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Bases of variation in resistance to β-lactams in *Klebsiella oxytoca* isolates hyperproducing K1 β-lactamase

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Nineteen isolates of *Klebsiella oxytoca* were examined, representing 18 distinct strains. All were from a 1994 survey of resistance amongst klebsiellae in intensive care units in Europe, and all had reduced susceptibility, or were resistant, to cefuroxime, ceftriaxone and aztreonam, suggesting hyperproduction of the chromosomal K1 β-lactamase. We sought to confirm this mechanism and to identify why the levels of resistance varied between isolates. Possible reasons for variation were differences in the quantity or subtype of the K1 enzyme or differences in this enzyme's interplay with permeability. Spectrophotometric assays showed that all 19 isolates had K1-like β-lactamases and that these were present at ≥15-fold higher levels than in β-lactam-sensitive *K. oxytoca* isolates. Fourteen of the 19 isolates had the OXY-2 form of K1 enzyme, while the remaining five had the OXY-1 form, as determined by isoelectric focusing and PCR amplification. Most isolates with the OXY-2 enzyme were more resistant than those with the OXY-1 subtype, but this difference partly reflected enzyme quantity rather than subtype. More generally, and irrespective of enzyme subtype, levels of resistance were broadly related to β-lactamase specific activity, and the degree of hyperproduction was a major determinant of the level of resistance. Nevertheless, other factors had a role too: several isolates had reduced susceptibility or were resistant to cefoxitin, which is not a substrate for K1 enzyme, and examination of outer membrane protein profiles revealed considerable strain-to-strain diversity in the molecular weight range typical of the major enterobacterial porins (40–48 kDa).

Introduction

Klebsiella oxytoca typically has a molecular class A chromosomal β-lactamase, often called the K1 or KOXY type. This enzyme, which is placed in group 2be in Bush's scheme[1] and in class IV of Richmond & Sykes' classification,[2] is distinct from the chromosomal SHV-1 β-lactamase typical of *Klebsiella pneumoniae*, having greater activity against oxyimino-cephalosporins and lesser affinity for oxacillins.[1] Recently, Fournier *et al.*[3] distinguished two forms of K1 enzyme, which they designated OXY-1 and OXY-2. These give distinct band patterns upon isoelectric focusing; and their genes can be distinguished using specific DNA probes.

Most *K. oxytoca* isolates have a low level of K1 enzyme and are resistant to ampicillin and carboxypenicillins. A few hyperproduce the enzyme and are also resistant to cefuroxime, ceftriaxone and aztreonam. Ceftazidime is largely spared, distinguishing K1 hyperproducers from klebsiellae with TEM- or SHV-derived extended-spectrum enzymes.[4] Hyperproducers may occasionally be selected during therapy,[5] though this is much rarer than the selection of *Enterobacter* mutants derepressed for AmpC β-lactamase.

During a survey of resistance at 35 European intensive care units in 1994, *K. oxytoca* isolates (n = 248) accounted for 25.6% of all klebsiellae collected.[6] Nineteen of these isolates had antibiograms suggesting hyperproduction of K1 enzyme. Nevertheless, MICs of individual β-lactams showed substantial scatter (Table), and the present study sought causes for this variation, seeking relationships with the quantity or subtype of K1 enzyme, or with the enzyme's interplay with cell impermeability.

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Materials and methods

Bacterial cultures

Nineteen isolates of *K. oxytoca* were examined. All were collected during a recent survey of klebsiellae from European intensive care units,[6] and were selected as being resistant or less susceptible (compared with typical *K. oxytoca*) to cefuroxime, ceftriaxone and aztreonam, as well as to penicillins, whilst remaining susceptible to ceftazidime and carbapenems. This pattern is typical of K1 hyperproducers.[4,7] Two cefuroxime- and aztreonam-susceptible strains (nos 710 and 1611, Table) from the same survey were included as controls, together with *K. oxytoca* strains KOSL781 and KOSL911 as reference producers of the OXY-1 and OXY-2 variants of K1 enzyme, respectively.[3]

Molecular typing

Where multiple isolates were from a single centre, they were compared by pulsed-field gel electrophoresis, so as to detect possible replicates. Isolates were inoculated into 10 mL volumes of Todd–Hewitt broth (Unipath, Basingstoke, UK) and incubated overnight. The cells were then pelleted, resuspended in 0.5 mL of 10 mM Tris–HCl pH 7.6 containing 1 M NaCl, mixed with equal volumes of pulsed-field certified agarose 2% (Bio-Rad, Poole, UK) at 50°C, and dispensed into plug moulds. These plugs were incubated overnight at 37°C in 10 mL of 6 mM Tris–HCl pH 7.6 containing 1 M NaCl, 100 mM disodium EDTA, 0.5% Brij 58, 0.2% deoxycholate, 0.5% *N*-lauroylsarkosine; 0.002% RNase and 0.01% lysozyme then overnight at 50°C in 5 mL of 0.5 M disodium EDTA containing 1% *N*-lauroylsarkosine and 0.005% proteinase K. Subsequently, the plugs were washed three times for 30 min in 10 mM Tris–HCl pH 7.5 containing 0.1 mM disodium EDTA. For digestion, three slices of these plugs were incubated overnight with 20 U of *Not*I restriction enzyme (Boehringer Mannheim, Lewes, UK) in 25 μL of the buffer supplied with the enzyme. Electrophoresis was on gels comprising 1% pulsed-field certified grade agarose in 0.5 × TBE (45 mM Tris–borate, 1 mM disodium EDTA buffer) and was run in a CHEF DRII apparatus (Bio-Rad) at 6 V/cm for 14 h with a switching time of 1–6 s. The gels were stained with ethidium bromide 0.5 mg/L and destained in water for 1 h before photography.

Antibiotics

Antimicrobial agents were obtained from suppliers as follows: aztreonam, Bristol Myers Squibb (Hounslow, UK); ceftazidime and cefuroxime, Glaxo (Stevenage, UK); cefotaxime, Roussel (Uxbridge, UK); clavulanate lithium, SmithKline Beecham (Betchworth, UK); ceftriaxone, Roche (Basel, Switzerland); cefoxitin, Merck Sharp and Dohme (Hoddesdon, UK); penicillin G, Sigma (St Louis, MO, USA); nitrocefin, BBL (Cockeysville, MD, USA).

MICs

MICs were determined by spotting 10^4 cfu from overnight nutrient broth cultures on to IsoSensitest agar (Unipath, Basingstoke, UK) containing doubling dilutions of antimicrobials. Results were read after overnight incubation at 37°C as the lowest concentrations to inhibit growth completely.

Isoelectric focusing

Cells from overnight cultures on nutrient agar plates were resuspended in 2 mL of 10 mM phosphate buffer pH 7.0, then disrupted by brief sonication. The supernatants, obtained after centrifugation at 12,000**g** rpm for 15 min, were examined for β-lactamases by isoelectric focusing[8] in polyacrylamide gels containing equal amounts of ampholines (Resolyte, BDH, Poole, UK) of pH ranges 3.5–10 and 5.0–8.0. β-Lactamase was detected by flooding the gel with 0.5 mM nitrocefin in 0.1 M phosphate buffer pH 7.0. β-Lactamases of known pIs were used as standards, and comprised TEM-1 (pI 5.4), TEM-2 (pI 5.6), PSE-2 (pI 6.1) and SHV-1 (pI 7.6).

PCR of the KOXY gene

Template DNA was prepared by suspending four colonies from overnight nutrient agar plate cultures in 0.1 mL of TE buffer (10 mM Tris pH 7.5, 0.1 mM EDTA), and heating at 100°C for 5 min. PCRs were performed in 100 μL with GeneAmp PCR Core Reagents (Perkin–Elmer Cetus, Warrington, UK), using 2.5 mM $MgCl_2$, 100 ng of each primer and 100 ng AmpliTaq polymerase together with 1 μL of template DNA. Primers for amplification of bla_{OXY-1} were as follows: CGTGGCGTAAAACCGCCCTG (corresponding to coordinates 99–118 in the sequence published by Arakawa *et al.*,[9] and GTCCGCCAAGGTAGCTAATC (coordinates 508–527 in the same sequence) giving an amplification product of 428 bp; those for bla_{OXY-2} were: AAGGCTGGAGATTAACGCAG (coordinates 595–614 in the sequence published by Fournier *et al.*[3]) and GCCCGCCAAGGTAGCCGATG (coordinates 731–750 in the same sequence) giving a product of 155 bp. The mixtures were heated to 95°C for 3 min, then subjected to 30 cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 1 min on a Hybaid Thermal Reactor (Teddington, UK). PCR products were analysed on 0.9% agarose minigels.

β-*Lactamase specific activity*

Bacterial cultures were grown overnight, with shaking, in 10 mL of nutrient broth at 37°C, then diluted 20-fold into 200 mL of pre-warmed nutrient broth. Incubation was continued for 4 h, after which the cells were harvested by centrifugation for 10 min at 5000**g** and 37°C, washed once in 10 mL of 0.1 M phosphate buffer pH 7.0, and resuspended in 1.5 mL of the same buffer. β-Lactamases were liberated by two bursts of sonication at amplitude 12 μm. Their ability to hydrolyse 1 mM penicillin G, cefuroxime and cefotaxime was examined by UV spectrophotometry at wavelengths of 235 nm, 264 nm and 255 nm, respectively. The light-path was 1 cm for penicillin G and 1 mm for the cephalosporins. All assays were at 37°C in 0.1 M phosphate buffer pH 7.0. β-Lactamase activities were standardized against protein content, as measured with the Micro BCA Protein Assay Reagent (Pierce, Rockford, IL, USA).

Outer membrane proteins

Starter cultures were grown overnight at 37°C with shaking in 100 mL of nutrient broth, then added to 1 L amounts of the same broth, pre-warmed to 37°C. Incubation was continued for 4 h, after which the cells were harvested at 5000**g** and 37°C, washed once in 100 mL of 30 mM Tris–HCl pH 8.0 and resuspended in 20 mL of the same buffer. These suspensions were chilled to 4°C and passed three times through a French pressure cell (SLM Aminco, Urbana, IL, USA) at 12,000 lb/in^2, then centrifuged at 5000**g** and 4°C for 10 min to remove debris. The supernatants were re-centrifuged at 100,000**g** for 30 min at 4°C to pellet the membranes. These were resuspended in 10 mL of 30 mM Tris–HCl pH 8.0, to which was added 1 mL of 20% sodium lauroyl sarkosinate. After standing for 20 min at room temperature, the preparations were centrifuged at 100,000**g** for 30 min at 4°C to pellet the outer membranes, which were resuspended in 2 mL of distilled water and stored at –20°C.

Before electrophoresis, the outer membrane preparations were adjusted to a protein concentration of 1 mg/mL and mixed with equal volumes of sample buffer, comprising 0.25 M Tris–HCl buffer pH 6.8, 20 % glycerol, 10% β-mercaptoethanol, 4% SDS and saturated 1% aqueous Bromophenol Blue. After heating to 100°C for 10 min, 15 μL volumes were loaded on to polyacrylamide gels prepared as described by Hancock & Carey,[10] and run with the buffers of Lugtenberg *et al.*[11] Electrophoresis was at 30 mA as the samples traversed the stacking gel, then at 60 mA/gel. Proteins were stained with Coomassie Blue.

Results

Molecular typing

Isolates 1757 and 1785, from the same Italian hospital, gave identical DNA profiles and probably were replicates (not shown); otherwise, multiple isolates from the same centre gave different profiles and were considered to be unrelated.

MICs

The 19 isolates studied varied widely in their degree of resistance, but were all 16- to 1024-fold less susceptible than the control strains to aztreonam, ceftriaxone and cefuroxime (Table), whereas susceptibility to ceftazidime was reduced ≤16-fold. The behaviour of cefotaxime was intermediate between that of ceftriaxone and ceftazidime, with the MICs for the present isolates up to 32-fold higher than those for the controls, but still ≤2 mg/L. Cefoxitin MICs were 2–32 mg/L, compared with 2–4 mg/L for the control organisms. Ceftazidime susceptibility was increased by up to four-fold by the addition of clavulanate.

MICs of aztreonam, ceftriaxone, cefuroxime, cefotaxime and ceftazidime, but not those of cefoxitin, were correlated, so that isolates with particularly high resistance to one of these agents were also resistant to the others.

β-*Lactamase identification*

Most of the 19 isolates gave multiple β-lactamase bands in isoelectric focusing but, generally single major bands could be identified, with isoelectric points (pIs) between 5.2 and 7.6 (Table). Isolates that gave a major band at pI 7.6 gave the predicted 428 bp amplification product in PCR with the OXY-1 primers, but no product with the OXY-2 primers, whereas isolates with principal β-lactamase bands at pIs 5.2, 5.6, 5.9, 6.1 or 6.5 gave the predicted 155 bp product with the OXY-2 primers, but no product with the OXY-1 primers (Figure 1).

β-*Lactamase specific activities*

All except two of the 19 aztreonam- and cefuroxime-resistant isolates had β-lactamase specific activities exceeding 3 μmol penicillin G hydrolysed/min/mg protein, whereas the specific activities of the cefuroxime- and aztreonam-susceptible isolates 710 and 1611, and the reference producers of OXY-1 and -2 enzymes, were below 0.020 μmol penicillin G hydrolysed/min/mg protein (Table). The remaining two aztreonam- and cefuroxime-resistant isolates, nos 864 and 1333, had β-lactamase specific activities of 1.2 and 0.3 μmol penicillin hydrolysed/min/mg protein respectively. Notably, these were the most cefoxitin-resistant isolates but they had relatively modest levels of resistance to cefuroxime (MIC 32 mg/L) and aztreonam (MIC 2–8 mg/L).

Relative hydrolysis rates for 1 mM cefuroxime ranged from 2.2 to 14.2% of the rate for penicillin G, whereas those for cefotaxime were 0.8–7.1% of that for penicillin G. The relative rates were unrelated to whether OXY-1 or OXY-2 enzyme was produced (Table).

Table. Antimicrobial susceptibility and β-lactamase production characteristics of the *K. oxytoca* isolates studied

Isolate	MIC (mg/L)							Specific activity *versus* PEN-G[b]	Relative activity[c]			β-Lactamase	
	AZT[a]	CTZ	CTZ + CLAV	CTR	CTX	CXM	FOX		PEN-G	CXM	CTX	pI	OXY gene
1757	2	0.12	0.06	1	0.25	32	1	3.4	100	8.6	–[d]	5.9	OXY-2
1785	2	0.12	0.06	2	0.25	32	2	3.9	100	9.8	2.1	5.9	OXY-2
635	2	0.12	0.03	1	0.5	32	2	10.5	100	2.4	1.9	7.6	OXY-1
1333	2	1	1	0.5	0.5	32	32	0.3	100	ND	ND	5.9 + 7.6	OXY-2
715	4	0.25	0.12	2	0.25	32	2	14.1	100	2.2	0.8	7.6	OXA-1
717	8	0.12	0.06	4	0.5	128	1	12.3	100	8	2	5.9	OXY-2
226	8	0.25	0.12	8	1	128	2	20.5	100	8.4	1.6	5.2	OXY-2
1005	8	0.25	0.12	4	1	128	2	11	100	7.2	0.7	6.1	OXY-2
1154	8	0.12	0.25	2	0.12	64	8	6.2	100	2.4	1	7.6	OXY-1
864	8	1	0.5	8	1	32	32	1.2	100	6	7.1	7.6	OXY-1
188	16	0.25	0.12	8	0.5	128	2	13	100	8	1.8	5.2	OXY-2
26	16	0.12	0.06	2	0.5	32	4	4.7	100	14.2	4.5	7.6	OXY-1
670	32	1	0.5	8	1	256	2	21.7	100	7.8	1.6	5.9	OXY-2
680	32	1	0.25	8	0.5	128	8	16.3	100	7.2	1.3	6.1	OXA-2
768	64	1	1	16	2	256	16	16.5	100	7.6	1.4	5.9	OXY-2
1923	128	2	0.5	16	0.12	256	8	13	100	7.5	1.3	5.2	OXY-2
1980	128	2	0.5	32	0.12	512	8	13	100	7.5	1.6	5.6 + 7.6	OXY-2
453	128	2	0.5	32	2	256	16	19	100	6.2	1.5	6.5–7.0	OXY-2
620	128	2	0.5	16	2	256	16	17	100	8.1	1	5.9	OXY-2
Controls													
710	0.06	0.25	0.25	0.25	0.06	4	4	⩽0.02	–	–	–	ND	ND
1611	0.12	0.5	0.25	0.03	0.06	2	4	⩽0.02	–	–	–	ND	ND
Mode[e]	0.12	0.12	0.06	0.06	ND	2	2	ND	ND	ND	ND	ND	ND

[a]Abbreviations: AZT, aztreonam; CLAV, clavulanic acid at 4 mg/L; CTR, ceftriaxone; CTX, cefotaxime; CTZ, ceftazidime; CXM, cefuroxime; FOX, cefoxitin; PEN-G, penicillin G; pI, isoelectric point; ND, not done.

[b]Units: μmol hydrolysed/min/mg protein.

[c]Relative to penicillin G, defined as 100%.

[d]Activity too low for accurate assay.

[e]Mode MICs for 248 *K. oxytoca* isolates collected in the survey of Livermore & Yuan.[6]

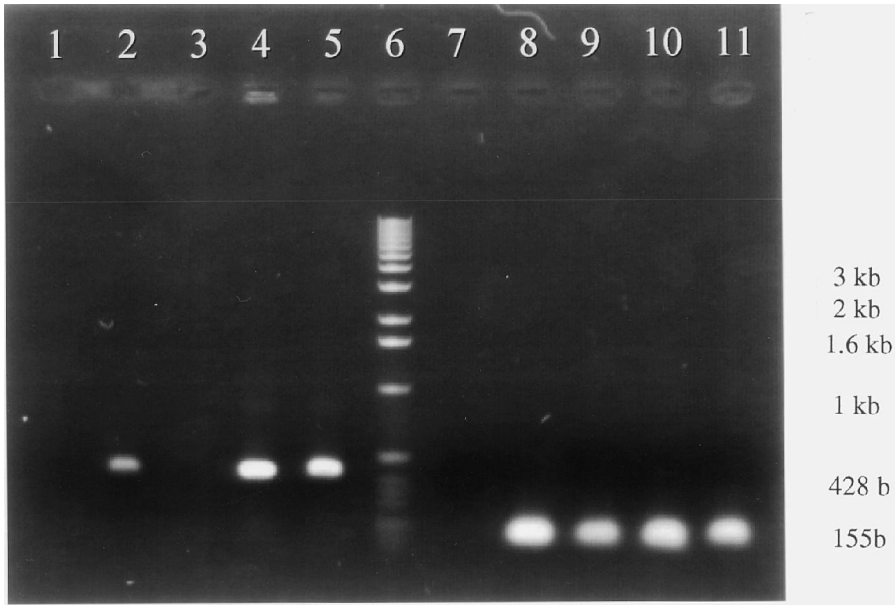

Figure 1. Gel of β-lactamase gene PCR products for representative isolates. Lanes contain PCR products as follows: 1, negative control with no DNA added; 2, reference producer of OXY-1 β-lactamase with bla_{OXY-1} primers; 3, reference producer of OXY-2 β-lactamase with bla_{OXY-1} primers; 4, isolate 715 with bla_{OXY-1} primers; 5, isolate 864 with bla_{OXY-1} primers; 6, 1 kb DNA Ladder (Gibco-BRL Life Technologies, Paisley, UK); 7, reference producer of OXY-2 β-lactamase with bla_{OXY-1} primers; 8, reference producer of OXY-2 β-lactamase with bla_{OXY-2} primers; 9, isolate 620 with bla_{OXY-2} primers; 10, isolate 670 with bla_{OXY-2} primers; 11, isolate 680 with bla_{OXY-2} primers.

Outer membrane proteins

Outer membrane protein (OMP) profiles of the isolates are shown in Figure 2, with panel (a) illustrating those for organisms with cefoxitin MICs of ≤4 mg/L and panel (b) showing those for organisms with MICs of ≥8 mg/L. For both groups there was some scatter in the OMP band patterns and intensities in the molecular weight range 40–48 kDa.

Discussion

During a recent survey of klebsiellae from European ICUs[6] we collected 19 *K. oxytoca* isolates with antibiograms suggesting hyperproduction of K1 β-lactamase. The present study aimed to confirm this mechanism and to determine why the isolates varied in their levels of resistance to substrates for K1 enzyme, specifically aztreonam, cefuroxime and ceftriaxone. MICs of these three agents were inter-related (Figure 3) and were also related to those of ceftazidime and ceftriaxone, which are weaker substrates for the K1 enzyme and which consistently retained antibacterial activity at ≤2 mg/L. By contrast, the MICs of cefoxitin, which is stable to K1 enzyme, remained independent of those of the other compounds (Figure 3). The greater resistance to substrates for K1 enzyme implied that this enzyme was the primary cause of resistance but did not explain why resistance varied quantitatively between isolates. Hypothetically, variation might reflect the amount or subtype of K1 enzyme, or its interplay with other resistance determinants, and these possibilities were investigated.

Each of the 19 isolates gave multiple β-lactamase bands in isoelectric focusing, and the principal bands from different isolates showed a wide scatter of pIs, from 5.2 to 7.6. Variable electrofocusing is typical of K1 β-lactamase[12] and complicates its recognition. Nevertheless, like Fournier *et al.*,[3] we found good agreement between electrofocusing behaviour and enzyme genotype, in this case determined by PCR. Isolates with principal β-lactamase bands at pI 7.6 gave an amplification product with bla_{OXY-1} primers but not with bla_{OXY-2} primers, whereas isolates with pI 5.2, 5.6, 5.9, 6.1, 6.5 enzymes gave a product with the bla_{OXY-2} primers but not with bla_{OXY-1} primers. Detection of bla_{OXY-1} or bla_{OXY-2} does not, of course, prove that K1 enzyme is hyperproduced—all *K. oxytoca* strains have one or other gene, which may be expressed strongly or not.[3,7] Evidence for hyperproduction of K1 enzyme did, however, come from the observation of high β-lactamase specific activities in most of the isolates, as compared with sensitive controls, coupled with the relative constancy of the hydrolysis rate ratios of penicillin G:cefuroxime:cefotaxime. The possibility that some strains had other β-lactamases besides K1 enzyme cannot be excluded; secondary activities might have been masked by the subsidiary bands of the K1 enzyme. Nevertheless, it is

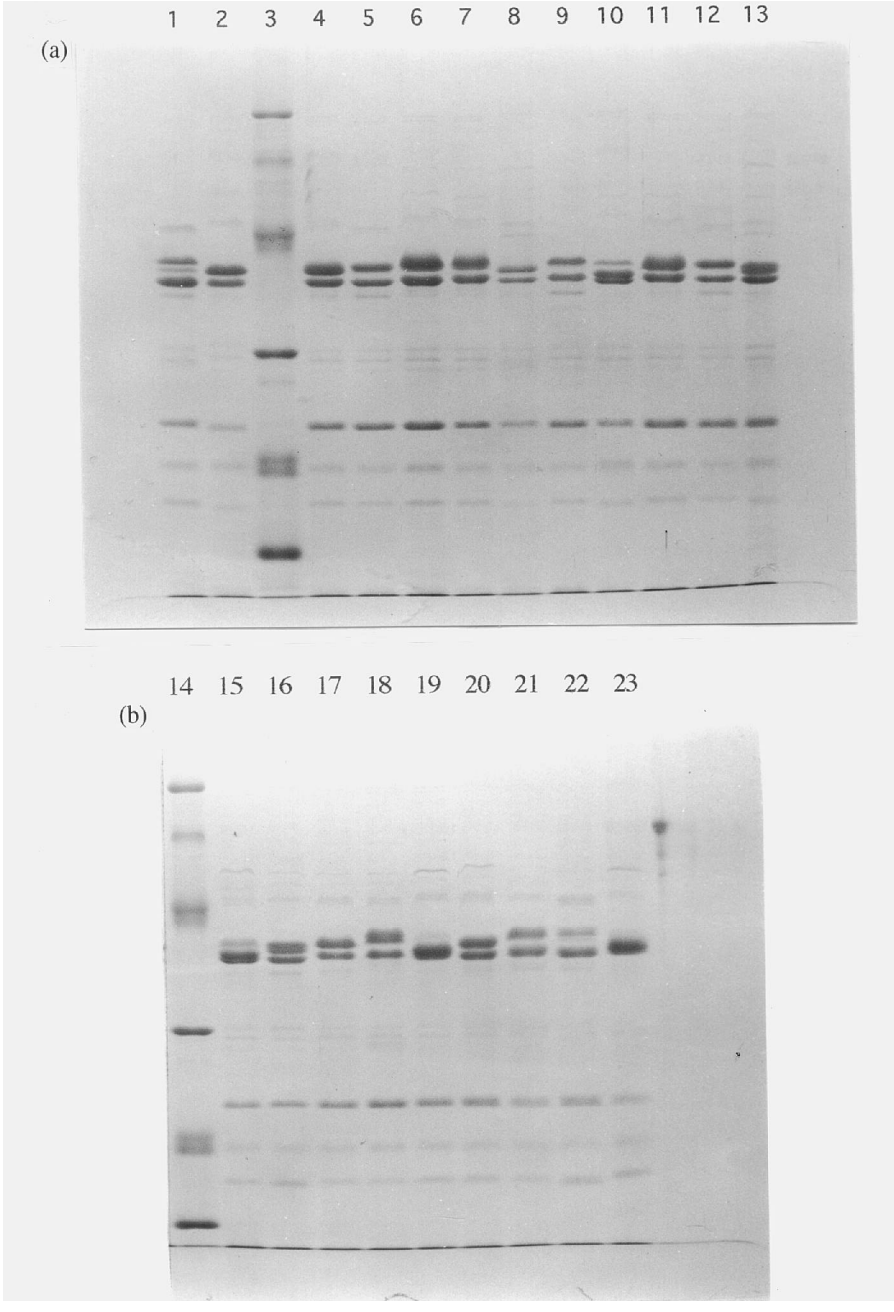

Figure 2. OMP profiles of *K. oxytoca* isolates in relation to cefoxitin MICs. (a) Isolates with cefoxitin MICs of ≤4 mg/L: lane 1, isolate 710; lane 2, isolate 1611; lane 4, isolate 717; lane 5, isolate 1757; lane 6, isolate 188; lane 7, isolate 226; lane 8, isolate 635; lane 9, isolate 670; lane 10, isolate 715; lane 11, isolate 1005; lane 12, isolate 1785; lane 13, isolate 26. (b) Isolates with cefoxitin MICs of ≥8 mg/L: lane 15, isolate 680; lane 16, isolate 1154; lane 17, isolate 1923; lane 18, isolate 1980; lane 19, isolate 453; lane 20, isolate 620; lane 21, isolate 768; lane 22, isolate 864; lane 23, isolate 1333. Lanes 3 and 14 contain the following molecular weight markers: rabbit muscle phosphorylase B (97,400), bovine serum albumin (66,200), ovalbumin (45,000), bovine carbonic anhydrase (31,000), soya bean trypsin inhibitor (21,500) and lysozyme (14,400).

unlikely that further enzymes contributed to resistance to newer β-lactams, both because of the consistency of the hydrolysis rate ratios (Table), and because of the lack of substantial resistance to ceftazidime, which is a good substrate for most extended-spectrum β-lactamases except K1 type.

Isolates with the OXY-1 variant were less resistant than those with OXY-2 (Figures 3 and 4), but comparison was complicated by the fact that only five of the 19 isolates had the OXY-1 enzyme and that the β-lactamase specific activities of all but one of these isolates were in the lower half of the distribution range (Figure 4). When only

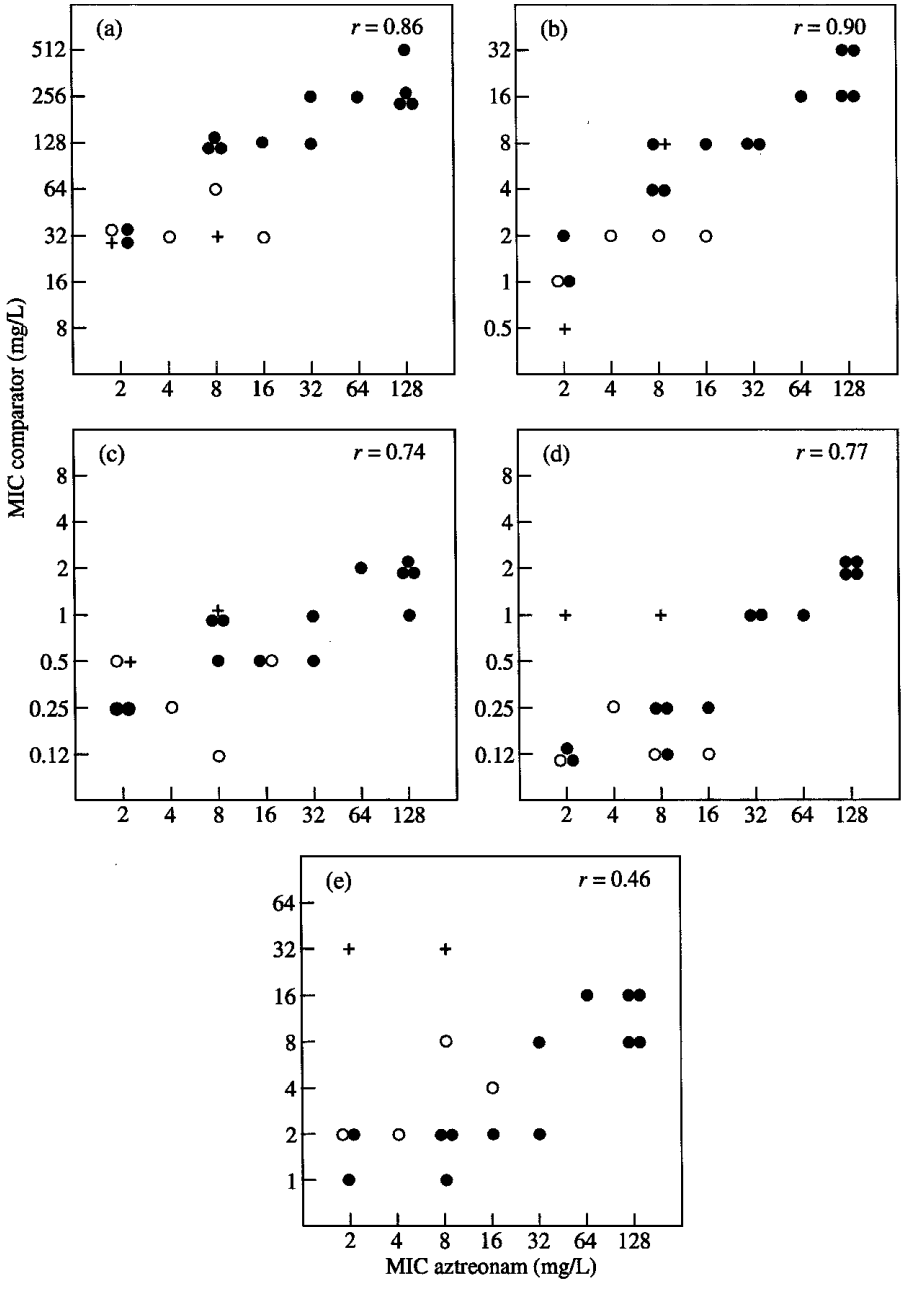

Figure 3. Scatter-gram showing correlation between MICs of aztreonam (which was taken as a reference compound) and (a) cefuroxime, (b) ceftriaxone, (c) cefotaxime, (d) ceftazidime and (e) cefoxitin. Symbols: ○, isolates hyperproducing OXY-1 β-lactamase; ●, isolates hyperproducing OXY-2 β-lactamase; +, isolates 864 and 1333, which had the lowest β-lactamase specific activities but which were amongst the most resistant to cefoxitin (Table). *r* is the correlation coefficient between the log MICs.

isolates with similar levels of β-lactamase activity were compared, the tendency of OXY-1 producers to be less resistant than those with OXY-2 enzyme was much less apparent. Generally, there was better correlation between β-lactamase specific activity, irrespective of enzyme type and level of resistance to substrate drugs, particularly cefuroxime and ceftriaxone (Figure 4), suggesting that the major determinant of the level of resistance was the amount of K1 enzyme manufactured. β-Lactamase quantity was not, however, the sole regulator of resistance level, as evidenced by the fact that the MICs of cefoxitin, which is not a substrate for K1 enzyme, varied 16-fold among the isolates (Table). Notably, isolates 864 and 1333, which had the lowest β-lactamase specific activities of all the test strains, had the highest levels of resistance to cefoxitin. It follows that some other mechanism—probably impermeability but maybe efflux—must have modulated resistance to cefoxitin, and it seems likely that this

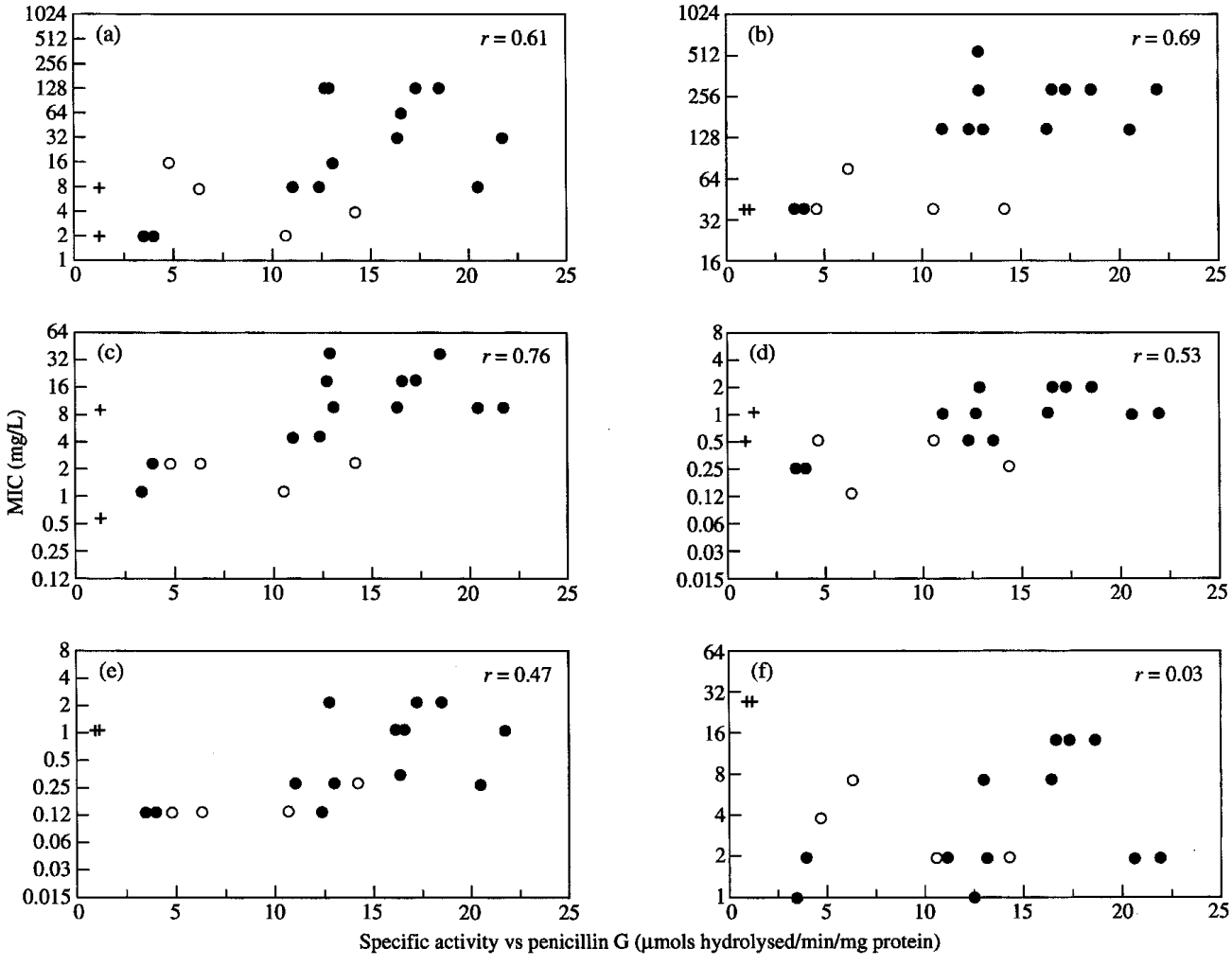

Figure 4. Correlation between β-lactamase specific activity *versus* penicillin G and MICs of (a) aztreonam, (b) cefuroxime, (c) ceftriaxone, (d) cefotaxime, (e) ceftazidime and (f) cefoxitin. Symbols as in Figure 3. *r* is the correlation coefficient between log MIC and specific activity.

mechanism would also have affected substrates for K1 enzyme. Attempts to show a relationship between level of cefoxitin resistance and OMP profile were only partly successful, as considerable strain-to-strain variation in OMP pattern was found in the molecular weight range typical for enterobacterial porins (40–48 kDa). No single protein loss was clearly associable with elevated resistance.

In summary, we investigated 19 *K. oxytoca* isolates, collected in a recent survey, that had cross-resistance to aztreonam, cefuroxime and ceftriaxone. Hyperproduction of K1 enzyme was inferred from antibiogram data and confirmed by measurement of enzyme quantity. The level of resistance to substrates, including aztreonam, cefuroxime and ceftriaxone, was related to the degree of hyperproduction of the enzyme, but was also modulated by other factors. Hyperproduction of K1 enzyme is frequent: the present 19 isolates represent 7.7% of the *K. oxytoca* isolates collected in the survey, and were obtained at 15 of the 35 centres that contributed cultures. This compares with only 15 (6%) of the *K. oxytoca* isolates, from six participating centres, that had extended-spectrum TEM or SHV β-lactamases.[6] Moreover, on at least one occasion, selection of a K1 hyperproducer during ceftriaxone therapy has been recorded in a patient initially infected by a strain that produced a low level of the enzyme.[5]

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