

## Multiple antibiotic-resistance mechanisms including a novel combination of extended-spectrum $\beta$ -lactamases in a *Klebsiella pneumoniae* clinical strain isolated in Argentina

Roberto Melano<sup>1</sup>, Alejandra Corso<sup>1</sup>, Alejandro Petroni<sup>1</sup>, Daniela Centrón<sup>2</sup>, Betina Orman<sup>2</sup>,  
Adriana Pereyra<sup>3</sup>, Noemí Moreno<sup>3</sup> and Marcelo Galas<sup>1\*</sup>

<sup>1</sup>Servicio Antimicrobianos, Dpto. Bacteriología, INEI-ANLIS 'Dr Carlos G. Malbrán', Av. Velez Sársfield 563 (1281), Buenos Aires; <sup>2</sup>Dpto. Microbiología, Facultad de Medicina, Universidad Nacional de Buenos Aires, Paraguay 2155, P. 12 (1120), Buenos Aires; <sup>3</sup>Servicio de Bacteriología, Hospital 'Governador Centeno', Calle 17 y 108, General Pico, Provincia de La Pampa, Argentina

Received 6 December 2002; returned 20 January 2003; revised 2 April 2003; accepted 7 April 2003

*Klebsiella pneumoniae* M1803, isolated from a paediatric patient with chronic urinary infection, presented nine antimicrobial resistance mechanisms harboured on two conjugative megaplasms, in addition to the chromosomally mediated SHV-1  $\beta$ -lactamase. These nine antimicrobial resistance mechanisms comprised two extended-spectrum  $\beta$ -lactamases (ESBLs) (PER-2 and CTX-M-2), TEM-1-like, OXA-9-like, AAC(3)-IIa, AAC(6')-Ib, ANT(3'')-Ia and resistance determinants to tetracycline and chloramphenicol. During fluoroquinolone treatment, a variant derived from M1803 (named M1826) was selected, with an overall increase of MICs, in particular of cefoxitin and carbapenems. No enzymic activity against these latter drugs was found. Mutations in the region analogous to the quinolone resistance-determining region were not found. Strain M1826 was deficient in OmpK35/36 expression, which produced the decrease in the susceptibility to cefoxitin, carbapenems and fluoroquinolones. The *bla*<sub>CTX-M-2</sub> gene was located in an unusual class 1 integron, which includes Orf513, as occurred in the recently described In35. In addition, Tn3 and Tn1331 were detected in both *K. pneumoniae* isolates. This is the first report of *in vivo* selection of an OmpK35/36 deficiency in a *K. pneumoniae* strain that produced a novel combination of two ESBLs (CTX-M-2 and PER-2) during fluoroquinolone treatment in a paediatric patient with chronic urinary infection.

Keywords: CTX-M-2, PER-2, In35, Tn3, Tn1331

### Introduction

*Klebsiella pneumoniae* is a common nosocomial pathogen causing severe morbidity and mortality in paediatric patients. Most *K. pneumoniae* isolates have a chromosomally encoded SHV-1  $\beta$ -lactamase.<sup>1</sup> Since 1983, plasmid-encoded extended-spectrum  $\beta$ -lactamases (ESBLs) derived from the TEM and SHV families have been extensively reported in Enterobacteriaceae, especially in *Klebsiella* spp.<sup>2,3</sup> Although ESBLs have been identified in Argentina, their prevalences are different from those observed in other countries. A national surveillance of extended-spectrum cephalosporin resistance in *Klebsiella* spp. was performed through the WHONET-Laboratory Network Argentina. The unique ESBLs detected among

the 200 isolates analysed in this surveillance were CTX-M-2-like (>60% of isolates) and PER-2-like, SHV-5-like and SHV-2-like (~10% of isolates in each case).<sup>4</sup>

*K. pneumoniae* producing two or more ESBLs have been reported scarcely, but when they are, in most cases they include TEM- and/or SHV-derived enzymes.<sup>5–11</sup> Herein we report the characterization of a multiresistant *K. pneumoniae* isolate that produces a novel combination of two ESBLs (CTX-M-2 and PER-2) and a derived variant of this strain, which was selected during ciprofloxacin treatment and showed alterations in the expression levels of outer membrane proteins (OMPs).

\*Corresponding author. Tel/Fax: +54-11-43032812; E-mail: mgalas@anlis.gov.ar

Materials and methods

Patient and bacterial strains

*K. pneumoniae* M1803 and M1826 were isolated from a 1-year-old patient who suffered chronic urinary tract infection due to a posterior urethral valve malformation and bilateral vesicourethral reflux. During a period of 5 months, the child was treated for successive urinary infections with amphotericin (Gador, Buenos Aires, Argentina), ceftriaxone (Roche), furazolidone (nitrofurantoin analogue; Schering-Plough), amoxicillin (Roche), imipenem (imipenem/cilastatin; Merck Sharp & Dohme) and ciprofloxacin (Bayer). During one of these infections, *K. pneumoniae* strain M1803 was isolated. After 10 days of ciprofloxacin therapy, *K. pneumoniae* strain M1826 was isolated.

*Escherichia coli* ER1793 [F<sup>-</sup> *fluA2* Δ(*lacZ*)*r1 glnV44 e14*<sup>-</sup> (*McrA*<sup>-</sup>) *trp-31 his-1 rpsL104(Str<sup>R</sup>) xyl-7 mtl-2 metB1* Δ(*mcrC-mrr*)114::IS10; New England Biolabs, Beverly, MA, USA], resistant to nalidixic acid and rifampicin, was employed as the recipient strain in conjugative assays.

Antimicrobial agents and susceptibility testing

The antimicrobial agents used for susceptibility testing were obtained from standard laboratory powders, and their sources were as follows: amikacin, aztreonam and cefepime (Bristol-Myers Squibb); amoxicillin and clavulanic acid (Smith Kline Beechman and Roemmers, Buenos Aires, Argentina); ampicillin (Temis Lostaló, Buenos Aires, Argentina); chloramphenicol (Parke Davis, Buenos Aires, Argentina); cefotaxime (Argentina, Buenos Aires, Argentina); ceftaxime and imipenem (without cilastatin) (Merck Sharp & Dohme); ceftazidime (Glaxo); ceftibuten, gentamicin, sulfamethoxazole and trimethoprim (Schering Plough); cefalothin, piperacillin and tazobactam (Wyeth Pharmaceuticals); tetracycline (Microsules and Bernabó, Buenos Aires, Argentina); meropenem (Astra Zeneca); and ciprofloxacin (Bagó, Buenos Aires, Argentina). The antimicrobial susceptibility of each isolate and its transconjugants was determined by the disc diffusion and agar dilution methods according to NCCLS guidelines.<sup>12-15</sup> When indicated, clavulanic acid (2 mg/L) or tazobactam (4 mg/L) was used in combination with β-lactams.

Table 1. Primers for PCR analysis of antimicrobial resistance mechanisms

Target	Primer	No. <sup>a</sup>	Oligonucleotide sequence	Ref.
<i>bla</i> <sub>CTX-M</sub>	<i>bla</i> <sub>CTX-M</sub> F <sup>b</sup>		CGGAATTCATGATGACTCAGAGCATTCG	16
	<i>bla</i> <sub>CTX-M</sub> R <sup>b</sup>		GCTCTAGATTATTGCATCAGAAACCGTG	
<i>bla</i> <sub>PER</sub>	<i>bla</i> <sub>PER</sub> F		GTAGTATCAGCCCAATCCCC	16
	<i>bla</i> <sub>PER</sub> R		CCAATAAAGGCCGTCCATCA	
<i>bla</i> <sub>TEM</sub>	OT-1		TTGGGTGCACGAGTGGGTTA	23
	OT-2		TAATTGTTGCCGGGAAGCTA	
<i>bla</i> <sub>SHV</sub>	OS-1		TCGGGCCGCGTAGGCATGAT	23
	OS-2		AGCAGGGCGACAATCCCGCG	
<i>aac</i> (3)-IIa	<i>aac</i> C2 F		CGCTAAACTCCGTTACC	this work
	<i>aac</i> C2 R		TAGCACTGAGCAAAGCC	
<i>aph</i> (3')-VIa	<i>aph</i> (3')-VIa F		GCCGATGTGGATTGCGAAAA	this work
	<i>aph</i> (3')-VIa R		GCTTGATCCCCAGTAAGTCA	
<i>gyrA</i>	GyrA F		ACGTATTGGCGAYGACTGGA	this work
	GyrA R		AAGAGACGGTCGATTTCGTTG	
Class 1 integrons <sup>c</sup>				
<i>bla</i> <sub>CTX-M</sub>	CTXM-F	1	CGTCGGGATATTCTGGC	30
	CTXM-R	2	TCACTTTATCGGGACCAC	
5'-CS <sup>d</sup>	5'-CS	3	GGCATCCAAGCAGCAAG	22
<i>ant</i> (2'')-Ia	<i>ant</i> (2'')-Ia	4	CCGCAGCTAGAATTTTG	22
	<i>ant</i> (2'')-Ia-COOH	5	ACGTTGAGGTCTTGCGT	this work
<i>sull</i>	<i>sull</i> R	6	TTTGAAGGTTTCGACAGC	30
<i>orf513</i>	ORF513F	7	ATGGTTTCATGCGGGTT	this work
3'-CS <sup>d</sup>	3'-CS	8	AAGCAGACTTGACCTGA	22
Tn1331 <sup>c</sup>				
<i>mpA</i>	Tn3F	9	AAGTTCATCGGGTTCGC	this work
<i>aac</i> (6')-Ib	<i>aac</i> (6')-Ib <sup>e</sup>	10	TGTGACGGAATCGTTGC	22
<i>bla</i> <sub>OXA-9</sub>	<i>bla</i> <sub>OXA-9</sub>	11	GAACACCAACATATGCA	this work
<i>ant</i> (3'')-Ia	<i>ant</i> (3'')-Ia <sup>e</sup>	12	TCGATGACGCCAACTAC	22
	<i>ant</i> (3'')-Ia-COOH <sup>e</sup>	13	CGCAGATCAGTTGGAAG	this work

F, forward; R, reverse.

<sup>a</sup>Numeration corresponding to primers employed in PCR mapping (see Figure 3).

<sup>b</sup>Used to obtain the sequenced amplicon.

<sup>c</sup>Different primer combinations were made in PCR cartography for detection of class 1 integrons and Tn1331 transposon.

<sup>d</sup>5' and 3' conserved segments of class 1 integrons.

<sup>e</sup>These primers were also used in the PCR cartography of class 1 integrons.<sup>22</sup>

Nalidixic acid and rifampicin (both for use in molecular biology applications) were from Sigma (St Louis, MO, USA).

#### Isoelectric focusing and microbiological method for detection of $\beta$ -lactamases

$\beta$ -Lactamase preparation and analytical isoelectric focusing (IEF) were performed as described previously.<sup>16</sup>  $\beta$ -Lactamase bands were visualized by the iodometric method described by Labia & Barthélémy.<sup>17</sup> For  $\beta$ -lactamase inhibition procedures the gel was covered with a piece of filter paper impregnated with 1 mM clavulanic acid, aztreonam or ceftoxitin as indicated, and incubated for 10 min at room temperature before developing as described above. Crude preparations from bacteria possessing  $\beta$ -lactamases of known isoelectric points (pI) were used as standards: TEM-1 (pI 5.4), PER-2 (pI 5.4), SHV-2 (pI 7.6), P99 (pI 7.8), CTX-M-2 (pI 7.9) and SHV-5 (pI 8.2).

The hydrolytic activity against ceftoxitin and carbapenems was determined by the microbiological method.<sup>18</sup>

#### OMP analysis

OMPs were isolated as sodium lauroyl sarcosinate-insoluble material from cell envelopes after sonication (20 pulses of 30 s in ice at 30 s intervals) and centrifugation of lysed cells at 100 000g for 1 h at 4°C.<sup>19</sup> Electrophoretic analysis of OMPs was performed as previously reported,<sup>19</sup> using 12% SDS-PAGE gels and broad molecular weight markers (unstained; Bio-Rad, Hercules, CA, USA) as standards.

#### Conjugative assays

Biparental conjugations were performed by mixing the donor and recipient strains on Luria–Bertani agar in a ratio of 5:1, and incubating the mixture for 18 h at 35°C. Transconjugants were selected on Mueller–Hinton agar supplemented with nalidixic acid (50 mg/L) plus cefotaxime (8 mg/L), or nalidixic acid plus ceftazidime (15 mg/L).

#### General molecular biology procedures

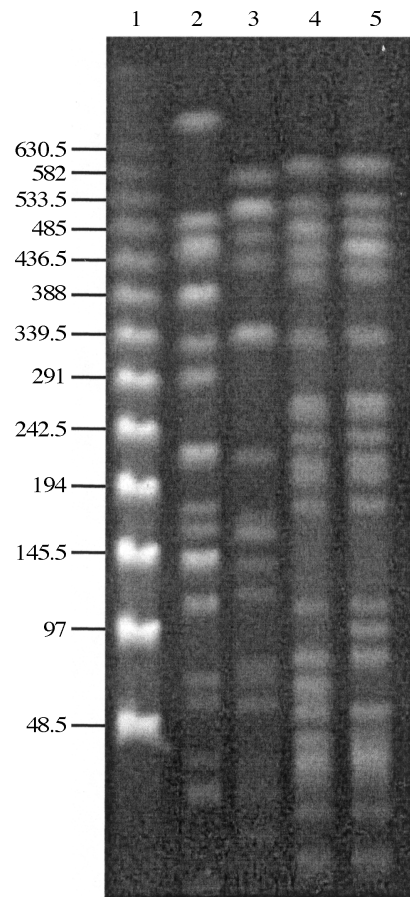
The relatedness of *K. pneumoniae* M1803 and M1826 was assessed by pulsed-field gel electrophoresis (PFGE) of the *Xba*I-digested DNA using conditions already described.<sup>20</sup> Plasmid DNA was extracted from *K. pneumoniae* and *E. coli* transconjugant cells as described previously,<sup>21</sup> and were analysed by electrophoresis in 0.7% agarose gels (Tris–acetate buffer), employing plasmids of known size as standards.

#### PCR assays

Detection of antimicrobial resistance genes was performed by PCR using the forward and reverse primers listed in Table 1. DNA templates were prepared by lysing one or two colonies of each *K. pneumoniae* isolate, or each *E. coli* transconjugant, in 50  $\mu$ L of boiling water, and 2  $\mu$ L was used for the PCRs. Amplifications were performed with a Biometra thermal cycler (Whatman Biometra GmbH, Göttingen, Germany) in a final volume of 50  $\mu$ L containing 20 pmol of each primer (purchased from Gibco-BRL, Gaithersburg, MD, USA), 25  $\mu$ M of each dNTP, 1.5 mM MgCl<sub>2</sub> and 2.5 U of *Taq* polymerase (Promega, Madison, WI, USA). Previously described conditions for cycling were used.<sup>16,22,23</sup>

#### DNA sequencing

DNA sequencing of amplicons obtained with primers specific to *bla*<sub>CTX-M</sub>, *bla*<sub>PER</sub> and *gyrA* [including the region analogous to the quinolone resistance-determining region (QRDR)] genes (Table 1) was performed on both strands by the method of Sanger *et al.*,<sup>24</sup> using the BigDye terminators methodology (Applied Biosystems/Perkin Elmer,



**Figure 1.** PFGE patterns of *K. pneumoniae* strains. Chromosomal DNA was digested with *Xba*I endonuclease. Lane 1,  $\lambda$  ladder; lane 2, *K. pneumoniae* ATCC 700603; lane 3, *K. pneumoniae* ATCC 10031; lane 4, *K. pneumoniae* M1803; lane 5, *K. pneumoniae* M1826.

Foster City, CA, USA). The sequences were analysed in an ABI Prism 377 DNA Sequencer (Applied Biosystems/Perkin Elmer).

## Results and discussion

#### Antibiotic susceptibility and plasmid profiles of *K. pneumoniae* M1803 and M1826, and *E. coli* transconjugants

*K. pneumoniae* M1803 and M1826 showed less than three differences according to PFGE profiling, indicating the clonal relationship between both isolates (Figure 1).<sup>25</sup> In contrast to M1803, isolate M1826 was resistant to ceftoxitin and ceftibuten, showing a 4–32 $\times$  increase in the MICs of extended-spectrum cephalosporins, carbapenems, aztreonam, piperacillin–tazobactam, chloramphenicol and ciprofloxacin. In addition, clavulanic acid produced almost no reduction in the MICs of extended-spectrum cephalosporins against isolate M1826 (Table 2).

For strains M1803 and M1826, two different types of *E. coli* transconjugants were obtained, named type 1 and type 2 (Table 2). Type 1 transconjugants (M3089 and M3090, derived from M1803 and M1826 strains, respectively) were selected with nalidixic acid plus ceftaxime, and were resistant to ceftaxime, cefepime and gentamicin. Type 2 transconjugants (M3129 and M3130, derived from M1803 and M1826 strains, respectively) were selected with nalidixic acid plus ceftazidime, and were resistant to ceftazidime, ceftibuten, chloramphenicol, tetracycline and gentamicin, and showed

## Novel ESBL combination in *K. pneumoniae*

**Table 2.** Antibiotic susceptibility [MIC (mg/L)], plasmid content, IEF of  $\beta$ -lactamases and PCR mapping of antimicrobial resistance mechanisms in *K. pneumoniae* isolates and *E. coli* transconjugants

	<i>K. pneumoniae</i>		<i>E. coli</i>		
	M1803	M1826	type 1 <sup>a</sup>	type 2 <sup>b</sup>	ER1793
(i) Antimicrobial susceptibility					
ampicillin	>1024	>1024	>1024	>1024	4
co-amoxiclav	16	32	16	8	4
cefalothin	>1024	>1024	>1024	512	8
cefoxitin	2	64	4	8	4
cefotaxime	64	512	256	4	<0.03
cefotaxime–clavulanic acid	0.12	256	0.12	0.03	<0.03
ceftazidime	64	256	8	>256	0.12
ceftazidime–clavulanic acid	0.25	64	0.25	0.25	0.12
cefepime	32	512	64	8	<0.5
cefepime–clavulanic acid	<0.5	128	<0.5	≤0.25	0.5
ceftibuten	16	128	2	64	<0.25
ceftibuten–clavulanic acid	<0.25	64	<0.25	≤0.12	<0.25
aztreonam	64	512	32	128	0.06
piperacillin	>1024	>1024	1024	256	8
piperacillin–tazobactam	64	512	1	ND	1
amikacin	16	32	0.25	4	0.25
gentamicin	256	256	128	16	≤0.12
imipenem	0.12	1	0.25	0.12	0.25
meropenem	0.03	1	≤0.015	0.015	≤0.015
tetracycline	256	256	1	128	1
SXT	128	>256	≤0.12	≤0.12	≤0.12
chloramphenicol	128	512	4	256	4
ciprofloxacin	0.032	0.25	0.25	ND	0.25
(ii) Plasmid content (kb)	140, 60, 4, 3	140, 60	140	140	–
(iii) IEF band (pI)	5.4, 7.6, 7.9	5.4, 7.6, 7.9	5.4, 7.9	5.4	–
(iv) PCR mapping					
<i>bla</i> <sub>SHV</sub> -like	+	+	–	–	–
<i>bla</i> <sub>TEM</sub> -like	+	+	+	+	–
<i>bla</i> <sub>PER-2</sub>	+	+	–	+	–
<i>bla</i> <sub>CTX-M-2</sub>	+	+	+	–	–
<i>ant</i> (2'')-Ia	+	+	+	–	–
<i>aac</i> (3)-IIa	+	+	+	+	–
Tn1331	+	+	–	+	–
Tn3	+	+	+	–	–

SXT, trimethoprim–sulfamethoxazole; ND, not determined.

<sup>a</sup>Features shown were the same for M3089 and M3090 transconjugants.

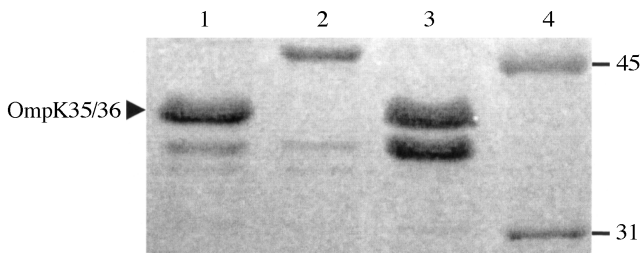
<sup>b</sup>Features shown were the same for M3129 and M3130 transconjugants.

decreased susceptibility to amikacin. The results of the plasmid profile analysis are summarized in Table 2. It is noteworthy that the segregation of different non- $\beta$ -lactam susceptibility profiles into type 1 and 2 transconjugants occurred in the absence of any selective pressure. The most probable explanation for this fact would be the co-existence of two different megaplasmids of the same size (i.e. 140 kb) in each of the two parental *K. pneumoniae* isolates, each carrying a different complement of  $\beta$ -lactam and non- $\beta$ -lactam resistance genes.

### Characterization of $\beta$ -lactamases produced by *K. pneumoniae* M1803 and M1826

By IEF analysis, with the  $\beta$ -lactamase bands being visualized by an iodometric method using ampicillin plus cefaloridine as substrate, three bands were detected in both *K. pneumoniae* strains, with pIs of 5.4, 7.6 (corresponding to the chromosomal SHV-1  $\beta$ -lactamase)

and 7.9 (Table 2). In contrast, when visualizing the  $\beta$ -lactamase bands with ceftazidime, two bands were detected, with pIs of 5.4 and 7.9. All bands were susceptible to clavulanic acid inhibition. The nature of a broad-spectrum  $\beta$ -lactamase activity with a pI of 5.4 could not be specifically determined in these standard IEF assays because of an overlapping ESBL band at the same pI. Accordingly, *in situ*  $\beta$ -lactamase inhibition using cefoxitin was performed on the IEF gel, as described previously.<sup>16</sup> The ESBL activity with a pI of 5.4 was inhibited with cefoxitin, whereas the broad-spectrum  $\beta$ -lactamase activity with a pI of 5.4 and the ESBL activity with a pI of 7.9 were not. This property, of cefoxitin inhibition of the ESBL band at pI 5.4, indicated it was most likely to be the PER-2 enzyme. These results are in agreement with those obtained by PCR assays, which yielded amplification products with primers specific to TEM, SHV, CTX-M and PER families of  $\beta$ -lactamases genes. Therefore, the presence of four  $\beta$ -lactamase genes, *bla*<sub>PER-2</sub>-like, *bla*<sub>CTX-M-2</sub>-like, *bla*<sub>SHV-1</sub>-like



**Figure 2.** OMP profiles of *K. pneumoniae* M1803 and M1826. Numbers on the right indicate molecular weight markers (in kDa); the arrow shows the relative mobility of OmpK35/36 porins. Lane 1, *K. pneumoniae* M1803; lane 2, *K. pneumoniae* M1826; lane 3, *K. pneumoniae* ATCC 10031; lane 4, molecular weight standard.

and  $bla_{TEM-1}$ -like, was confirmed. These results were replicated using both types of transconjugant strains to determine which  $\beta$ -lactamase gene is encoded for on which plasmid (Table 2). The identity of  $bla_{PER-2}$  and  $bla_{CTX-M-2}$  genes was unequivocally established by sequencing of the corresponding amplicons (data not shown).

#### Non-transferable antibiotic resistance caused by an OmpK35 and OmpK36 deficiency in *K. pneumoniae* M1826

*K. pneumoniae* isolates that are resistant to ceftioxin have been described previously, with resistance caused by either the production of a plasmid-mediated AmpC-like  $\beta$ -lactamase<sup>26,27</sup> or the loss of OMPs, such as OmpK35 and OmpK36.<sup>19,28</sup> Indeed, reduced expression of both porins has been reported in *K. pneumoniae* strains with high MICs of ceftioxin (>32 mg/L).<sup>28</sup> Moreover, increase in the levels of resistance to extended-spectrum cephalosporins, carbapenems and also fluoroquinolones other than ceftioxin, has been associated to the loss of both OmpK35 and OmpK36.<sup>19,29,30</sup>

In this study, the genetic determinants of *K. pneumoniae* M1826 conferring resistance to ceftioxin and carbapenems could not be transferred to *E. coli* by conjugation. Furthermore, incubation of the IEF gels with aztreonam (an AmpC-like  $\beta$ -lactamase inhibitor), prior to  $\beta$ -lactamase visualization using ampicillin plus cefaloridine, did not result in inhibition of any of the  $\beta$ -lactamase bands produced by *K. pneumoniae* M1826 (data not shown). This fact suggests the absence of enzymic activity against ceftioxin in this isolate. Indeed, using a microbiological method, no enzymic activity against ceftioxin and carbapenems was detected in *K. pneumoniae* M1826. In this assay, the parallel control was a crude extract containing the known AmpC MIR-1, and this showed a positive result for ceftioxin hydrolysis. All this information suggests the absence of AmpC-type enzymes in *K. pneumoniae* M1826, and that ceftioxin resistance was chromosomal.

Accordingly, OMP analysis for *K. pneumoniae* M1803 and M1826 (ceftioxin MICs of 2 and 64 mg/L, respectively) was performed and showed that the latter was deficient in OmpK35/36 expression (Figure 2). Our results are in agreement with the previous reports mentioned above, suggesting that the deficiency in OmpK35/36 expression is responsible for the higher overall resistance levels displayed by the M1826 isolate (particularly to ceftioxin and carbapenems) as compared with those of the pretreatment M1803 isolate (Table 2).

The partial sequencing of *gyrA* from *K. pneumoniae* M1826, including the QRDR, did not reveal mutations at codons 83 and 87, indicating that the increased levels of fluoroquinolone resistance in this mutant are not associated with these target site alterations, and

are probably a result of the observed loss of OmpK35/36. Increased expression of efflux mechanisms cannot be completely discounted, however.

#### Detailed analysis of the transferable resistance genes carried by *K. pneumoniae* M1803 and M1826

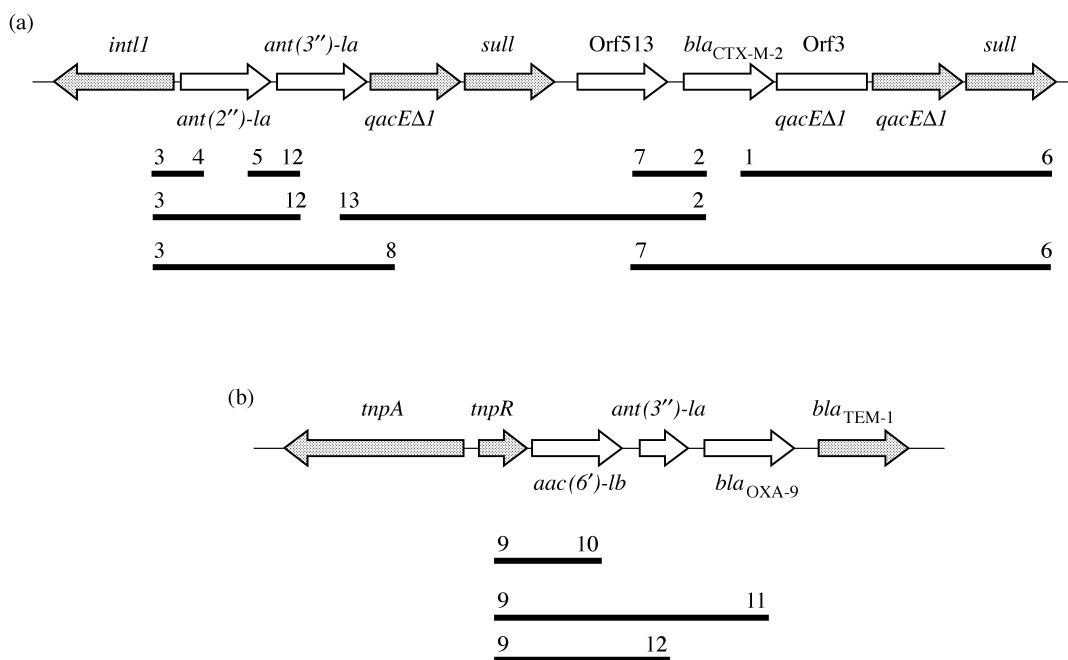
Very recently, the  $bla_{CTX-M-2}$  gene has been reported as being located in unusual class I integrons In35 and InS21, in clinical isolates of *Proteus mirabilis* and *Salmonella enterica* ser. Infantis, respectively.<sup>30,31</sup> This unusual class I integron contains a partial duplication of the 3' conserved segment, and a region that includes  $bla_{CTX-M-2}$  and the Orf513 between both 3' conserved segments. In addition, In35-related integrons having  $bla_{CTX-M-2}$  in conjunction with different resistance cassettes such as  $aac(6')-Ib-ant(3'')-Ia$ , *orfD*,  $ant(2'')-Ia-ant(3'')-Ia$ ,  $ant(3'')-Ia$  and  $aac(6')-Ib$  within the variable region of class I integrons were detected in clinical isolates of Enterobacteriaceae.<sup>30</sup> In the present work, by PCR mapping of both *K. pneumoniae* isolates and the type 1 transconjugants, the  $bla_{CTX-M-2}$  gene was located in an unusual class I integron having  $ant(2'')-Ia-ant(3'')-Ia$  gene cassettes within the variable region (Figure 3a). The co-existence of  $bla_{CTX-M-2}$  and  $ant(2'')-Ia$  genes in the same genetic structure (a complex *sull*-type integron) and harboured, in turn, in a conjugative megaplasmid, may explain the high prevalence in our bacterial population of both extended-spectrum cephalosporin and gentamicin resistance. The widespread distribution of CTX-M-2-like enzymes among clinical isolates of *K. pneumoniae* in Argentina (60%)<sup>4</sup> could be explained by the fact that ceftioxin and cefotaxime are the most used cephalosporins in our country. Another gene conferring resistance to gentamicin,  $aac(3)-IIa$ , was found in both type 1 and type 2 transconjugants. The finding of two gentamicin resistance genes [ $ant(2'')-Ia$  and  $aac(3)-IIa$ ] in type 1 transconjugants correlated well with an MIC of gentamicin 8-fold higher than that observed for type 2 transconjugants (Table 2).

TEM-1 is the most widespread plasmid-mediated  $\beta$ -lactamase around the world, because its structural gene is harboured on the Tn3 transposon. Tn1331 is a transposable element belonging to the Tn3 family that carries, in addition to  $bla_{TEM-1}$ , another  $\beta$ -lactamase gene ( $bla_{OXA-9}$ ) and two genes encoding aminoglycoside-modifying enzymes:  $aac(6')-Ib$  and  $ant(3'')-Ia$  (Figure 3b).<sup>32</sup> By PCR mapping, three amplification products of Tn1331 were produced using templates from both *K. pneumoniae* isolates in this study (Table 2; Figure 3b). Interestingly, Tn1331 was only detected in the type 2 transconjugants, while in the type 1 transconjugants, the  $bla_{TEM-1}$ -like gene was located in Tn3 by PCR with primers to *tnpA* and  $bla_{TEM}$  (OT-2) genes (Table 2). These data also support the hypothesis of the existence of two different megaplasmids of the same size in each of the two parental *K. pneumoniae* isolates (one of them harbouring Tn1331 and the other Tn3), which can be separately transferred by conjugation into *E. coli*. The presence of the Tn1331-encoded  $aac(6')-Ib$  gene conferring amikacin resistance only in type 2 transconjugants may explain the 16-fold increase in the MIC of amikacin observed for type 2 as compared with those for type 1 (Table 2). The  $aph(3')-VIa$  gene, which also confers amikacin resistance, was not detected by PCR in the *K. pneumoniae* parental isolates.

#### Conclusions

Generally, in *Vibrio cholerae*<sup>16</sup> and Enterobacteriaceae<sup>4,33,34</sup> from Argentina, ESBLs and TEM-1-like enzymes are encoded on the

## Novel ESBL combination in *K. pneumoniae*



**Figure 3.** PCR mapping strategy of relevant resistance determinants. Numbers represent the primers employed (see Table 1). (a) *bla*<sub>CTX-M-2</sub> gene and its surrounding regions inner of In35; (b) Tn1331.

same megaplasmid, which, additionally, harbours genetic determinants conferring resistance to other kinds of antibiotics, such as aminoglycosides, trimethoprim–sulfamethoxazole, chloramphenicol and tetracycline. Therefore, the use of any of these drugs in nosocomial acquired infections may lead to cross-selection of extended-spectrum cephalosporin resistance.

Worldwide, few descriptions of strains with two or more ESBLs have been reported, and all of those that have been reported involve TEM- or SHV-derived enzymes. We identified here two ESBLs, CTX-M-2 and PER-2, in a single multiresistant isolate, the first report of this ESBL combination in the same strain. The fact that *bla*<sub>CTX-M-2</sub> and *bla*<sub>PER-2</sub> were harboured in two different megaplasmids suggests a differential spread of these genes, which correlates well with their different prevalences (60% versus 10%, respectively<sup>4,33,34</sup>) observed in the Argentinean isolates. After successive antibiotic treatments, the last of them with ciprofloxacin, *K. pneumoniae* M1826 could be selected. The selection of these multiresistant bacteria in the hospital environment could be due, amongst other factors, to excessive and/or inappropriate antibiotic use, particularly of empirically prescribed broad-spectrum agents.

## Acknowledgements

This paper is dedicated to the memory of Dr Alicia Rossi, who directed our Service and guided this work. We thank Diego Faccone, Cecilia Ormazábal and Moira Ruiz O'Neill for their assistance in technical work. We thank Fernando Pasterán and Paola Ceriana for critical reading of the manuscript.

## References

1. Babini, G. S. & Livermore, D. M. (2000). Are SHV beta-lactamases universal in *Klebsiella pneumoniae*? *Antimicrobial Agents and Chemotherapy* **44**, 2230.

2. Du Bois, S., Marriott, M. & Amyes, S. (1995). TEM- and SHV-derived extended-spectrum  $\beta$ -lactamases: relationship between selection, structure and function. *Journal of Antimicrobial Chemotherapy* **35**, 7–22.

3. Jacoby, G. A. & Medeiros, A. A. (1991). More extended-spectrum  $\beta$ -lactamases. *Antimicrobial Agents and Chemotherapy* **35**, 1697–1704.

4. Galas, M., Rapoport, M., Pasterán, F. *et al.* (1999). High distribution of CTX-M-2  $\beta$ -lactamase among *Klebsiella* spp. isolates in an Argentinean extended spectrum  $\beta$ -lactamase (ESBLA) surveillance program. In *Program and Abstracts of the Thirty-ninth Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 1999*. Abstract 1474, p. 165. American Society for Microbiology, Washington, DC, USA.

5. Bradford, P. A., Cherubin, C. E., Idemyor, V. *et al.* (1994). Multiply resistant *Klebsiella pneumoniae* strains from two Chicago hospitals: identification of the extended-spectrum TEM-12 and TEM-10 ceftazidime-hydrolyzing  $\beta$ -lactamases in a single isolate. *Antimicrobial Agents and Chemotherapy* **38**, 761–6.

6. Essack, S., Hall, L., Pillay, D. *et al.* (2001). Complexity and diversity of *Klebsiella pneumoniae* strains with extended-spectrum  $\beta$ -lactamases isolated in 1994 and 1996 at a teaching hospital in Durban, South Africa. *Antimicrobial Agents and Chemotherapy* **45**, 88–95.

7. Liu, P., Tung, J. C., Ke, S. C. *et al.* (1998). Molecular epidemiology of extended-spectrum  $\beta$ -lactamase-producing *Klebsiella pneumoniae* isolates in a district hospital in Taiwan. *Journal of Clinical Microbiology* **36**, 2759–62.

8. Pai, H., Lyu, S., Lee, J. *et al.* (1999). Survey of extended-spectrum  $\beta$ -lactamases in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*: prevalence of TEM-52 in Korea. *Journal of Clinical Microbiology* **37**, 1758–63.

9. Kim, J., Kwon, Y., Pai, H. *et al.* (1998). Survey of *Klebsiella pneumoniae* strains producing extended-spectrum  $\beta$ -lactamases: prevalence of SHV-12 and SHV-2a in Korea. *Journal of Clinical Microbiology* **36**, 1446–9.

10. Soilleux, M., Morand, A., Arlet, G. *et al.* (1996). Survey of *Klebsiella pneumoniae* producing extended-spectrum  $\beta$ -lactamases: prevalence of TEM-3 and first identification of TEM-26 in France. *Antimicrobial Agents and Chemotherapy* **40**, 1027–9.

11. Yuan, M., Hall, L., Hoogkamp-Korstanje, J. *et al.* (2001). SHV-14, a novel  $\beta$ -lactamase variant in *Klebsiella pneumoniae* isolates from Nijmegen, The Netherlands. *Antimicrobial Agents and Chemotherapy* **45**, 309–11.
12. National Committee for Clinical Laboratory Standards. (2000). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically—Fifth Edition: Approved Standard M7-A5*. NCCLS, Wayne, PA, USA.
13. National Committee for Clinical Laboratory Standards. (2002). *Performance Standards for Antimicrobial Susceptibility Testing—Twelfth Informational Supplement: MIC Testing, M100-S12*. NCCLS, Wayne, PA, USA.
14. National Committee for Clinical Laboratory Standards. (2000). *Performance Standards for Antimicrobial Disk Susceptibility Test—Seventh Edition: Approved Standard M2-A7*. NCCLS, Wayne, PA, USA.
15. National Committee for Clinical Laboratory Standards. (2002). *Performance Standards for Antimicrobial Susceptibility Testing—Twelfth Informational Supplement: Disk Diffusion M100-S12*. NCCLS, Wayne, PA, USA.
16. Petroni, A., Corso, A., Melano, R. *et al.* (2002). Plasmidic extended-spectrum  $\beta$ -lactamases in *Vibrio cholerae* O1 El Tor in Argentina. *Antimicrobial Agents and Chemotherapy* **46**, 1462–8.
17. Labia, R. & Barthélémy, M. (1979). L'enzymogramme des  $\beta$ -lactamases: adaptation en cel de la methode iodometrique. *Annales de Microbiologie (Paris)* **130B**, 295–304.
18. Masuda, G., Tomioka, S. & Hasegawa, M. (1976). Detection of  $\beta$ -lactamase production by gram-negative bacteria. *Journal of Antibiotics (Tokyo)* **29**, 662–4.
19. Martínez-Martínez, L., Hernández-Allés, S., Abertí, S. *et al.* (1996). In vivo selection of porin-deficient mutants of *Klebsiella pneumoniae* with increased resistance to cefoxitin and expanded-spectrum cephalosporins. *Antimicrobial Agents and Chemotherapy* **40**, 342–8.
20. Foodborne and Diarrheal Diseases Branch, Centers for Disease Control and Prevention. (1998). *Standardized Molecular Subtyping of Foodborne Bacterial Pathogens by Pulsed-Field Gel Electrophoresis: A Training Manual*. Centers for Disease Control and Prevention, Atlanta, GA, USA.
21. Nakamura, M., Sato, S., Ohya, T. *et al.* (1986). Plasmid profile analysis in epidemiological studies of animal *Salmonella typhimurium* infection in Japan. *Journal of Clinical Microbiology* **23**, 360–5.
22. Levesque, C., Piche, L., Larose, C. *et al.* (1995). PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrobial Agents and Chemotherapy* **39**, 185–91.
23. Arlet, G. & Philippon, A. (1989). Construction by polymerase chain reaction and intragenic DNA probes for three main types of transferable  $\beta$ -lactamases (TEM, SHV, CARB). *FEMS Microbiology Letters* **82**, 19–26.
24. Sanger, S., Micklen, S. & Coulson, A. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, USA* **74**, 5463–7.
25. Tenover, F. C., Arbeit, R. D., Goering, R. V. *et al.* (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of Clinical Microbiology* **33**, 2233–9.
26. González-Leiza, M., Pérez-Díaz, J., Ayala, J. *et al.* (1994). Gene sequence and biochemical characterization of FOX-1 from *Klebsiella pneumoniae*, a new AmpC-type plasmid-mediated  $\beta$ -lactamase with two molecular variants. *Antimicrobial Agents and Chemotherapy* **38**, 2150–7.
27. Papanicolaou, G. A., Medeiros, A. A. & Jacoby, G. A. (1990). Novel plasmid-mediated  $\beta$ -lactamase (MIR-1) conferring resistance to oxyimino- and alpha-methoxy  $\beta$ -lactams in clinical isolates of *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy* **34**, 2200–9.
28. Ardanuy, C., Liñares, J., Domínguez, M. *et al.* (1998). Outer membrane profiles of clonally related *Klebsiella pneumoniae* isolates from clinical samples and activities of cephalosporins and carbapenems. *Antimicrobial Agents and Chemotherapy* **42**, 1636–40.
29. Martínez-Martínez, L., Pascual, A., Hernández-Allés, S. *et al.* (1999). Roles of  $\beta$ -lactamases and porins in activities of carbapenems and cephalosporins against *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy* **43**, 1669–73.
30. Arduino, S. M., Roy, P. H., Jacoby, G. A. *et al.* (2002). *bla*<sub>CTX-M-2</sub> is located in an unusual class 1 integron (In35) which includes Orf513. *Antimicrobial Agents and Chemotherapy* **46**, 2303–6.
31. Di Conza, J., Ayala, J. A., Power, P. *et al.* (2002). Novel class 1 integron (InS21) carrying *bla*<sub>CTX-M-2</sub> in *Salmonella enterica* serovar Infantis. *Antimicrobial Agents and Chemotherapy* **46**, 2257–61.
32. Tolmasky, M. & Crosa, J. (1993). Genetic organization of antibiotic resistance genes (*aac(6)-Ib*, *aadA*, and *oxa9*) in the multiresistance transposon Tn1331. *Plasmid* **29**, 31–40.
33. Galas, M. F., Pasterán, F. G., Melano, R. G. *et al.* (1998). Unusual distribution of enzymatic resistance to third-generation cephalosporin (TGC) in *E. coli* in Argentina. In *Program and Abstracts of the Thirty-eighth Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, CA, 1998*. Abstract E-109, p. 201. American Society for Microbiology, Washington, DC, USA.
34. Pasterán, F. G., Melano, R. G., Galas, M. F. *et al.* (1999). High proportion of extended spectrum  $\beta$ -lactamases (ESBLA) among AmpC producers enterobacteria in Argentina. In *Program and Abstracts of the Thirty-eighth Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 1999*. Abstract 1475, p. 166. American Society for Microbiology, Washington, DC, USA.