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# *Klebsiella pneumonia* strains moderately resistant to ampicillin and carbenicillin: characterization of a new β-lactamase

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*Klebsiella pneumoniae* strain 11-03, moderately resistant to ampicillin and carbenicillin, produces one constitutive β-lactamase with an isoelectric point of 7·10 and a molecular weight of 20,000 ± 500. The enzymatic activity is directed primarily against the penicillins, ampicillin being the best substrate. Some cephalosporins are also hydrolyzed to some extent but the affinity of the enzyme for these antibiotics is low (high Km values). It has not been possible to determine whether the biogenesis of this β-lactamase is mediated by the bacterial chromosome or by a non-transferable plasmid. This β-lactamase appears to be different, on the basis of isoelectric focusing, from an enzyme produced by the *K. pneumoniae* strain GN422 (Sawai *et al.*, 1973), but similar to the latter in several properties.

## Introduction

Among the plasmid-mediated β-lactamases, R-TEM is the most well known (Datta & Richmond, 1966) and probably the most widespread. It usually expresses a high resistance level to ampicillin and carbenicillin in recipient strains without interfering very much with the sensitivity to the cephalosporins. Several studies have dealt with closely related β-lactamases, the 'TEM-like' enzymes which are related to class III of Richmond & Sykes (1973). For example in 1970, Sawai *et al.* (1970), described variants of penicillinase mediated by an R. plasmid in *Escherichia coli*, with enzymes, characterized as: Ia, pI = 5·1 and Ib, pI = 6·9. In 1972, Pitton characterized 'type 1' and 'type 2' β-lactamases, according to their immunological properties and isoelectric points (Pitton, 1972, 1973). More recently, Matthew & Hedges (1976) characterized a TEM-1 and TEM-2.

Very often, clinical isolates of *Klebsiella pneumoniae* appear moderately resistant to ampicillin and carbenicillin but sensitive to most cephalosporins [a low level 'TEM-like' phenotype]. This study describes the properties of the β-lactamase produced by a *K. pneumoniae* strain with such a 'TEM-like' phenotype.

## Material and methods

### *Bacterial strains*

*K. pneumoniae* strain 11-03 is a clinical isolate, moderately resistant to ampicillin (32 μg/ml) and carbenicillin (128 μg/ml), but sensitive to the usual cephalosporins and

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the other major antibiotics. *K. pneumoniae* strains GN118 and GN422 were kindly provided by Dr Sawai (Sawai, Yamagishi & Mitsuhashi, 1973). *E. coli* strains P111 and P453 were previously described (Labia *et al.*, 1974); they produce respectively, type 1 (pI = 5·4, mol. wt. 23,000) and type 2 (pI = 7·7, mol. wt. 21,500) which are R plasmid-mediated β-lactamases. *E. coli* strain RP4 (Sykes & Matthew, 1976) produces a TEM-2 (pI = 5·6) enzyme, and *E. coli* strain PUB 5451 (Petrochelou, Sykes & Richmond, 1977) produces a 'novel' R-factor mediated β-lactamase.

*Bacterial cultures and purification of β-lactamase*

Bacterial cultures were grown as previously described (Kazmierczak, Philippon, Chardon, Labia & Le Goffic, 1973) with or without induction by penicillin G (final concentration 100 or 500 μg/ml). Bacterial suspensions were disrupted by sonification 4 times for 20 seconds at 20 kHz (Branson Sonifier B12) and centrifuged (30 min, 20,000 g, 4°C). Streptomycin sulphate was added (final concentration 2% w/v) to the clear supernatant containing the crude enzymatic extract, and after 4 h incubation at 4°C, the precipitated nucleic acids were eliminated by centrifugation (30 min, 20,000 g, 4°C). The resulting supernatant fluid was dialysed 24 h against distilled water, ultrafiltered on Diaflo membranes (UM 10, Amicon) and the ultrafiltrate submitted to preparative isoelectric focusing as previously described (Barthelemy, Guionie & Labia, 1978).

*Determination of kinetic constants*

The Michaelis-Menten constants (Km and Vm) were determined by microacidimetry using a Mettler pH-stat coupled to a Wang 600 mini-computer (Kazmierczak *et al.*, 1973; Labia, Andrillon & Le Goffic, 1973). The Km is expressed in μM units, and Vm as a percentage with 100 for penicillin G. At low concentrations enzymatic stability ($\tau$ = Km/Vm) is proportional to the half-life of the antibiotic and is also expressed in relationship to penicillin G ($\tau$ = 100) for each substrate (Labia, 1974). The unit of β-lactamase is defined as the quantity of enzyme which hydrolyzes one micromole of penicillin G per min at pH 7 and 37°C. Protein contents were determined directly by ultraviolet spectrophotometry at 280 nm or according to Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin (Calbiochem) as reference. The specific activity is then expressed as the number of milli-units of β-lactamase per mg of total protein (mU/mg).

*Analytical determinations*

Analytical isoelectric focusing on polyacrylamide gels was carried out by the technique recently described (Barthelemy *et al.*, 1978; Labia, Barthelemy & Masson, 1976) in order to avoid possible artifacts in pI measurements.

Molecular weights were estimated by gel filtration on Sephadex G-75 by the method of Andrews (1964), using ovalbumin (Calbiochem), deoxyribonuclease (Worthington Biochemical Co.), chymotrypsinogen (Sigma Chemical Co.) and myoglobin (Calbiochem) as standard proteins.

The immunoprecipitation properties were determined by immunodiffusion (Ouchterlony) and immunoelectrophoresis using specific type 1, type 2 and type 30 antibodies (Heitz & Pitton, 1975; Pitton, 1972).

*Transferability*

Transferability was tested according to Anderson & Lewis (1965) and Anderson (1965), the recipient strains being *S. typhi*, *S. paratyphi B*, *S. typhimurium* and *E. coli*K12F$^-$lac$^-$ Nx$^r$; the plasmids used to 'mobilise' the A determinant of *K. pneumoniae* 11-03 are listed in Table I.

**Table I.** Reference plasmids used for compatibility experiments

| Incompatibility group | Plasmid number | Antibiotic resistance | References |
|---|---|---|---|
| $F_{II}$ | RI-16 | K | Hedges & Datta (1972) |
| | 240 | T *fi*$^+$ | Grindley *et al.* (1971) |
| $F_{IV}$ | TP 129 | T | Anderson (unpublished) |
| $I_1$ | R 112 | K col Ib | Bouanchaud & Chabbert (1969) Chabbert *et al.* (1972) |
| | T-Δ | T | Anderson & Lewis (1972) |
| | T-Δdrpl | T | Grindley *et al.* (1972) |
| $I_2$ | TP 114 | K | Grindley *et al.* (1972) |
| com 10 | R 72 | K | Scavizzi (1973) |
| B | TP 113 | K | Grindley *et al.* (1972) |
| $H_1$ | TP 123 | CSSuT | Anderson & Smith (1972) |
| $H_2$ | TP 116 | CSSu | Anderson & Smith (1972) Grindley *et al.* (1972) |
| N | R 113 | T | Bouanchaud & Chabbert (1969) Chabbert *et al.* (1972) |
| J | R 391 | K | Coetzee *et al.* (1972) |
| T | Rts 1 | K | Coetzee *et al.* (1972) |
| W | RS-a | CKSSu | Hedges & Datta (1972) |

*Elimination of the resistance determinant*

Curing experiments were performed with acridine dyes following the method described by Chabbert, Scavizzi, Witchitz, Gerbaud & Bouanchaud (1964).

## Results

The specific activity of crude extracts obtained from non-induced cultures of *K. pneumoniae* strain 1103 and cultures induced by 100 μg/ml of penicillin G do not present significant differences. We obtained values of 20 and 15 mU/mg respectively. If a higher concentration of inducing agent is used (500 μg/ml) the specific activity of the extracts diminished, which is related to some bacterial growth inhibition.

Analytical isoelectric focusing of crude extracts from *K. pneumoniae* strain 1103 shows only a single β-lactamase. The pI of this enzyme is estimated at 7·10 in a sucrose gradient. Plate 1 shows a comparison of few well known β lactamases, very often associated with ampicillin and carbenicillin resistance. They are *1*, TEM-1 (pI = 5·4); *2*, TEM-2 (pI = 5·6); *3*, Pitton's type 2 (pI = 7·7); *4*, strain GN118 (pI = 7·7); *5*, strain GN422 (pI = 7·05) and *6* 11-03 (pI = 7·10). The mobility difference between strains GN422 and 1103 is not clearly revealed in Plate 1, but is in Plate 2 where the individual positions for the samples are closer to each other. Positions 1,3,5,7 are mixtures of enzyme from strains 1103 and Pitton's type 2, in order to emphasize the isoelectric shift and its

reproducibility between these strains. When Pitton's type 2 is omitted, the same pI difference between strains 1103 and GN422 is shown. In another set of experiments, we found that the β-lactamase produced by *E. coli* PUB 5451 shows the same pI that those produced by *E. coli* P453 (pI = 7·7).

The method used for purification allows an enrichment of approximately 100 fold over the crude extract. Kinetic constants (Km and Vm) for both penicillin G and cephalothin are practically the same before and after purification. For this reason, the determination of the kinetic constants Km, Vm and τ for the different penicillins and cephalosporins was subsequently done with crude extracts. These results are shown in Table II.

**Table II.** Kinetic constants (Km Vm, and τ of the β-lactamase from *K. pneumoniae* 11-03. Vm and τ are expressed as a percentage of corresponding values for penicillin G

| | Km (μM) | Vm (rel.) | τ (rel.) |
|---|---|---|---|
| Penicillin G | 13·0 | 100 | 100 |
| Penicillin V | 10·6 | 92 | 89 |
| Ampicillin | 27·1 | 136 | 153 |
| Epicillin | 23·1 | 130 | 137 |
| Amoxycillin | 21·5 | 126 | 131 |
| Carbenicillin | 5·0 | 8·6 | 447 |
| Ticarcillin | 5·7 | 9 | 487 |
| Sulbenicillin | 3·7 | 2 | 1420 |
| Cephalothin | 41 | 23 | 1371 |
| Cephaloridine | 420 | 104 | 3106 |
| Cefazolin | 460 | 10·7 | 33,100 |
| Cefamandole | 152 | 17·5 | 6680 |
| Cephalexin | — | <0·5 | — |
| Cefoxitin | Ki>200 | <0·3 | — |
| Cloxacillin | Ki = 1·6 | <0·1 | — |

The β-lactamase activity is very high with penicillin G and V, as well as with ampicillin, epicillin and amoxycillin which are the best substrates. Carbenicillin, ticarcillin and sulbenicillin are hydrolyzed more slowly. The cephalosporins are also hydrolyzed. In addition, we note that the cephalosporins always exhibit lower affinity for the enzyme (high value of Km) with the exception of cephalothin. Cephalexin, cefoxitin and cloxacillin are not hydrolyzed to a significant extent. These latter two molecules were tested as inhibitors; cefoxitin is a very poor inhibitor whereas cloxacillin is relatively better.

The β-lactamase has a molecular weight of 20,000 ± 500. No precipitation with antibodies of types 1, 2 or 30 is observed by immunoelectrophoresis or immunodiffusion (Plate 3).

We were not able to demonstrate 'mobilization' of the ampicillin resistance determinant of strain 11-03 for conjugal transfer by plasmids of different groups of compatibility. It is interesting to note that, with the exception of plasmid R 72 (com 10) and TP 113 (group B), all the plasmids used can be transferred into strain 11-03 and can coexist with the A determinant.

Elimination of the resistance character by acridine dyes was never observed whatever the concentration used.

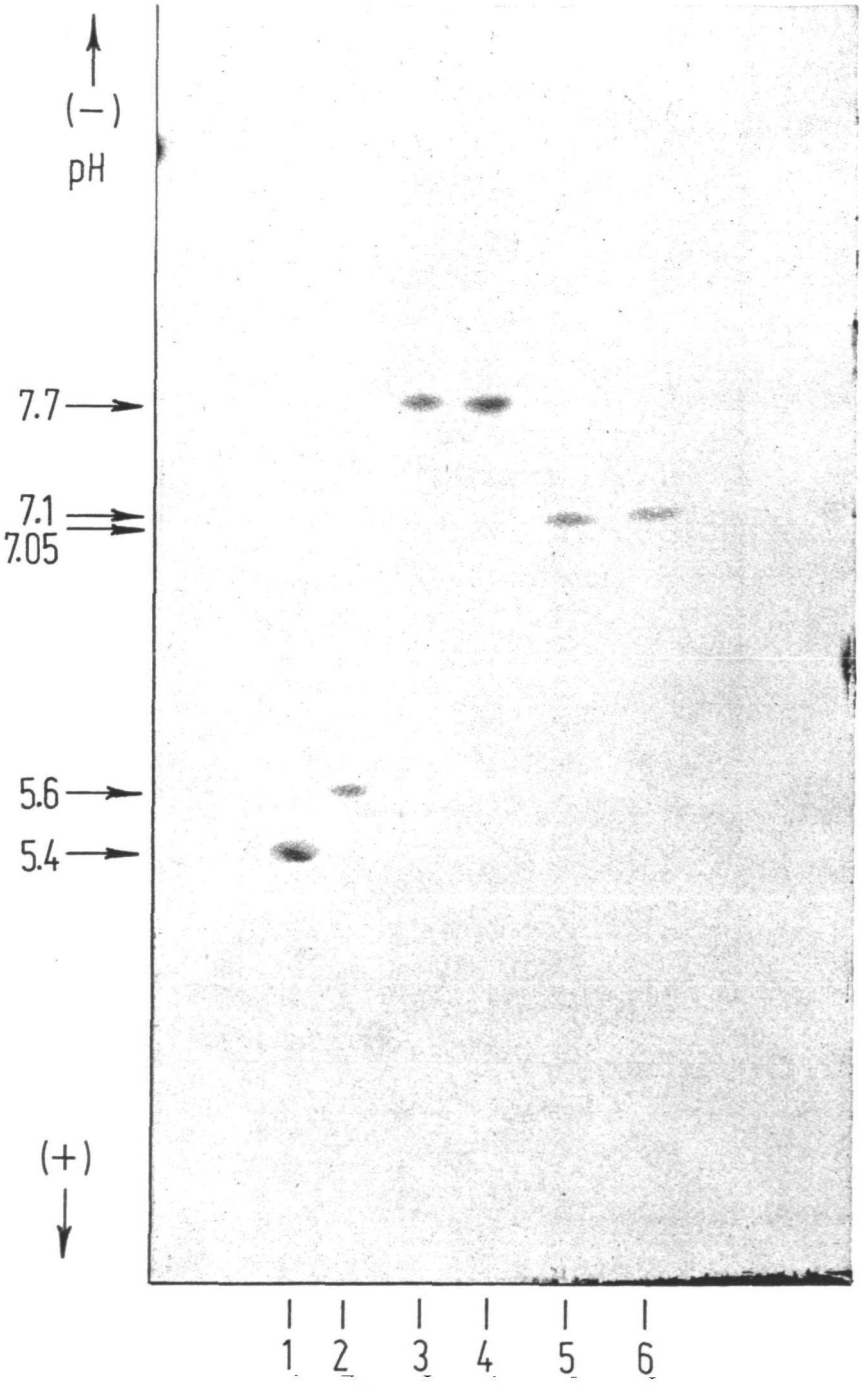


Plate 1. Analytical isoelectric focusing of β-lactamases from *E. coli* strain P111, 1; strain RP4, 2; strain P453, 3; *K. pneumoniae* strain GN 118, 4; strain GN 422, 5; and strain 1103, 6.

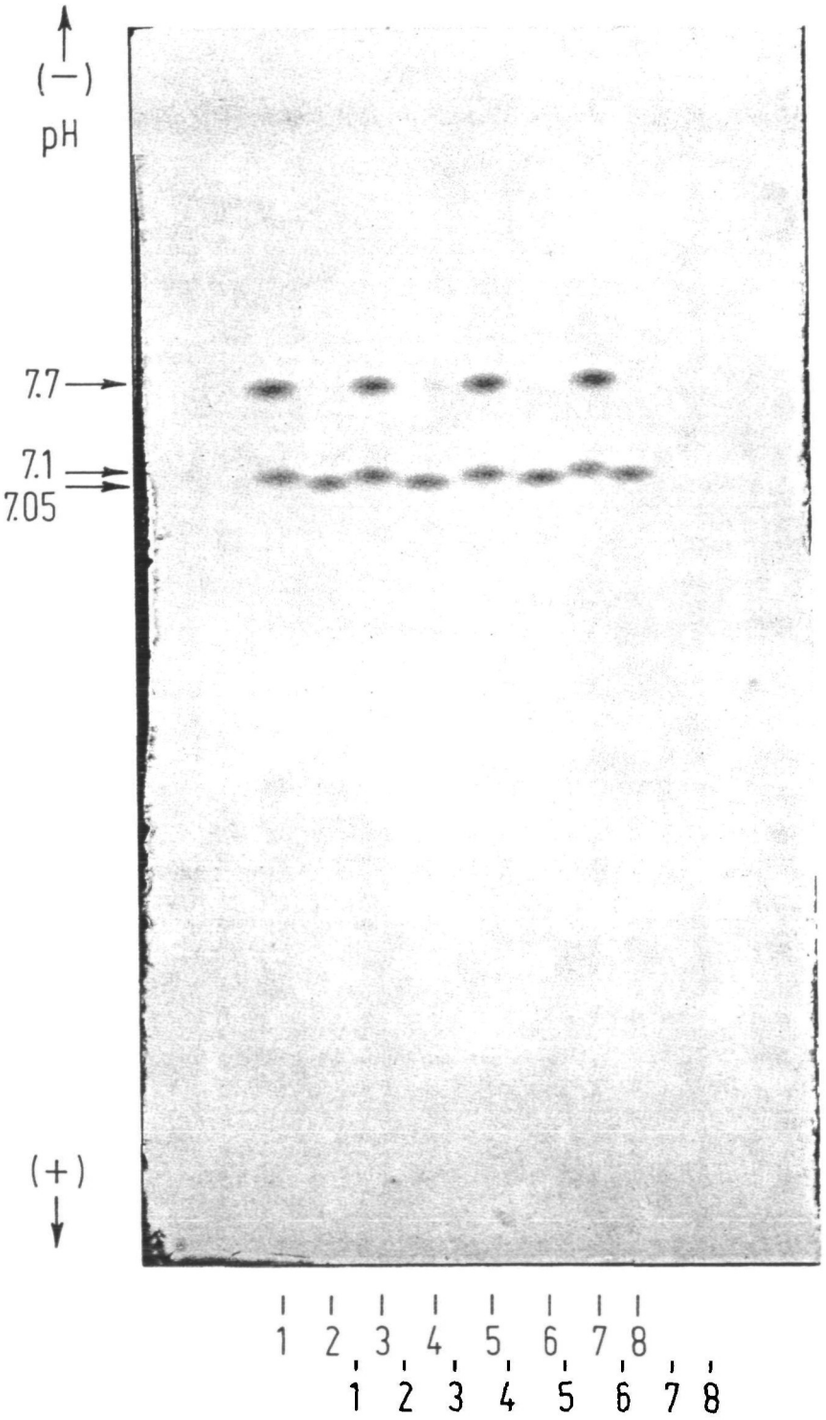


*Plate 2.* Analytical isoelectric focusing of β-lactamases. *Slots* 1,3,5,7: mixture of strains P453 and 11-03; slots 2,4,6,8: strain GN422.

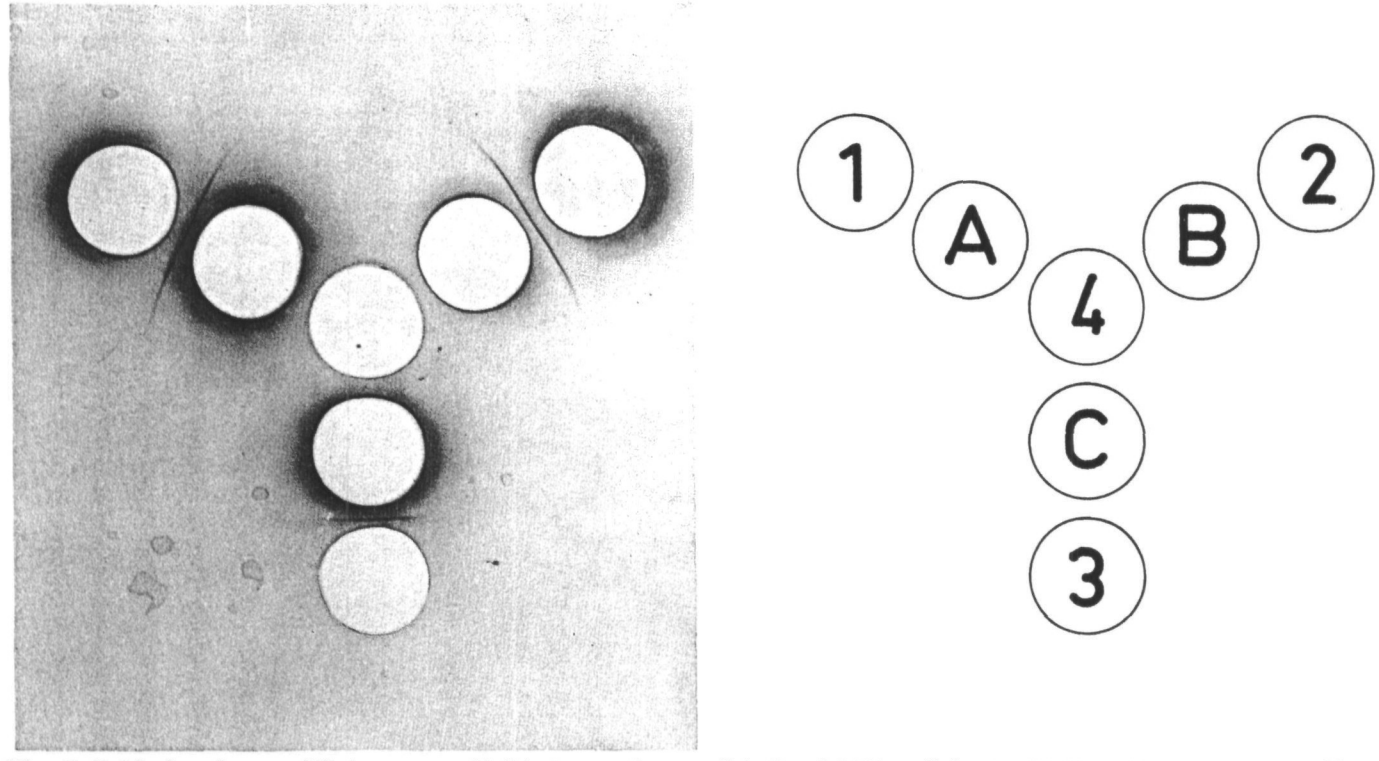


Plate 3. Outcherlony immunodiffusion assays with β-lactamases from: well 1, *E. coli* P111; well 2, *E. coli* P453; well 3, *K. oxytoca* R 30; well 4, *pneumoniae* 11-03. Antiserums were as follows, well A strain P111, well B, strain P453; well C, strain R30.

## Discussion

*K. pneumoniae* 11-03 produces only one β-lactamase, as the kinetic constants do not vary to any significant extent during purification; moreover, analytical isoelectric focusing on acrylamide gels of the crude extracts from induced or non-induced cultures shows only one band of β-lactamase activity (Plate 1). This β-lactamase appears to be constitutive and exhibits a low level of activity (20 mU/mg) (crude extracts) compared with the usual activities found with other similar β-lactamases (for example in crude extracts *E. coli*, TEM-1 shows about 2000 mM/mg, TEM-2 about 4000 mU/mg and type 2 about 150 mU/mg). This low level of activity of strain 11-03 β-lactamase may be related to the low level of resistance of the producing strain to ampicillin and carbenicillin.

From the genetic point of view, it was important to determine whether the biogenesis of this enzyme was governed by the chromosome or by a plasmid. All attempts to transfer as well as those to eliminate the possible plasmid with acriflavine have failed. These experiments do not discriminate between the two hypotheses, although they tend to support the argument in favour of a chromosomal-mediated biogenesis, with either a plasmid integrated into the chromosome or a non-transferable R-determinant coding for biosynthesis of the enzyme.

It is also noteworthy to examine the kinetic constants of the β-lactamase of strain 11-03 (Table II). This enzyme shows its main activity with penicillins; it is observed that penicillins G and V exhibit similar activity, as do ampicillin, epicillin and amoxycillin. With these latter three antibiotics the enzyme activity correlates with the resistance. With cephaloridine a high Vm (104) is also demonstrated which permitted this β-lactamase to be classified by Sykes & Matthew (1976) among the 'broad-spectrum' enzymes. We conclude however that this interpretation is not altogether correct: the high Km value for cephaloridine entails, in low concentrations of the antibiotic, an increased half-life ($\tau$). Since the $\tau$ value of cephaloridine is elevated, it is therefore one of the bases of the sensitivity of *K. pneumoniae* 11-03 to this antibiotic. Carbenicillin and ticarcillin present very similar Km and Vmax values, as well as similar $\tau$ values. It is surprising to note that sulbenicillin presents a half-life approximately three times higher than that of carbenicillin or ticarcillin, thus reaching that of cephalothin. However, *K. pneumoniae* 11-03 is sensitive to cephalotin and resistant to sulbenicillin.

This gives support to the idea that the presence of β-lactamase is not the only event involved in resistance to β-lactams. Richmond & Sykes (1973) invoked 'crypticity', which corresponds to a selective modification of the permeability of the bacterial wall, as well as 'intrinsic factors'.

It is interesting to compare the plasmid-mediated β-lactamase type 2 described by Pitton (1973) with that of strain 11-03, particularly in respect to the pI (7·7) for type 2 described by Pitton (1972) and Philippon, Paul, Labia & Nevot (1976). This β-lactamase type 2 is often observed in *K. pneumoniae*.

Table III summarizes some properties of various β-lactamases which are active with ampicillin and carbenicillin and derivatives as described in the literature, but does not include the enzymes highly active on the isoxazolylpenicillins. It is possible that some of these enzymes are identical. In this table, the most interesting fact to observe is perhaps the isoelectric points: an important group of enzymes presents pIs between 5·1 and 5·6, while another one presents pIs between 7·1 and 7·9. All the Km values for penicillin G are very similar.

Table III. Comparison of some β-lactamases from literature data (a, Sawai *et al.*, 1973; b, Heitz & Pitton, 1975; c, Pitton, 1973; d, Pétrochelou *et al.*, 1977; e, Sykes & Matthew, 1976; f, Sawai *et al.*, 1970) and the enzyme from *K. pneumoniae* 11-03. GN118, P453 and PUB-5451 showed the same pI by analytical isoelectric focusing (pI = 7·7) as do P111, RTEM, RGN14 (pI = 5·4) and RP4, RGN823 (pI = 5·6)

| Bacterial strain | pI | Transferability | Mol. weights | Pen. G Km (μM) |
|---|---|---|---|---|
| *K. pneumoniae* | | | | |
| 11-03 | 7·1 | — | 20,000 | 13 |
| GN 69 (a) | 7·8 | — | 17,400 | 11 |
| GN118 (a) | 7·76 | — | 18,300 | 22 |
| GN1103R⁻ (a) | 7·87 | — | 18,100 | 15 |
| GN422 (a) | 7·2 | — | 20,000 | 28 |
| *K. oxytoca* | | | | |
| R30 (b) | 5·8 | — | 29,000 | 21·8 |
| *E. coli* | | | | |
| P 453 (c) | 7·7 | + | 21,500 | 10·6 |
| PUB5451 (d) | 7·5 | + | — | — |
| PIII (c) | 5·4 | + | 23,000 | 21 |
| R-TEM(TEM-1) (e) | 5·4 | + | 22,000 | 22 |
| RP4(TEM-2) (e) | 5·6 | + | 23,500 | — |
| R-GN14(Ia) (f) | 5·1 | + | 20,600 | 27 |
| R-GN823(Ib) (f) | 6·9 | + | 22,600 | 24 |

Thus *K. pneumoniae* 11-03 β-lactamase appears very similar to the *K. pneumoniae* GN 422 enzyme described previously by Sawai, Takahashi, Yamagishi & Mitsuhashi (1970). In the same units, with crude extracts, they respectively show specific activities of 20 and 33 mU/mg (data from Sawai *et al.*, 1973). Both of them are constitutive and have the same molecular weights and kinetic constants. Only analytical isoelectric focusing gives a small but significant difference between their isoelectric points.

One could think that a small pI shift between the two proteins could be of little importance as the other biochemical parameters are similar. The difference may be of importance in indicating that the two proteins are not the same, and possess different primary structures.

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