ANTIMICROBIAL AGENTS

Back mutations to the TEM-1 β -lactamase from TRC-1 lead to restored sensitivity to clavulanic acid

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Summary. Back mutations from the TRC-1 β -lactamase to the TEM-1 enzyme were selected *in vitro*. The revertant β -lactamase was obtained from *Escherichia coli* strain J62.2 carrying plasmid pUK901 which encodes the TRC-1 β -lactamase. The revertant was obtained after repeated subculture of *E. coli* J62.2 (pUK901) in amoxycillin 512 mg/L for 5 days. The revertant β -lactamase had the same pI as TEM-1 (5.4) and had restored inhibition by clavulanic acid (ID₅₀ reduced from 4.2 μ M to 0.15 μ M). The prevalence of these β -lactamases in the clinical population may be the result of a two-way flux, with mutations in both forward and backward directions.

Introduction

The most important mechanism of resistance to β -lactam antibiotics is the production of β -lactamases that catalyse hydrolysis of the β -lactam ring.^{1,2} Two strategies have been employed to overcome resistance mediated by β -lactamases. First, the development of new β -lactam antibiotics which, as a result of modifications in structure, are stable to hydrolysis by β -lactamases. Second, the use of β -lactamase inhibitors which, when administered with the β -lactam, protect it from hydrolysis by the β -lactamase.^{3,4}

The TEM-1 enzyme is the most important β -lactamase encountered in gram-negative bacilli, both clinical isolates and normal flora.^{5,6} The ability of the later generation cephalosporins to select TEM-1 β -lactamase mutants with the ability to hydrolyse these cephalosporins is well documented.⁷ The vast majority of TEM-derived extended spectrum β -lactamases remain sensitive to inhibition by the β -lactamase inhibitors sulbactam, tazobactam and clavulanic acid,⁸ and it was thought unlikely that the TEM-1 enzyme would be capable of mutation to form new enzymes with increased resistance to β -lactamase inhibitors. However, TRC-1, a novel TEM-derived β -lactamase with increased resistance to β -lactamase inhibitors, was identified in a urinary *Escherichia coli* isolate in Edinburgh,⁹ and similar β -lactamases, termed TRI β -lactamases, have been found in clinical isolates in France.¹⁰ The TRC-1 β -lactamase can be selected from TEM-1 directly with a combination of amoxycillin and clavulanic acid; however, it has been demonstrated

that increased resistance to β -lactamase inhibitors is associated with a decrease in resistance to amoxycillin alone.¹¹

The unexpected appearance of TEM β -lactamases with increased resistance to β -lactamase inhibitors has led to speculation about the impact that such enzymes may have on the successful clinical use of β -lactam plus β -lactamase inhibitor combinations. At present TRC-1 appears to be rare, although it is not clear if this is simply because few studies have looked specifically for this β -lactamase. Strains encoding this enzyme have only a moderate increase in resistance to co-amoxiclav, and may therefore be missed in routine sensitivity testing.¹¹ Alternatively, the rarity of this enzyme may result from reduced activity of the β -lactamase against amoxycillin. To investigate if this latter point may have a role in determining the clinical success of the TRC-1 β -lactamase, the effect of amoxycillin challenge on the bacteria producing TRC-1 was examined.

Materials and methods

Bacterial strain

The strain used in the challenge experiments was *E. coli* strain J62.2 (pUK901). This strain is the TRC-1 producing transconjugant derived from the original TRC-1 producing clinical isolate (*E. coli* strain E307).⁹

Challenge experiments

E. coli strain J62.2 (pUK901) was grown in a series

Fig. 1. β -Lactamase IEF profile of purified isolates. The IEF gel contained a 50:50 mixture of ampholines (pH range 3.5–10 and pH range 4–6). Tracks: A–F and I–L, β -lactamase from purified isolates obtained after challenge with amoxycillin 512 mg/L for 5 days; G, TEM-1 control strain; H, TRC-1 control strain.

of Oxoid IsoSensitest broths containing amoxycillin at concentrations of 0, 128, 256 and 512 mg/L and incubated with agitation at 37°C. At 24-h intervals, each culture was subcultured into a further series of IsoSensitest broths containing the same range of amoxycillin concentrations. This procedure was repeated five times. Also, after each 24-h period, bacteria were harvested from 10 ml of the culture by centrifugation (4500 *g*; 15 min) in a Heraeus Christ Bactifuge. The cell pellet was resuspended in 10 ml of single strength Davis Mingioli medium,¹² and 0.1 ml was spread on to IsoSensitest agar plates containing amoxycillin at the same concentration as the broth from which the culture was derived. Ten single colonies were purified on the same medium. The auxotrophic requirements of the colonies (L-proline, L-histidine and L-tryptophan dependency) were checked¹¹ on Davis Mingioli minimal medium plates to confirm that they were *E. coli* J62.2.

Plasmid analysis

Plasmid DNA was extracted from 4.5 ml of an overnight IsoSensitest broth culture by alkaline denaturation as described by Takahashi and

Nagano.¹³ The final DNA pellet was resuspended in 60 μ l of RNAase (Sigma) solution (50 mg/L in sterile H₂O) and 18- μ l portions were digested with *Eco*RI (BRL), used according to the manufacturer's instructions. After electrophoresis at 60 v for 18 h in agarose 0.8% w/v gels, DNA was visualised by staining with ethidium bromide 5 mg/L and examination on a flat bed UV source.

β -Lactamase studies

β -Lactamases for iso-electric focusing (IEF) were prepared from bacterial cells grown overnight on nutrient agar slopes. IEF was by the method of Matthew *et al.*,¹⁴ with ampholine ranges as described in Results. β -Lactamase for inhibition studies was prepared from bacteria grown for 15 h in 1 L of nutrient broth. Bacteria were harvested by centrifugation (5000 *g*; 15 min), washed and then resuspended in 5 ml of 50 mM sodium phosphate buffer, pH 7.0, disrupted by sonication and the cell debris was removed by centrifugation (20000 *g*; 30 min). One-ml of this crude β -lactamase preparation was applied to a previously calibrated Sephadex G75 column (2 cm² $\times$ 90 cm) and eluted with 50 mM sodium

phosphate buffer. The collected fractions (2 ml) were assayed for β -lactamase activity by spot testing with the chromogenic cephalosporin nitrocefin (Glaxo). Fractions showing peak β -lactamase activity were pooled and used for further analysis. All β -lactamase preparations were stored at -20°C until required.

Spectrophotometric assay was performed as described previously.¹⁵ The decrease in absorbance of nitrocefin at 384 nm was monitored at 37°C in 50 mM sodium phosphate buffer, pH 7.0, in a Perkin Elmer Lambda-2 Double-Beam UV Spectrophotometer. Inhibition studies with clavulanic acid were performed with and without a 5-min pre-incubation of the inhibitor with the β -lactamase.

Results

Challenge experiment

E. coli J62.2 (pUK901) was challenged in IsoSensitest broth containing amoxycillin at concentrations of 0, 128, 256 and 512 mg/L. Growth was observed at all concentrations. Ten purified colonies from each challenge were analysed for their auxotrophic requirements on Davis-Mingioli minimal medium plates; all were confirmed to be *E. coli* J62.2.

β -Lactamase analysis of challenged strains

β -Lactamase production by the challenged strains was analysed by screening cleared lysates of bacterial sonicates with IEF on polyacrylamide gels containing ampholines of pH range 3.5–10, with TEM-1 and TRC-1 β -lactamases as controls. At amoxycillin concentrations of 0, 128 and 256 mg/L, TRC-1 was produced by every isolate tested throughout the 5-day period. However, at an amoxycillin concentration of 512 mg/L, four apparent revertants to TEM-1 were detected on day 4, and by day 5, isolates producing this β -lactamase predominated (six of 10 isolates).

Analysis of strains isolated on day 5 after challenge with amoxycillin 512 mg/L

The 10 purified isolates from the challenge with amoxycillin 512 mg/L were examined in more detail. Initial IEF data suggested that six of these isolates

Table. ID₅₀ of clavulanic acid for TRC-1 and putative TEM-1 β -lactamases obtained from isolates pre- and post-challenge with amoxycillin 512 mg/L for 5 days

β -Lactamase	ID ₅₀ (μM) of clavulanic acid	
	No pre-incubation	With pre-incubation*
TRC-1 control	50	4.2
TRC-1 post-challenge	50	5.0
TEM-1 control	0.54	0.01
TEM-1 revertant	0.80	0.15

*Pre-incubation of clavulanic acid with the β -lactamase for 5 min.

Fig. 2. *EcoRI* restriction endonuclease digests of purified plasmid DNA from strains encoding putative TEM-1 and TRC-1 isolated after challenge with amoxycillin 512 mg/L for 5 days. Tracks: A, λ -HindIII digest; B, *E. coli* (pUK901) unchallenged parent strain; C, as B; D, post-challenge isolate producing putative TEM-1; E, as D; F, post-challenge isolate producing TRC-1; G, as F.

were no longer producing TRC-1, but may have reverted to producing TEM-1. The β -lactamase produced by each isolate was examined on a narrow range IEF gel containing a mixture of ampholines of pH range 3.5–10 and 4–6 (fig. 1). This confirmed that six of the isolates produced a β -lactamase that co-focused with TEM-1.

Plasmid DNA was extracted from the parent *E. coli* J62.2 (pUK901) strain and from isolates producing TRC-1 and TEM-1 after challenge with amoxycillin 512 mg/L for 5 days. Restriction endonuclease analysis of purified plasmid DNA demonstrated that both TEM-1 and TRC-1 producers contained the same plasmid as the parent strain (fig. 2).

Biochemical analysis of TRC-1 and TEM-1 revertants

Biochemical properties of TRC-1 and TEM-1 isolated from post-challenge strains were determined and compared to TRC-1 and TEM-1 from controls. The ID₅₀ values for clavulanic acid were determined and demonstrated that reversion to TEM-1 was accompanied by a loss of resistance to clavulanic acid, with the ID₅₀ decreasing from 4.2 µM for TRC-1 to 0.15 µM for TEM-1 revertants (table).

Discussion

The use of β -lactam antibiotics in combination with β -lactamase inhibitors has proved to be one of the most effective ways of combating β -lactamase-mediated resistance.^{3,4} It was suggested initially that the only likely cause of resistance was from strains that hyperproduce the TEM-1 β -lactamase.^{16,17} However, while such strains do indeed have increased resistance to β -lactam plus β -lactamase inhibitor combinations, the frequency of such strains does not appear to have increased since the introduction of inhibitor combinations into clinical practice.¹⁸ It had been thought unlikely that the TEM-1 β -lactamase would be capable of mutation to resistance to suicide inhibitors, but the TEM β -lactamase has shown a remarkable capacity to evolve to overcome changes in β -lactam design. TRC-1 was the first clinically isolated TEM β -lactamase derivative that showed an increase in resistance to β -lactamase inhibitors.⁹ There have been reports of similar β -lactamases in France,¹⁰ and

possibly from other centres in the UK.¹⁹ Sequence analysis has demonstrated that resistance can result from changes of arginine-244 to cysteine or serine, or a change of methionine-69 to leucine.²⁰ Mutation to increased resistance of TEM-1 to β -lactamase inhibitors is, however, accompanied by a decrease in efficiency against simple penicillins when measured both by kinetic parameters and by MIC.^{11,21}

At present, TEM enzymes resistant to β -lactamase inhibitors appear to be rare, and this may result from their decreased efficiency against penicillins. The findings of this study demonstrate for the first time that it is possible for back-mutations to the parent TEM β -lactamase to be selected. It may be that similar changes are possible *in vivo*, and that the prevalence of these β -lactamases is the result of a two-way flux, with mutations in both the forward and backward directions. In this respect it is tempting to speculate on the role that the relative ratios of inhibitor to β -lactam agent have on the process; it is possible that the greater the amount of inhibitor, the greater the pressure towards TRC-1, and *vice versa*. β -Lactam plus β -lactamase inhibitor combinations are available in the UK only in fixed proportions, although these are reformulated periodically. However, β -lactamase-inhibitors are available in some other countries as separate compounds. The influence of this availability on the selection of resistance has not as yet been addressed.

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