Journal of Antimicrobial Chemotherapy (2005) **55**, 461–465
doi:10.1093/jac/dkh556
Advance Access publication 2 March 2005

JAC

Description of In116, the first *bla*$_{CTX-M-2}$-containing complex class 1 integron found in *Morganella morganii* isolates from Buenos Aires, Argentina

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Received 16 September 2004; returned 28 October 2004; revised 11 November 2004; accepted 27 November 2004

***Objectives*: We analysed the architecture and probable origin of a class 1 integron from cefotaxime-resistant *Morganella morganii* isolates.**

***Methods*: *bla* genes and class 1 integron elements were detected by PCR and DNA–DNA hybridization in a *M. morganii* strain isolated in 1996. PCR-mapping and sequencing of different fragments were carried out to determine the integron's architecture.**

***Results and conclusions*: A class 1 integron (In116), strongly related to the In6/In7 family, was detected in a plasmid from an oxyimino-cephalosporin-resistant *M. morganii* strain, producing CTX-M-2 β-lactamase. The variable region of In116 contains *aacA4*, *bla*$_{OXA-2}$ and *orfD* cassettes. Downstream of the 3′-conserved-segment (3′-CS), an *orf513*-containing common region is followed by *bla*$_{CTX-M-2}$ and flanking regions, having 96–99% nucleotide identity with *Kluyvera ascorbata*'s *kluA-1* and neighbouring sequences. Some of the evidence supporting the incorporation of foreign DNA is as follows: a partial deletion in a second 3′-CS (3′-CS2), and the absence of 59-base element or IS-like structures upstream of *bla*$_{CTX-M-2}$.**

Keywords: *Kluyvera ascorbata*, In6/In7, *orf513*, *aacA4*, *bla*$_{OXA-2}$

Introduction

Plasmid-borne extended-spectrum β-lactamases (ESBLs) are associated with different genetic elements like transposons and integrons. Integrons are able to capture and mobilize different gene cassettes, whose expression is controlled by a suitably orientated promoter, by an integrase-mediated site-specific recombination, usually occurring at the *attI* recombination site.[1]

Currently, nine integron classes are recognized, class 1 integrons being the most frequently reported and distributed among Gram-negative clinical isolates.[2]

The general organization of a class 1 integron includes: a 5′-conserved-segment (5′-CS), containing the divergently-transcribed *intI1* gene, most of *attI1*, and the promoter region; the 3′-conserved-segment (3′-CS), including at least two genes, *qacEΔ1* (basal-level resistance to quaternary-ammonium compounds) and *sul1* (sulphonamide resistance); and a variable region (between both conserved domains) harbouring the gene cassettes with the corresponding *attC* or 59-base element (59-be).[1]

Unusual class 1 integrons, having a partial duplication of 3′-CS, and a 2.1 kb region lying between both 3′-CSs, called the common region (CR), have been reported. Adjacent to CR resides a unique region containing an antibiotic resistance gene, not resembling a gene cassette. The first integrons of this family were In6 and In7,[3,4] harbouring *catA2* (chloramphenicol resistance) and *dfrA10* (trimethoprim resistance), respectively. A growing number of these integrons have been reported in different regions and species,[5,6] including In34 and InS21 (*bla*$_{CTX-M-2}$) from *Proteus mirabilis* and *Salmonella* Infantis, respectively, isolated in Argentina.[7,8]

We have previously detected the presence of a plasmid-encoded CTX-M-2-type cefotaximase in six oxyimino-cephalosporin-resistant *Morganella morganii* isolates, collected during

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1996–1997, being the first report of a cefotaximase-mediated resistance in the species.[9] In this study, we establish the association of this cefotaximase with a complex class 1 integron, named In116, in a cefotaxime-resistant *M. morganii* strain isolated during 1996.

Materials and methods

Bacterial strains and DNA

Morganella morganii PP16, isolated from a urinary tract infection during 1996 in Buenos Aires, was previously characterized as a producer of a pI 8.2 cefotaximase (cefotaxime MIC 64 mg/L) and TEM-1.[9] *Escherichia coli* CAG12177 (*E. coli* Genetic Stock Center, USA) and *Escherichia coli* Top10F′ (Invitrogen, USA) were used as hosts for conjugation and transformation assays, respectively. Control strains: *E. coli* 1353 (bla_{TEM-1}, $bla_{CTX-M-2}$) kindly provided by M. Radice (Universidad de Buenos Aires); *E. coli* J53(RGK) (bla_{TEM-1}) and *E. coli* J53.2 86-1 (bla_{SHV-2}) kindly provided by A. Medeiros; *Salmonella* Infantis S21 ($bla_{CTX-M-2}$, bla_{OXA-2}, class 1 integron).[8]

Plasmid DNA from *M. morganii* PP16 (pM16), vectors and genomic DNA were purified by conventional methods,[10] or by commercial systems (GFx Micro Plasmid Prep Kit; Amersham Biosciences, USA).

PCR screening

Screening for β-lactamase-encoding genes (*bla*) and class 1-integron elements was carried out by PCR, using pM16 as template, 0.8–1 μM primers (Table 1), and 1.25 U of *Taq* DNA polymerase (Amersham Biosciences, USA) or 1.5 U of *Pfu* polymerase (Promega, USA), as required. PCR-amplicons were resolved in 1–1.3% agarose gels, using commercial standards (1 kb DNA Ladder; MBI-Fermentas, Lithuania).

DNA–DNA hybridization

Overnight *M. morganii* colonies, transferred to nitrocellulose membranes, and DNA dot-blots were treated as described previously.[10] Gene probes were generated by PCR from control strains and ^{32}P-labelled by random priming.[10] Hybridization was carried out overnight at 60°C, and detected by radioautography, using Kodak BioMax MS films and revealing solutions (Kodak, USA).

Conjugative transfer of pM16

The conjugative mobilization of pM16 was attempted by both liquid- and solid-medium mating, to recipient *E. coli* CAG12177.[10] Selection was carried out on Luria–Bertani or MacConkey agar plates containing cefotaxime (10 mg/L) and tetracycline (20 mg/L).

Table 1. Oligonucleotides used for both PCR amplification and DNA sequencing

No.	Primer	Nucleotide sequence (5′ → 3′)	Target DNA	Accession no.
1	I5	ACC GCC AAC TTT CAG CAC AT	*intI1* gene	M95287
2	I3	GCG TTC GGT CAA GGT TCT GG	*intI1* gene	M95287
3	5CS	GGC ATC CAA GCA GCA AGC	gene cassettes region	M73189
4	3CS	AAG CAG ACT TGA CCT GAT	gene cassettes region	M73189
5	qacEΔ1F	ATC GCA ATA GTT GGC GAA GT	3′-CS (*qacEΔ1*)	X15370
6	qacEΔ1B	CAA GCT TTT GCC CAT GAA GC	3′-CS (*qacEΔ1*)	X15370
7	Sul1F	CTT CGA TGA GAG CCG GCG GC	3′-CS (*sul1*)	X12869
8	Sul1B	GCA AGG CGG AAA CCC GCG CC	3′-CS (*sul1*)	X12869
9	Oxa2A	CCT GCA TCG ACA TTC AAG ATA	bla_{OXA-2}	X03037
10	Oxa2B	CTC AAC CCA TCC TAC CCA CCA	bla_{OXA-2}	X03037
11	Oxa2F	ACT TCC TCC AGT CAG AAC	bla_{OXA-2}	this study
12	Oxa2-59	CTA CCC ACC AAC CCA TAC	bla_{OXA-2}	this study
13	IC1	AAT GCC TCG ACT TCG CTG	*aac(6′)-Ib*	this study
14	AAC4	TCA CCA AGA TCC AAA CGG	*aac(6′)-Ib*	this study
15	ORFD-59	ATT CTG CGG TCG GCT TAC	*orfD* cassette	this study
16	ORFend	CCG TTA AGC TCT TAT GTG GG	*orf513*	L06418
17	F12D	GTA TTG CGC CGC TCT TAG AC	*sul1+orf513*	this study
18	F12R	AAA CCA GCA TGG TTG GCT AC	*sul1+orf513*	this study
19	blaI	TTA ATG ATG ACT CAG AGC ATT	$bla_{CTX-M-2}$	X92507
20	blaII	GAT ACC TCG CTC CAT TTA TTG C	$bla_{CTX-M-2}$	X92507
21	blaIII	CTG TTC GCC TGC GCA TGC AGC	$bla_{CTX-M-2}$	X92507
22	blaIV	TAC CCA ACC GGA GCA GAA GG	$bla_{CTX-M-2}$	X92507
23	blaUp	GGC TTC CAG CTG CTG TTG CAC	$bla_{CTX-M-2}$	this study
24	blaDn	TTG ACT GTC GAC CCC AAA TCC	$bla_{CTX-M-2}$	this study
25	IV16D	TGG ATA AGG AGG GGA CAA TG	*orf3*	this study
26	blaTEM-1	ATG AGT ATT CAA CAT TTC CG	bla_{TEM}	J01749
27	blaTEM-II	CCA ATG CTT AAT CAG TGA GC	bla_{TEM}	J01749
28	SHV-1A	ATG ATG AGC ACC TTT AAA GTA	bla_{SHV}	M59181
29	SHV-1B	ATT TCG CTC GGC CAT GCT CGC	bla_{SHV}	M59181
30	blaPER-I	TGT GTT TTC ACC GCT TCT GCT CTG	bla_{PER-2}	X93314
31	blaPER-II	CAG CTC AAA CTG ATA AGC CGC TTG	bla_{PER-2}	X93314

Restriction endonuclease digestions

Standard reactions were carried out for 2 h at the corresponding temperature, and fragments resolved as above. Restriction endonucleases used: *Eco*RV, *Eco*RI, *Pst*I, *Eco*47III, *Hin*dIII, *Hin*cII (New England Biolabs, USA), *Sph*I, *Not*I, *Pvu*II, *Ava*I and *Sac*I (Pharmacia, USA).

PCR mapping of In116 and DNA sequencing

PCR amplifications, using different pairs of primers (Table 1 and Figure 1), were carried out to assess the architecture of the class 1 integron. PCR products were cloned into pGEM-T (Promega, USA) or pUC18 (Amersham Pharmacia, USA) vectors, according to manufacturers' recommendations, and sequenced in both strands, from recombinant plasmids, by the automated Sanger method.[11] Sequences were analysed with the NCBI (http://www.ncbi.nlm.nih.gov/) and EBI (http://www.ebi.ac.uk/) tools.

Nucleotide sequence accession number

The sequence of In116 has been submitted to the EMBL database under the accession no. AJ621187.

Results and discussion

Plasmid detection and screening of bla genes

The presence of the following *bla* genes was detected in a 54 kb plasmid (pM16) from *M. morganii* PP16: bla_{TEM} (0.85 kb), $bla_{CTX\text{-}M\text{-}2}$-type (0.9 kb), and bla_{OXA} (0.5 kb). Therefore, plasmid-borne β-lactamases would be responsible for the acquired resistance to β-lactams in this strain, oxyimino-cephalosporin resistance being mediated by the CTX-M-2-type enzyme. No bla_{SHV} or $bla_{PER\text{-}2}$ could be detected, indicating that *M. morganii* PP16 is not an SHV- or PER-producer.

Conjugative transfer of pM16

We failed to transfer pM16 plasmid to a recipient *E. coli* strain, both by solid- and liquid-medium reactions. This was probably due to a very low efficient conjugation, an incomplete mobilization because of the size of pM16, or even a host species not able to allow the conjugation. Though a *Proteus mirabilis* strain, phylogenetically closer to *M. morganii*, could have been used, the multiresistant *Morganella* strain made it extremely difficult to find the proper conditions for selection of transconjugant cells.

Sequencing and analysis of ESBL-encoding gene and upstream region

The sequence of a 1.4 kb fragment containing $bla_{CTX\text{-}M}$ and its upstream region showed a strict identity with the CTX-M-2-encoding gene. The deduced protein has a single amino acid shift (Arg-246 → Ser) compared with Toho-1 (99% identity). According to a theoretical 3D model of CTX-M-2 against Toho-1 (not shown), this shift would not influence the catalytic activity, and similar kinetic properties are expected. Moreover, these two cefotaximases are presumably derived from

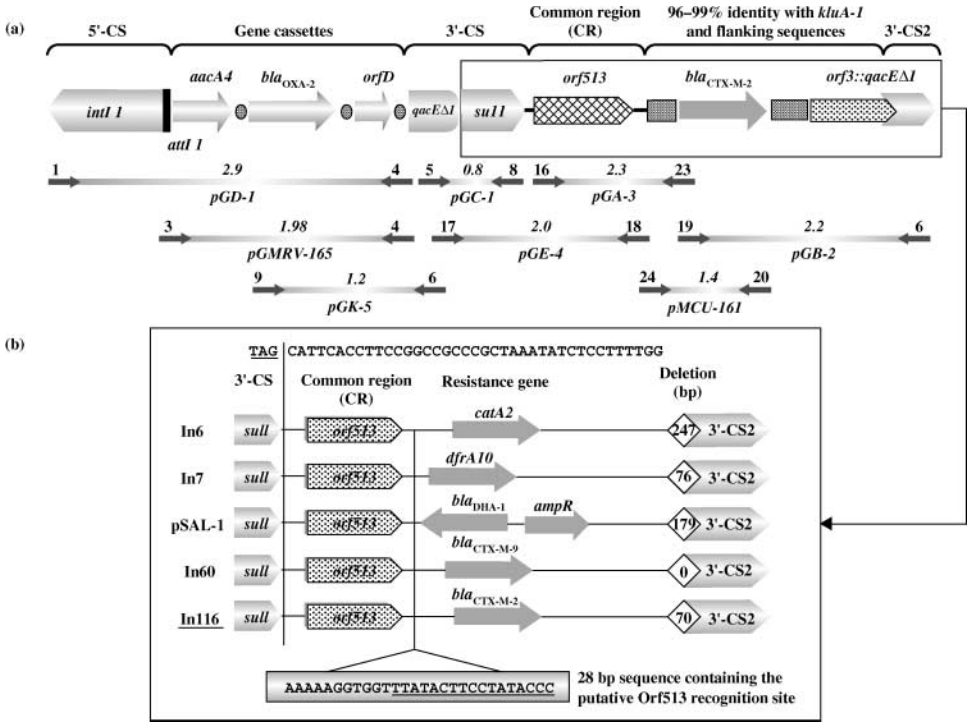

Figure 1. (a) Architecture of In116 (not to scale) deduced by a PCR mapping strategy. Genes are represented by bold arrows, indicating the direction of transcription; circles represent 59-be. Numbers in italic correspond to sizes (in kb) of the PCR products. For simplification, only those PCR products cloned for sequencing are shown. Primers are represented as arrows and numbers (Table 1). *orf3* and *qacEΔ1* seem to exist as a gene fusion (*orf3::qacEΔ1*), as proposed elsewhere.[7] (b) Detail of the squared region from (a): schematic comparison of common regions (CR) and 3′-CS2 from In6/In7-related integrons. Top: the CR (bold nucleotides) starts at +24 bp from the *sul1* stop codon (underlined 'TAG'). Bottom: 28 bp region containing the putative Orf513 recognition site (underlined 17 bp). Deletions in 3′-CS2 are represented as squared numbers, except for In60 (no deletion). Note that only deletions in 3′-CS2 5′ end are indicated.

chromosome-encoded β-lactamases from infrequent human pathogens, such as *Kluyvera* species.[12]

A putative promoter was predicted upstream of $bla_{CTX\text{-}M\text{-}2}$ in pM16, in good agreement with the experimental evidence for a $bla_{CTX\text{-}M\text{-}2}$ promoter in a *Salmonella enterica* strain (J. Di Conza, unpublished results). This 506 bp upstream region could be divided into a proximal 266 bp segment having 97% identity with an equivalent region upstream of *Kluyvera ascorbata kluA-1* (AJ272538), reinforcing the hypothesis of their phylogenic relationship, and a distal 240 bp segment, showing strict identity with the CR, reported in In6/In7 integrons,[4] including InS21 and In35, among others.[7,8] The last 17 bp of this 240 bp region were postulated as a putative recombination site for insertion of resistance genes.[13] It is worth noting the absence of IS*Ecp1* in *M. morganii* PP16, associated with $bla_{CTX\text{-}M\text{-}9}$ and $bla_{CTX\text{-}M\text{-}1}$ sub-families,[14,15] including a chromosome-encoded $bla_{CTX\text{-}M\text{-}3}$ from a *K. ascorbata* strain.[16]

These features suggest the acquisition of $bla_{CTX\text{-}M\text{-}2}$ via 'non-IS-mediated' mechanisms, although the presence of a hypothetical recombination site could indicate an earlier transposition, followed by the loss of the IS, probably by recombination events.

Detection and analysis of class 1 integron elements

Typical elements of class 1 integrons were detected by PCR and sequenced: *intI1* from 5′-CS, *sul1* and *qacEΔ1* from 3′-CS, and a 2 kb variable region (Figure 1a), including the following gene cassettes: *aacA4*-cassette, encoding an aminoglycoside acetyltransferase, which probably mediates the aminoglycoside resistance in this strain;[9] $bla_{OXA\text{-}2}$-cassette, encoding an OXA-2 β-lactamase; and an open reading frame (*orfD*) of unknown function. Notably, this gene-cassette arrangement was also observed in four other *M. morganii* strains (data not shown), in InS21 and In35,[7,8] and in a *Klebsiella pneumoniae* isolate from Uruguay (R. Vignoli, Universidad de la República, Montevideo, personal communication), suggesting a common origin of these integrons.

Description of In116 architecture

The map of In116, obtained by a PCR-based strategy and sequencing, is showed in Figure 1(a). In116 has 99.7% identity with InS21, and a similar structure to the In6/In7 family, reinforcing the idea of their common origin and probably a recent dissemination. Twenty nucleotide differences were detected in In116, compared with InS21, including the 'TGAACT' −10 consensus sequence in the P_{ANT} promoter ('TAAACT' in InS21).

A 2.1 kb segment having 99% identity with the CR from In6/In7-type integrons was present downstream of 3′-CS.[3,4,7,8] This extension includes an orf513, followed by a 28 bp conserved sequence containing a putative 17 bp signal for the hypothetical recombinase Orf513, which probably leads to the incorporation of resistance genes (Figure 1b). A resistance gene, depending on the integron, is located downstream of the 28 bp region: $bla_{CTX\text{-}M\text{-}2}$ (In116), *catA2* (In6),[4] *dfrA10* (In7),[3] $bla_{DHA\text{-}1}$ (pSAL-1 integron),[5] $bla_{CTX\text{-}M\text{-}9}$ (In60),[6] etc. (Figure 1b). Notably, associated resistance genes and flanking sequences from In116 and the pSAL-1 integron have high homology with chromosomal DNA from other species: *K. ascorbata* and *M. morganii*, respectively. A 96–99% identity between $bla_{CTX\text{-}M\text{-}2}$ and flanking sequences with a homologous sequence including *K. ascorbata kluA-1* suggests their incorporation was probably Orf513-mediated and used a 28 bp target sequence. This feature endorses the hypothesis of the chromosomal origin of plasmid-borne β-lactamases, probably mobilized from different microorganisms to more promiscuous structures (integrons, transposons or plasmids).

As in In6/In7, In116 showed a partial duplication of 3′-CS (3′-CS2), lacking the first 70 bp. A 3′ 28 bp deletion also occurred in *orf3*, probably allowing the continuity of *orf3* and *qacEΔ1* from 3′-CS2, thus encoding a putative fusion protein, Orf3::QacEΔ1. These deletions (variable depending on the integron, Figure 1b) might have participated in recombination events implicated in the acquisition of foreign associated resistance genes, probably replacing less useful regions of DNA.

In summary, pieces of evidence supporting the hypothesis of the incorporation of $bla_{CTX\text{-}M\text{-}2}$ and flanking sequences from *K. ascorbata* in In116 are: (i) the absence of *attC*-like structures, and the presence of a putative recombinase-encoding gene (*orf513*) suggesting the acquisition of the resistance gene by a non-site-specific recombination mechanism; (ii) a partial deletion in 3′-CS2, probably associated with the incorporation of foreign DNA (see above); and (iii) the absence of IS-like structures (IS*Ecp1* or related) upstream of $bla_{CTX\text{-}M\text{-}2}$, suggesting a different mechanism, or even an earlier transposon-mediated incorporation followed by a loss of any 'trace' from that event. If the latter had happened, In116 and related integrons could be more recent than the IS-associated ones.

So far, only $bla_{CTX\text{-}M}$ and $bla_{DHA\text{-}1}$ genes have directly been associated with chromosome-borne genes. We could hypothesize that perhaps other In6/In7-associated resistance genes have been derived from a variety of chromosomal sources, probably using a similar mechanism: an initial collection from parental microorganisms by a still unknown strategy (probably transposon- or phage-mediated), followed by the acquisition of the whole fragment in the integron environment, presumably using a recombinase (Orf513) activity and not fully determined target sequences.

Acknowledgements

We are grateful to Iris Thamm (CIP, University of Liege, Belgium) for DNA sequencing. This work was supported by grants from UBACyT, ANPCyT and Beca 'Carrillo Oñativia' (Ministerio de Salud) to G.G., and grant LSHM-CT-2003-503335 from the European Union to J.A.A. G.G. is a member of 'Carrera del Invertigador' CONICET.

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