHI, H. & MATSUHASHI, M. (1988). Initiation of induction of chromosomal β -lactamase by binding of inducing β -lactam antibiotics to low molecular weight penicillin-binding proteins. In *Antibiotic Inhibition of Bacterial Cell Surface and Assembly*, pp. 494–501. Edited by P. Actor, L. Daneo-Moore, M. L. Higgins, M. R. J. Salton & G. D. Shockman. Washington, DC: American Society for Microbiology.
- MERRICK, M. J., GIBBINS, J. R. & POSTGATE, J. R. (1987). A rapid and efficient method for plasmid transformation of *Klebsiella pneumoniae* and *Escherichia coli*. *Journal of General Microbiology* **133**, 2053–2057.
- NAYLOR, P. G. D. (1964). The effect of electrolytes or carbohydrates in a sodium chloride deficient medium on the formation of discrete colonies of *Proteus* and the influence of these substances on growth in liquid culture. *Journal of Applied Bacteriology* **27**, 422–431.
- OTTOLENGHI, A. C. (1993). In *Enterobacter cloacae* alterations induced by glycine and D-amino acids in the composition and structure of peptidoglycan are accompanied by induction of chromosomal β -lactamase. A model involving fisZ and septation. In *Bacterial Growth and Lysis – Metabolism and Structure of the Bacterial Sacculus*, pp. 347–354. Edited by M. A. de Pedro, J. V. Hölte & W. Löffelhardt. New York: Plenum Publishing Co.
- ROUSSET, A., NGUYEN-DISTECHE, M., MINCK, R. & GHUYSEN, J. M. (1982). Penicillin-binding proteins and carboxypeptidase/transpeptidase activities in *Proteus vulgaris* P18 and its penicillin-induced stable L-forms. *Journal of Bacteriology* **152**, 1042–1048.
- SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- SCHMIDT, H. (1991). *Defektmutationen im Schlüsselen ampG der β -Laktamase-Induktion bei Escherichia coli – Kartierung, Klonierung und Sequenzierung von Mutantengen*. PhD thesis, Technische Hochschule Darmstadt, Germany.
- SIMON, R. (1989). High frequency mobilization of Gram-negative bacterial replicons by the *in vitro* constructed Tn5-Mob transposon. *Molecular and General Genetics* **196**, 413–420.
- SMITH, J. T. (1969). R-factor gene expression in Gram-negative bacteria. *Journal of General Microbiology* **55**, 109–120.
- SYKES, R. B. & SMITH, J. T. (1979). Biochemical aspects of β -lactamases from Gram-negative organisms. In *Beta-Lactamases* pp. 369–401. Edited by J. M. T. Hamilton-Miller & J. T. Smith. London: Academic Press.
- TAUBENECK, U. (1962a). Untersuchungen über die L-Form von *Proteus mirabilis* Hauser. I. Die penicillin-induzierten large bodies (Sphäro-

- plasten) und ihre Bedeutung für die Entstehung der L-Form. *Zeitschrift für Allgemeine Mikrobiologie* **2**, 56–76.
- TAUBENECK, U. (1962*b*). Untersuchungen über die L-Form von *Proteus mirabilis* Hauser. II. Entwicklung und Wesen der L-Form. *Zeitschrift für Allgemeine Mikrobiologie* **2**, 132–156.
- TRIPPEN, B., HAMMES, W., SCHLEIFER, K. H. & KANDLER, O. (1976). Die Wirkung von D-Aminosäuren auf die Struktur und Biosynthese des Peptidoglycans. *Archives of Microbiology* **109**, 247–261.
- TUOMANEN, E., LINDQUIST, S., SANDE, M., GALLEN, M., LIGHT, K., GAGE, D. & NORMARK, S. (1991). Coordinate regulation of β -lactamase induction and peptidoglycan composition by the *amp* operon. *Science* **251**, 201–204.
- WARD, J. B. (1975). Peptidoglycan synthesis in L-phase variants of *Bacillus licheniformis* and *Bacillus subtilis*. *Journal of Bacteriology* **124**, 668–678.
- WIRTH, R., FRIESENEGGER, A. & FIEDLER, S. (1989). Transformation of various species of Gram-negative bacteria belonging to 11 different genera by electroporation. *Molecular and General Genetics* **216**, 175–177.