Characterisation of a unique ceftazidime-hydrolysing β -lactamase, TEM-E2

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Summary. A strain of *Klebsiella oxytoca*, originally isolated in Liverpool in 1982, has been found to produce a novel transferable β -lactamase, TEM-E2. This enzyme confers resistance to ceftazidime and focused as a doublet band with an iso-electric point (pI) of 5.3. The strain also produced the TEM-1 β -lactamase. Both TEM-1 and TEM-E2 β -lactamases were encoded by a transferable 103 kb plasmid; these two enzymes also had similar molecular weights, were inhibited by clavulanic acid, and hydrolysed ampicillin, carbenicillin and cephaloridine at similar rates. However, unlike the TEM-1 enzyme, the TEM-E2 β -lactamase hydrolysed ceftazidime and cefotaxime with similar efficiency, although it conferred much greater resistance to ceftazidime in the host strain. This is the earliest documented example of a TEM-like enzyme which confers transferable resistance to ceftazidime and related cephalosporins.

Introduction

When cefotaxime and ceftazidime were first introduced, they were resistant to hydrolysis by most of the plasmid-mediated β -lactamases that were known at that time, including the TEM-1 and TEM-2 enzymes.^{1,2} However, there have since been several reports of β -lactamases, which differ from TEM-1 and TEM-2 by only a few amino acids, that are able to hydrolyse these and related cephalosporins.³ Between 1984 and June 1987, 490 strains producing TEM-3 (which exhibits high activity against cefotaxime) were isolated in France.⁴ In 1987 and 1988 the novel enzymes TEM-4, 5, 6, 7, 9 and CAZ-2 were identified;^{5–7} all appear to be related to the TEM group of β -lactamases. We now describe a ceftazidime-resistant strain of *Klebsiella oxytoca*, isolated in 1982, which produces a β -lactamase that hydrolyses ceftazidime and cefotaxime and shows strong similarities with TEM-1.

Materials and methods

Bacterial strains

K. oxytoca strain 5445 was isolated from the blood and cerebrospinal fluid of a baby in the Neonatal Intensive

Care Unit of Liverpool Maternity Hospital on Feb. 5, 1982. Rifampicin-resistant *Escherichia coli* J53-2 was used as the standard recipient strain in the conjugation experiments.⁸ *E. coli* J53-2 transconjugants were selected on agar containing rifampicin 100 mg/L and ceftazidime 4 mg/L after overnight incubation of a mixture of the donor and recipient strains.⁹

Antibiotic sensitivities and plasmid analysis

Minimum inhibitory concentrations (MICs) of β -lactam antibiotics were determined by the agar incorporation method in Iso-Sensitest Agar (Oxoid), with a bacterial inoculum of 10^5 cfu. In certain experiments, clavulanic acid or sulbactam was included in the medium at a concentration of 2 mg/L. The method of Takahashi and Nagano¹⁰ was used to extract, separate and visualise plasmid DNA.

β -Lactamase studies

Bacteria were grown overnight in 1 L of Nutrient Broth No. 2 (Oxoid) containing ceftazidime 4 mg/L, and β -lactamase preparations were obtained from sonicated extracts of the concentrated bacterial pellet.¹¹ The β -lactamases were identified by analytical iso-electric focusing (IEF) of these extracts;¹² β -lactamases PSE-4, TEM-1, TEM-2 and TEM-7 were used as standard pI markers. β -Lactamase activity, substrate profile, Michaelis-Menten kinetics and the effect of inhibitors were determined by spectrophotometric assays according to

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published methods.¹³⁻¹⁵ The relative molecular mass (M_r) of the β -lactamase was determined on a Sephadex G-75 column (2 cm² × 90 cm) eluted with 25 mM phosphate buffer (pH 7.0) at a flow rate of 16 ml/h.¹⁶ Ovalbumin, chymotrypsinogen and cytochrome C were used as standard M_r markers.

Purification of the TEM-E2 β -lactamase by electrodialysis

A crude β -lactamase solution, prepared from the *E. coli* J53-2 transconjugant of *K. oxytoca* 5445, was spread on to an analytical IEF polyacrylamide gel containing pH 6–8 and pH 4–6 ampholines (LKB Pharmacia) in a 1:1.5 ratio. Care was taken not to cover the area of the gel where the β -lactamase bands focused. After focusing, a 1-cm wide strip of filter paper soaked in a solution of nitrocefin (0.5 mg/ml) was placed from the cathode to the anode of the gel to identify the β -lactamase bands. The portion of the gel containing the TEM-E2 enzyme was cut out and placed in dialysis tubing with a minimal amount of 25 mM sodium phosphate buffer (pH 7.0). The dialysis sack was placed in the cathode reservoir of a Bio Rad Mini Sub Cell with 25 mM phosphate buffer (pH 7.0) as running buffer. A charge of 150 V was applied for 10 min. The dialysis sack was then removed and the polyacrylamide gel was discarded. The purity of the enzyme remaining in the sack was analysed by IEF.

Results

Conjugation experiments

The ceftazidime-resistant transconjugants of *E. coli* J53-2 were obtained by conjugation with *K. oxytoca* 5445 at 37°C for 6 h. Analysis of the plasmid DNA in the transconjugant strains showed a single large plasmid band of 103 kb, designated pUK721, similar to that found in the original clinical strain.

Antibiotic sensitivities

K. oxytoca 5445 and the *E. coli* J53-2 transconjugant were resistant to ceftazidime but sensitive to cefotaxime and ceftriaxone. Indeed, ceftazidime resistance was the most significant additional β -lactam resistance conferred by plasmid pUK721 when compared with a TEM-1 β -lactamase producing strain (table I). Susceptibilities to ceftazidime were restored in the presence of clavulanic acid (2 mg/L) or sulbactam (2 mg/L). However, clavulanic acid was more efficient than sulbactam in reducing MICs of ampicillin.

Table I. Antibiotic sensitivities of test strains

Antibiotic	Minimum inhibitory concentrations (mg/L)			
	<i>K. oxytoca</i> 5445	<i>E. coli</i> J53-2 × 5445	<i>E. coli</i> J53-2 2136E*	<i>E. coli</i> J53-2
Ampicillin	>250	>250	>250	4
+ clav	16	8	2	2
+ sulb	125	250	>250	4
Carbenicillin	>250	>250	>250	8
Cephaloridine	250	64	8	4
Cephalexin	16	8	4	4
Cefoxitin	16	4	1	2
Cefuroxime	16	4	2	2
Ceftazidime	64	32	<0.06	0.13
+ clav	1	0.25	<0.06	0.13
+ sulb	2	0.5	<0.06	<0.06
Cefotaxime	0.5	0.25	<0.06	<0.06
+ clav	<0.06	<0.06	<0.06	<0.06
+ sulb	<0.06	<0.06	<0.06	<0.06
Ceftriaxone	0.5	0.13	<0.06	<0.06
Aztreonam	2	1	<0.06	<0.06
Imipenem	0.25	0.25	0.13	0.25

+ clav, in combination with clavulanic acid 2 mg/L;

+ sulb, in combination with sulbactam 2 mg/L.

*Strain 2136E produces TEM-1.

β -Lactamase studies

Analytical IEF with a broad range ampholine (pH 3.5–10) revealed that *K. oxytoca* 5445 and the *E. coli* J53-2 transconjugant produced β -lactamases of low pI. IEF with a 1:1 mixture of pH 4–6 and pH 3.5–10 ampholines showed that the clinical isolate and the *E. coli* transconjugant each produced a band which co-focused with TEM-1 and a novel β -lactamase which focused as a doublet band at pI 5.3 (figure). This novel β -lactamase, TEM-E2, was clearly distinguishable from any of the other ceftazidime-hydrolysing β -lactamases.¹⁷ The TEM-E2 doublet band produced by *K. oxytoca* 5445 was separated from the TEM-1 band by electrodialysis from the IEF polyacrylamide gel (figure).

The $V_{\max}$ and K_m values of the TEM-E2 and TEM-1 enzymes are shown in table II. Neither enzyme had any measurable activity against cefuroxime. TEM-1 had no activity against ceftazidime but showed some affinity for cefotaxime. TEM-E2 showed activity against both substrates but had a greater affinity for cefotaxime. Both enzymes showed similar relative efficiency of hydrolysis of ampicillin, carbenicillin and cephaloridine (table II). However, the novel enzyme paradoxically hydrolysed ceftazidime less efficiently than cefotax-

Figure. IEF of β -lactamases. **A**, *E. coli* J53-2 producing TEM-1; **B**, TEM-E2 β -lactamase purified from TEM-1 by electro dialysis from IEF gel; **C**, *E. coli* J53-2 transconjugant of *K. oxytoca* 5445 producing TEM-1 and TEM-E2; **D**, *K. oxytoca* 5445 producing TEM-1 and TEM-E2.

ime. Hydrolysis of nitrocefin by TEM-E2 was inhibited by clavulanic acid (ID_{50} $0.8 \mu M$). The M_r of TEM-E2, purified by electro dialysis from an analytical IEF gel, was 23 500. Under the same experimental conditions, the M_r of the TEM-1 enzyme was 22 000. However, this difference may not be significant. The crude TEM-E2 enzyme preparation (i.e., with the TEM-1 enzyme) was analysed by gel filtration and fractions were collected from either side of the peak of β -lactamase activity; examination of each of these fractions by IEF showed that they all contained the TEM-1 band and the doublet band, thus confirming their identical M_r .

Discussion

The novel β -lactamase, TEM-E2, is different from any of the previously reported enzymes that hydrolyse cephalosporins of the cefotaxime type. Its kinetic characteristics appear similar to those of other TEM-like enzymes such as TEM-E1¹⁸ and TEM-7¹⁹ in that it hydrolysed cefotaxime and ceftazidime with similar, low efficiencies although it conferred resistance only to ceftazidime. The novel enzyme showed similarities with TEM-1, from which it could be separated by electro dialysis. An enzyme with very similar properties to TEM-E2 has been obtained from a TEM-1 producing strain by spontaneous mutation.¹⁷ This strongly suggests that TEM-E2 has been derived from the TEM-1 β -lactamase. Moreover, as the mutant strain exhibited virtually no TEM-1 activity, it is apparent that the TEM-1 enzyme did not contribute towards ceftazidime resistance. The first example of plasmid-mediated resistance to cefotaxime was identi-

Table II. Kinetic constants of TEM-E2 compared to those of TEM-1

Antibiotic	TEM-E2			TEM-1		
	K_m (μM)	V_{max}^*	$V_{max}/K_m^\dagger$	K_m (μM)	V_{max}^*	$V_{max}/K_m^\dagger$
Ampicillin	119	100	100	167	100	100
Carbenicillin	83	23	32	100	11	18
Cephaloridine	500	87	20	167	23	23
Cefuroxime	UM	UM	UM	UM	UM	UM
Cefotaxime	181	0.84	0.55	286	0.06	0.04
Ceftazidime	500	0.87	0.21	UM	UM	UM

UM, unmeasurable.

*Relative to ampicillin as 100%.

†Efficiency of hydrolysis relative to ampicillin as 100%.

fied in Germany in 1983.²⁰ This transferable resistance was later found to be conferred by the SHV-2 β -lactamase.²¹ Since the *K. oxytoca* 5445 strain discussed in this study was isolated in 1982, TEM-E2 is the earliest example of a TEM-like enzyme which can hydrolyse ceftazidime, and also the first plasmid-encoded β -lactamase to confer resistance to any of the new broader-spectrum

cephalosporins. The study of this enzyme has also illustrated the use of a simple technique for the separation and purification of β -lactamases. The method may be particularly useful when the host strain produces enzymes with similar pI values.

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