Nonspecific Induction of β -lactamase in *Enterobacter cloacae*

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Induction of β -lactamase was monitored in a strain of *Enterobacter cloacae* exhibiting high resistance to most β -lactam antibiotics. Large amounts of the enzyme were induced not only in the presence of β -lactams, but also in the presence of other bicyclic molecules such as folic acid, thiamin, tryptophan or haemin. Moreover, complex media (such as Trypticase soy broth and Schaedler's broth) and various body fluids (serum, pleural fluid and cerebrospinal fluid) also possessed considerable induction potency. Neither 'specific' induction (by β -lactams) nor 'non-specific' induction (by other bicyclic compounds) could be augmented by addition of exogenous cAMP. These findings indicate that inducible β -lactamases deserve more attention, above all with respect to the development of resistance against third-generation cephalosporins.

INTRODUCTION

Since the introduction of the newer β -lactam antibiotics, chromosomally mediated, inducible β -lactamases have become increasingly important (Sanders, 1983). It is well established that most β -lactam antibiotics appear to be affected by these enzymes either by hydrolysis or by the non-hydrolytic barrier mechanism (Gootz & Sanders, 1983; Sanders, 1983). In some cases, these enzymes are produced constitutively; however, induction generally occurs (Gootz & Sanders, 1983). Large amounts of enzyme are often produced by strains belonging to the genera *Enterobacter*, *Serratia* or *Citrobacter*, thus leading to a high degree of resistance against a wide variety of β -lactam antibiotics. As these strains sometimes produce several enzymes, it is of major interest to investigate which structural features of β -lactam antibiotics may be responsible for enzyme induction, and also whether induction potency can be attributed exclusively to the β -lactam nucleus. There are several reports in the literature that media or body fluids influence β -lactamase induction (Bondi *et al.*, 1954; Gootz & Sanders, 1983). Recently, it was observed that the expression of β -lactamase was more markedly enhanced in Trypticase soy broth than in Mueller–Hinton broth (Gootz & Sanders, 1983). Moreover, it could be demonstrated that in animals (granuloma pouch model) large amounts of β -lactamase were produced – even in those animals that were not treated with β -lactam antibiotics (Dalhoff, 1982). This was why we investigated whether β -lactamase induction is restricted to β -lactam compounds or whether other cyclic compounds also exhibit induction potency.

A preliminary report of some of our findings has already been presented (Cullmann *et al.*, 1983a).

METHODS

Strain. β -Lactamase induction was studied in an *Enterobacter cloacae* strain (966/81) recently isolated from a clinical specimen. The strain was identified using the API 20 system (API-Bio Mérieux, Nürtingen, FRG).

Determination of minimal inhibitory concentrations. Antibiotic sensitivity was measured by the microdilution procedure with twofold serial dilutions. An inoculum of 5×10^5 bacterial ml⁻¹ was used in all experiments. All minimal inhibitory concentrations were determined in Mueller–Hinton broth (Merck).

Monitoring of β -lactamase induction. Isosensitest broth (Oxoid) was used for all induction experiments. A minimal medium was prepared as described elsewhere (Gootz & Sanders, 1983) containing K_2HPO_4 (14 g l^{-1}), KH_2PO_4 (6 g l^{-1}), $NH_4CO_2CH_3$ (2 g l^{-1}), $MgSO_4$ (anhydrous) (0.2 g l^{-1}) and glucose (10 g l^{-1}); the pH was adjusted to 7.0. Mueller–Hinton broth (Merck), Trypticase soy broth, Brain–heart infusion broth and Schaedler's broth (all from Oxoid) were prepared according to the manufacturer's instructions. One batch of each was used throughout the experiments. For the study of enzyme induction an overnight culture of strain 966/81 was diluted 100-fold into Isosensitest broth containing the inducer and incubated at 37°C with shaking (120 r.p.m.).

Enzyme induction was monitored either for 24 h (samples were taken after 2, 3, 4, 6, 8, 10, 12, 16, and 24 h) or for 4 h (samples were taken after 90, 150, and 240 min); in the latter experiments, each assay series was run with inducer concentrations ranging from 20 to 500 mg ml^{-1} ; control assays without inducer were included in each series. Cells were harvested at the above-mentioned time intervals by centrifugation at 5000 g for 10 min (Christ Minifuge, Osterode, FRG) and washed once with $NaCl$ (9 g l^{-1}). The cells were resuspended in a corresponding volume of distilled water and subjected to ultrasonic disruption (Branson Sonic Power-Ultrasonics, Plainview, NY, USA) for 2 min at 4°C . Cell debris was removed by centrifugation at 5000 g for 15 min at 4°C . The cleared sonic extracts were immediately frozen at -20°C and analysed within 10 d. The effect on enzyme induction of added cAMP (5 mmol l^{-1}) was concurrently studied in cells grown either in Isosensitest medium containing 10 mg cefoxitin l^{-1} or in Schaedler's broth without drug.

β -Lactamase assay. The enzyme activity of cleared sonic extracts was determined by a spectrophotometric assay. For this purpose the chromogenic cephalosporin compound Centa was employed; it is less affected by pH and the protein matrix than nitrocefin (Jones *et al.*, 1982; O'Callaghan *et al.*, 1972). Increase in absorbance was monitored at 412 nm for 2 min. All experiments were carried out in 0.05 M -triethanolamine buffer adjusted to pH 7.6 with a final substrate concentration of $3.3 \times 10^{-4}\text{ M}$ at 37°C . Total protein content of the cell-free supernatants was determined by the method of Markwell *et al.* (1978). Enzyme activity was expressed in units (mg protein^{-1}); one enzyme unit is defined as the amount of enzyme hydrolysing $1\text{ }\mu\text{mol}$ substrate min^{-1} .

Isoelectric focusing was performed as previously described (Cullmann *et al.*, 1982). Gel sheets containing ampholine pH 3.5–9.5 were used (LKB). Enzyme activity was detected with the chromogenic cephalosporin nitrocefin because of its marked colour change (O'Callaghan *et al.*, 1972). In these experiments the strain was grown for 4 h in the presence of the inducers.

Reagents. The antibiotics used in this study were obtained from the following sources: cefoxitin and *N*-formimidoylthienamycin (imepenem) from Merck, Sharp & Dohme (Munich, FRG); moxalactam (latamoxef) from Eli Lilly (Giessen, FRG); mezlocillin from Bayer (Leverkusen, FRG); azthreonam (aztreonam) from Squibb–Heydn (Munich, FRG); clavulanic acid from Beecham–Wuelfing (Neuss, FRG); the penem compound Sch 29 482 from Byk–Essex (Munich, FRG); fusidic acid from Thomae Pharm. (Biberach a.d. Riss, FRG); 6-aminopenicillanic acid from Fluka (Buchs, Switzerland). The chromogenic cephalosporins were kindly supplied by Hoechst, Frankfurt, FRG (Centa) and by Cascan–Glaxo, Wiesbaden, FRG (nitrocefin).

cAMP was from Boehringer, and all other reagents, of analytical grade, were from Sigma.

RESULTS

Susceptibility of *E. cloacae* strain 966/81 to antimicrobial compounds. The following minimal inhibitory concentrations were obtained: 256 mg l^{-1} for cefoxitin; 128 mg l^{-1} for 6-aminopenicillanic acid and mezlocillin; 64 mg l^{-1} for moxalactam, azthreonam and clavulanic acid; 2 mg l^{-1} for the penem Sch 29 482; and 1 mg l^{-1} for *N*-formimidoylthienamycin.

Influence of various media on β -lactamase induction. To discover whether various media influenced enzyme inductions, the following were investigated: Isosensitest, Mueller–Hinton broth, Trypticase soy broth, Brain–heart infusion broth, Schaedler's broth, and the minimal medium described in Methods (Fig. 1). In all these experiments no β -lactam antibiotics were added. Schaedler's broth had by far the greatest induction potency, exceeding that of Trypticase soy broth, Mueller–Hinton broth, and Brain–heart infusion broth. On the other hand, the induction potency of Isosensitest broth was only marginal as compared to the minimal medium, in which good growth also occurred (Fig. 1). A repeat of the experiment three months later revealed negligible discrepancies between the two assay series: β -lactamase levels were reproducible with about 5% error. Isoelectric focusing confirmed the above findings (see Fig. 4b). The increase of induction potency was associated with the appearance of further bands. The poorly visible bands were probably 'satellite bands' (Matthew *et al.*, 1975). At present this phenomenon cannot be entirely explained (Simpson & Plested, 1983).

Specific induction of β -lactamases by various β -lactam compounds. In order to compare enzyme induction by the media with that of β -lactam antibiotics, eight different agents were evaluated.

Fig. 1. 'Nonspecific' induction of β -lactamase in *E. cloacae* strain 966/81 by culture media. Enzyme activity was quantified with the chromogenic cephalosporin Centa. ●, Schaedler's broth; □, Trypticase soy broth; ×, Mueller-Hinton broth; ■, Brain-heart infusion broth; ○, Isosensitest broth; ▲, minimal medium of Gootz & Sanders (1983) (control).

Fig. 2. Induction of β -lactamase in *E. cloacae* strain 966/81 by the addition of various β -lactam compounds. All experiments were carried out in Isosensitest medium. (a) ○, 6-Aminopenicillanic acid (10 mg l^{-1}); ■, moxalactam (10 mg l^{-1}); □, cefoxitin (10 mg l^{-1}); ●, mezlocillin (10 mg l^{-1}). (b) ◇, Penem Sch 29 482 (0.5 mg l^{-1}); ◆, *N*-formimidoylthienamycin (0.5 mg l^{-1}); ▲, clavulanic acid (10 mg l^{-1}); △, azthreonam (10 mg l^{-1}).

Usually the compound was added at a concentration of 10 mg l^{-1} to the Isosensitest medium; 0.5 mg l^{-1} was added in the case of *N*-formimidoylthienamycin and the penem compound Sch 29 482. 6-Aminopenicillanic acid, the oxacephem compound moxalactam, and the penem Sch 29 482 exceeded by about twofold, and clavulanic acid equalled, the induction potency of Schaedler's broth (Fig. 2*a, b*). Cefoxitin, the monobactam azthreonam and mezlocillin were poorer inducers. In the case of *N*-formimidoylthienamycin, a rapid increase followed by a strong decrease of enzyme activity was observed (Fig. 2*b*). Isoelectric focusing revealed enzyme patterns confirming the above findings (see Fig. 4*a*). Assays at different time intervals exhibited similar enzyme patterns.

Induction potency of various cyclic compounds. To establish which structural features might be responsible for 'nonspecific' induction of β -lactamase in *E. cloacae* strain 966/81, a variety of cyclic compounds was screened (Table 1). Tryptophan, folic acid, thiamin and haemin were inducers, whereas other amino acids such as phenylalanine or histidine were not. All inducers

Fig. 3. Induction of β -lactamase in *E. cloacae* strain 966/81 in the presence of various body fluids, each at 25% (v/v). ●, Heat-inactivated serum; ■, heat-inactivated pleural fluid; △, cerebrospinal fluid; ○, control assay in Isosensitest broth. β -Lactamase was quantified with the chromogenic compound Centa.

Table 1. Nonspecific induction of β -lactamase in *Enterobacter cloacae* strain 966/81

Inducer	Concn (mg l ⁻¹)	β -Lactamase activity relative to control assay (= 1.0)		
		after 90 min	after 150 min	after 240 min
Tryptophan	20	1.19	1.06	0.92
	100	1.12	1.43	1.96
	500	1.01	1.57	3.77
Indol-3-carbinol	500	1.15	0.38	0.31
Indican	500	1.02	1.03	0.61
Phenylalanine	500	1.50	1.11	1.02
Histidine	500	1.78	1.05	1.08
Adenine	500	1.27	1.03	0.91
Riboflavin	100	0.28	0.25	0.10
Thiamin	20	0.96	0.93	1.60
	100	1.12	1.15	2.56
	500	1.10	1.25	4.90
Folic acid	20	0.94	0.93	1.80
	100	1.00	1.10	3.13
	500	1.27	1.30	3.70
Haemin	20	0.97	1.10	2.95
	100	1.00	1.10	3.20
2-Aminothiazole	100	1.08	1.11	0.99
Thiophene	500	1.06	0.93	0.96
Piperazine	100	1.04	0.96	1.03
Menadione	25	1.04	0.93	0.96
Cortisone	100	0.85	0.68	0.73
17 α -Hydroxyprogesterone	100	0.73	1.16	1.37
β -Oestradiol	100	0.73	0.88	1.59
Cholecalciferol (D ₃)	100	1.04	0.95	1.40
Fusidic acid	100	0.81	0.58	0.49

showed a good correlation between their concentration and their induction potency. Addition of riboflavin and fusidic acid even resulted in a decrease of enzyme activity (Table 1). Cyclic molecules (such as thiophene, 2-aminothiazole, piperazine), which are often part of side-chains of β -lactams, revealed no induction potency. Although tryptophan was an inducer, its analogues indole-3-carbinol and indican were not (Table 1).

Enzyme induction by various body fluids. From the above-mentioned findings it seemed likely that body fluids may induce β -lactamases. When 25% (v/v) of heat-inactivated serum or pleural

Fig. 4. Isoelectric focusing of inducible β -lactamase in *E. cloacae* 966/81. (a) The following compounds served as inducers: 1, mezlocillin (10 mg l^{-1}); 2, 6-aminopenicillanic acid (10 mg l^{-1}); 3, cefoxitin (10 mg l^{-1}); 4, moxalactam (10 mg l^{-1}); 5, clavulanic acid (10 mg l^{-1}); 6, azthreonam (10 mg l^{-1}); 7, penem Sch 29 482 (0.5 mg l^{-1}); 8, *N*-formimidoylthienamycin (0.5 mg l^{-1}).

(b) The following media served as inducers: 1, minimal medium; 2, Mueller-Hinton broth; 3, Brain-heart infusion broth; 4, Schaedler's broth; 5, Isosensitest broth; 6, Trypticase soy broth.

(c) Induction occurred in the presence of the following body fluids (25%, v/v): 1, serum; 2, pleural fluid; 3, cerebrospinal fluid; 4, urine.

fluid was added to the Isosensitest medium, enzyme activities comparable to those obtained with cefoxitin as the inducer resulted (Fig. 3). Addition of cerebrospinal fluid resulted in somewhat lower enzyme activity (Fig. 3), as did addition of urine (data was not shown). The results of isoelectric focusing again corresponded well with these findings (Fig. 4).

Effect of cAMP on enzyme induction. In many cases enzyme induction is subject to catabolite repression. Thus we evaluated the effect of added cAMP (5 mmol l^{-1}) on β -lactamase induction. The enzyme was induced either by addition of cefoxitin (10 mg l^{-1}) or by further growth in Schaedler's broth. In both experiments, cAMP did not affect β -lactamase induction (data not shown).

DISCUSSION

To the best of our knowledge, 'nonspecific' induction of β -lactamase in *E. cloacae* has not been described in the literature by other workers. Some 20 years ago, there was a report that considerable penicillinase activity could be detected in kidney homogenates of *Staphylococcus aureus*-infected mice, which had not received penicillin (Dineen, 1961). The relevance of the media employed was pointed out as early as 1954 (Bondi *et al.*, 1954). These authors observed that *S. aureus* penicillinase was best expressed either in a casein hydrolysate or in an amino acid medium containing cyclic amino acids. However, they did not assume an induction, but an inhibition of enzyme production by aliphatic amino acids. Moreover, high concentrations of bacitracin were shown to induce β -lactamase synthesis in *Bacillus cereus* 569 (Citri, 1978).

Recently, marked discrepancies in β -lactamase production depending on the nutrient medium employed were observed for *E. cloacae* (Gootz & Sanders, 1983). Cefoxitin (a cephamycin derivative) proved to be a more potent inducer in Trypticase soy broth than in Mueller-Hinton broth. In most cases the inducing effect appears transient; this has to be ascribed to the stability of the β -lactam compound against breakdown by the induced enzyme (Minami *et al.*, 1980; Gootz & Sanders, 1983) or to the metabolic removal of the inducer

(tryptophan). The failure of cAMP to influence enzyme induction in *E. cloacae* had already been reported (Gootz & Sanders, 1983).

In order to identify the molecules responsible for 'nonspecific' induction, approximately 20 cyclic molecules were screened. It can be concluded that cyclic molecules exhibiting induction potency have some structural relationship with the β -lactam nucleus, i.e. they are bicyclic, whereas monocyclic compounds lacked induction potency. Loss of the aliphatic residue of tryptophan (in indole-3-carbinol and indican) was linked with the loss of induction potency. This observation may explain the ineffectiveness of purine derivatives in β -lactamase induction. Thus, the induction of β -lactamase by the media employed or by body fluids can be explained. On the other hand, addition of fusidic acid or riboflavin even resulted in the loss of enzyme activity. For fusidic acid an interaction of the enzyme with the drug can be assumed – consistent with the observation that fusidic acid was found to inhibit *S. aureus* penicillinase (Godtfredsen & Jahnsen, 1969). At present there is no report indicating an interaction of riboflavin with β -lactamases.

In a study on 78 ampicillin-resistant clinical isolates of *E. cloacae*, *Serratia marcescens* and *Citrobacter freundii*, 'nonspecific' induction of large amounts of β -lactamase could be detected in 24 isolates associated with a marked increase of resistance against most β -lactam compounds (Cullmann *et al.*, 1983*b*). With respect to *in vivo* conditions, recently published observations deserve attention: production of large amounts of β -lactamase could be detected in *Morganella morganii* independently of the administration of β -lactam antibiotics (Dalhoff, 1982). As the bacteria grown in haemorrhagic exudate were directly examined without subculture, it can be assumed that β -lactamase was produced *in vivo*.

At present many questions remain open, e.g. there is no information available as to whether chromosomally mediated enzymes interact with other bicyclic molecules than β -lactams (such as the inducers tryptophan, thiamin, etc.).

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