Recognition of two genetic groups in the *Klebsiella oxytoca* taxon on the basis of chromosomal β -lactamase and housekeeping gene sequences as well as ERIC-1R PCR typing

Sophie A. Granier,^{1,2} Laetitia Plaisance,³ Véronique Leflon-Guibout,¹ Evelynne Lagier,⁴ Serge Morand,³ Fred W. Goldstein² and Marie-Hélène Nicolas-Chanoine¹

Correspondence

Marie-Hélène Nicolas-Chanoine
marie-helene.nicolas-chanoine@
apr.ap-hop-paris.fr

¹Service de Microbiologie-Hygiène, Hôpital Ambroise Paré AP-HP, Université Versailles-Saint-Quentin-en-Yvelines-UFR Médicale Paris-Ile-de-France-Ouest, 9 avenue Charles de Gaulle, 92100 Boulogne-Billancourt, France

²Laboratoire de Microbiologie Médicale, Fondation Hôpital Saint-Joseph, Paris, France

³Centre de Biologie et d'Ecologie Tropicale et Méditerranéenne, UMR 5555, Université de Perpignan, France

⁴Service de Microbiologie, Centre Hospitalier du Pays d'Aix, Aix en Provence, France

Whilst searching for a molecular method to identify the different species of *Raoultella* and *Klebsiella oxytoca*, it was observed that the OXY-1 and OXY-2 β -lactamase-producing *K. oxytoca* isolates displayed two distinguishable enterobacterial repetitive intergenic consensus (ERIC)-1R profiles. It was hypothesized that the two groups of chromosomal β -lactamases might correspond to two groups of strains in the *K. oxytoca* taxon. To confirm this hypothesis, clinical isolates and reference strains of *K. oxytoca* were studied by determination of the sequence of their *bla*_{OXY} genes, and of a partial fragment of their 16S rRNA (387 bp) and *rpoB* (512 bp) genes. The sequence data were phylogenetically analysed by using the parsimony method. Four clinical isolates possessed a *bla*_{OXY-1} gene and nine possessed a *bla*_{OXY-2} gene. The mean percentage of *rpoB* and 16S rRNA gene identity was >99% within each group of strains, whereas it was $96.56 \pm 0.24\%$ for *rpoB* genes and $97.80 \pm 0.22\%$ for 16S rRNA genes between the group of strains harbouring the *bla*_{OXY-1} gene and the group harbouring the *bla*_{OXY-2} gene. The phylogenetic tree resulting from combined analysis of the 16S rRNA and *rpoB* datasets showed that the *K. oxytoca* isolates were monophyletic and separated into two clades; these clades included strains with either the *bla*_{OXY-1} gene or the *bla*_{OXY-2} gene. This result was supported with high bootstrap values of 97 and 99%, respectively. Moreover, the two groups of strains displayed distinct ERIC-1R profiles, with bands characteristic of each profile. Thus, the chromosomal *bla*_{OXY} gene sequence is able to delineate not only two groups of β -lactamases in *K. oxytoca*, but also two clades in the *K. oxytoca* taxon, in a manner similar to the sequence of housekeeping genes. These results suggest that *K. oxytoca* should be divided into two genetic groups, group OXY-1 represented by *K. oxytoca* strain SL781 (= CIP 104963) and group OXY-2 by *K. oxytoca* strain SL911 (= CIP 106098).

INTRODUCTION

Klebsiella oxytoca is an opportunistic pathogen that has been involved in various nosocomial infections (Boyeldieu *et al.*, 1999; Jeong *et al.*, 2001; Reiss *et al.*, 2000) and also

in antibiotic-associated diarrhoea (Benoit *et al.*, 1992; Högenauer *et al.*, 1998; Terrier *et al.*, 1995).

Methods routinely used for species identification are not able to differentiate *K. oxytoca* from the other indole-positive

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Abbreviations: ERIC, enterobacterial repetitive intergenic consensus; RAPD, randomly amplified polymorphic DNA.

Alignments of consensus DNA sequences of the 16S rRNA and *rpoB* genes are available as supplementary data in IJSEM Online.

Klebsiella species: *Klebsiella ornithinolytica*, *Klebsiella planticola* and *Klebsiella terrigena* (Monnet *et al.*, 1991). However, recent molecular studies have definitively demonstrated that *K. oxytoca* is phylogenetically distant from the other indole-positive *Klebsiella* species, on the basis of 16S rRNA and *rpoB* gene sequence analysis (Drancourt *et al.*, 2001). For this reason, the indole-positive *Klebsiella* species other than *K. oxytoca* have recently been renamed as *Raoultella* species (Drancourt *et al.*, 2001).

Both *K. oxytoca* and *Raoultella* species are naturally resistant to amino- and carboxypenicillins, but are susceptible to the amoxicillin-clavulanate combination (Stock & Wiedemann, 2001). Until now, the chromosomal β -lactamase responsible for this β -lactam-resistance phenotype has been defined only for *K. oxytoca*, and was shown to belong to class A of the β -lactamases. Although its production is not regulated, up-mutations induce its overproduction, resulting in the resistance of isolates to certain third-generation cephalosporins (ceftriaxone and cefotaxime) and aztreonam (Fournier *et al.*, 1994).

Fournier *et al.* (1996b) have determined two groups of chromosomal β -lactamase in *K. oxytoca*, OXY-1 and OXY-2, with isoelectric points ranging from 7.1 to 8.8 and 5.2 to 6.8, respectively. The corresponding genes, *bla*_{OXY-1} and *bla*_{OXY-2}, display a nucleotide sequence identity of 87%. Whilst searching for a molecular method to identify the different species of *Raoultella* and *K. oxytoca*, we observed that the OXY-1 β -lactamase-producing and the OXY-2 β -lactamase-producing isolates displayed two distinct profiles generated by the enterobacterial repetitive intergenic consensus (ERIC)-1R PCR typing system, and that each profile had characteristic bands. Faced with such a result, we hypothesized that the two groups of β -lactamases (OXY-1 and OXY-2) of *K. oxytoca* might correspond to two groups of strains in the *K. oxytoca* taxon. We confirmed this hypothesis by studying the sequence of the *bla*_{OXY}, 16S rRNA and *rpoB* genes in epidemiologically non-related clinical isolates of *K. oxytoca* and reference strains.

To our knowledge, this study is the first to show that chromosomal β -lactamase genes are able to identify clades within a given species, as do housekeeping genes such as 16S rDNA and *rpoB*.

METHODS

Strains. Thirteen non-redundant clinical isolates of *K. oxytoca* were studied in comparison with reference strains SL781 (=CIP 104963) and SL911 (=CIP 106098), producing OXY-1 and OXY-2 β -lactamases, respectively. Those clinical isolates ($n=10$: SG12, SG49, SG337, SG344, SG43, SG69, SG77, SG9, SG4 and SG62) that displayed an extended spectrum of resistance to certain β -lactam molecules (ceftriaxone, cefotaxime and aztreonam) had been collected between 1984 and 1998, in six French hospitals representing the northern and southern regions of France and the Paris area. The three remaining isolates (SG74, SG81 and SG176) were susceptible to

third-generation cephalosporins and aztreonam, and were obtained from three patients hospitalized in the Saint Joseph hospital in 1999 and 2000.

Mating-out assays and plasmid content analysis. Transfer of β -lactam resistance to *Escherichia coli* K-12C600S1000 (Str^R) was attempted by liquid and solid mating-out assays. The recipient and donor cells were mixed in a ratio of 1:1 and incubated in broth with moderate shaking at 37 °C for 1 h. Five hundred microlitres of each mixture was plated out on tryptic soy agar (TSA) plates and incubated overnight at 37 °C. Transconjugants were selected on Drigalski agar containing ampicillin (20 mg l⁻¹) and streptomycin (100 mg l⁻¹). Clinical isolates of *K. oxytoca* were examined for their plasmid DNA content by the procedure of Birnboim (1983).

DNA preparation. DNA templates were prepared by suspending a freshly grown colony in 500 μ l lysis buffer (20 mM Tris/HCl pH 8.3, 50 mM KCl, 0.1% Tween 20) and heating at 94 °C for 10 min. DNA preparations were stored at -20 °C.

Gene amplification and sequencing. To amplify the promoter and the coding regions of the *bla*_{OXY} genes, two primers were designed, one upstream from the promoter region (OXY-A: 5'-TCGGTAACTGTGACGGGA-3') and one downstream from the coding region (OXY-B: 5'-CCGAATTTCCGGAAGCCA-3'), on the basis of the *bla*_{OXY} sequences published previously. These primers generate a fragment of 1040 bp. The PCR mixture contained 20 mM Tris/HCl pH 8.0, 100 mM KCl, 1.5 mM MgCl₂, 500 μ M each dNTP, 0.2 μ M each primer and 0.0025 U *Taq* DNA polymerase μ l⁻¹. The amplification reaction consisted of a pre-PCR stage (at 94 °C for 5 min, at 51 °C for 1 min and at 72 °C for 1 min), and then 45 cycles at 94 °C for 30 s, at 51 °C for 30 s and at 72 °C for 30 s, and a final extension step at 72 °C for 10 min.

16S rDNA was amplified with two primers complementary to highly conserved regions in Gram-negative rRNA (A12, 5'-AAGCCTGATGCAGCCA-3'; A13, 5'-TTTCGCACCTGAGCGT-3'). The amplified fragment, which displays a great nucleotide variability in Gram-negative bacteria, starts at position 381 and ends at position 767 (387 bp) relative to the *E. coli* 16S rDNA sequence. The PCR mixture was identical to that described above, except for the MgCl₂ concentration, which was higher (2.5 mM). The reaction was performed with a pre-denaturation step for 5 min at 95 °C, then 35 cycles for 1 min at 95 °C, for 1 min at 45 °C and for 1 min at 72 °C, followed by a final extension step at 72 °C for 10 min.

The *rpoB* gene was partially amplified (512 bp) by using the primers CM32B and CM81B, together with the conditions described by Mollet *et al.* (1997). The first nucleotide of the fragment studied corresponds to codon 500 of the 1342 aa coding region in *E. coli*.

PCR products were sequenced by using an automated cycle sequencing system, with the same primers as for the PCR. However, for the *bla*_{OXY} gene, two additional internal primers were selected, OXY-C (5'-TATTAACACAGAG-3') and OXY-D (5'-ATTAGAGTCCGGA-3'), to obtain two additional priming sites for sequencing.

Sequence data analysis. Single sequence alignments were performed by using BLAST 2 sequence software (Tatusova & Madden, 1999), whereas multiple sequence alignments were performed with DIALIGN 2 software (Morgenstern, 1999). The *bla*_{OXY} gene sequences of our isolates were compared with those of reference strains SL781 (*bla*_{OXY-1}) and SL911 (*bla*_{OXY-2}) whose GenBank accession numbers are Z30177 and Z49084, respectively. The *rpoB* and 16S rDNA consensus sequences were constructed by using CAP software (Huang, 1992).

Tree reconstruction. Phylogenetic analyses were based on parsimony and were performed using version 4.0b5 of PAUP* (Swofford, 1998). Separate analysis was performed for each of the *rpoB* and 16S

rDNA sequence datasets. Both datasets were then grouped in a combined analysis according to the total-evidence method (Huelsenbeck, 1996). The analysis used a branch-and-bound search on all equally weighted characters. Gaps were considered to be missing data. Branch support was estimated using bootstrap analysis (1000 replicates). We added four external taxa: *E. coli* ATCC 25922^T (GenBank accession numbers for *rpoB* and 16S rDNA: U77436 and AF233451, respectively); *Raoultella ornithinolytica* ATCC 31898^T (AF129447 and AF129441, respectively); *Raoultella planticola* ATCC 33531^T (AF129449 and AF129443, respectively); and *Klebsiella pneumoniae* ATCC 13883^T (U77444 and AF130981, respectively), to test the monophyly of the *K. oxytoca* strains. The tree was rooted using *Rickettsia conorii* ATCC VR-141^T (*rpoB* and 16S rDNA GenBank accession numbers are AF076435 and L36105, respectively) as the outgroup.

Arbitrarily amplified DNA polymorphism. The primer used was the Enterobacterial Repetitive Intergenic Consensus sequence corresponding to ERIC-1R: 5'-ATGTAAGCTCCTGGGGATTAC-3' (Jones *et al.*, 1999). Amplification was performed with the Ready-To-Go RAPD Analysis Bead kit (Amersham Biosciences) under the conditions recommended by the manufacturer.

GenBank accession numbers. The sequences determined in this study have been submitted to GenBank and their corresponding accession numbers are presented in Table 1.

RESULTS

Mating-out assays and plasmid content analysis

A plasmid was detected in only two strains, SG81 and SG176. The transfer of these plasmids into *E. coli* resulted in transconjugants resistant to ampicillin. The gene encoding ampicillin resistance was not amplified with the primers specific for *bla*_{OXY} genes, but with primers specific for *bla*_{TEM} genes as published previously (Granier *et al.*, 2002b).

As the majority of *K. oxytoca* isolates were plasmid-free, and as repeated mating-out experiments failed to transfer

*bla*_{OXY} genes, these genes were assumed to be located on the bacterial chromosome.

*bla*_{OXY} gene sequences

According to the nucleotide sequences of the *bla*_{OXY} genes of the 13 clinical isolates in comparison with those of reference strains SL781 and SL911, four isolates possessed a *bla*_{OXY-1} gene and nine possessed a *bla*_{OXY-2} gene (Tables 2 and 3). All the *bla*_{OXY-1} genes of clinical isolates differed from that of the reference strain SL781 by a single nucleotide substitution (G8A) in the promoter region. They also differed from each other and from the reference *bla*_{OXY-1} gene within the coding region, either by point mutations leading to amino acid substitutions (Ala237Gly, Ala230Val), silent substitutions (G658A), or by a deletion of the first three or last three nucleotides of the palindromic sequence GCCG, starting at position 71. This resulted in the deletion of the Ala residue at position 12 according to the numbering of Ambler *et al.* (1991) (Table 2). Similar features were found in the *bla*_{OXY-2} genes of clinical isolates with respect to reference strain SL911 (Table 3). Six of the nine *bla*_{OXY-2} genes had substitutions in the promoter region, which consisted not only of G8A but also G12T. Five nucleotide substitutions located in the coding region led to amino acid substitutions (Gly20Ser, Asp35Ala, Asp197Asn, Asp255Asn and Val260Ile), whereas two were silent substitutions (A602G and C728A). The deletion of the Ala residue at position 12, which also occurred in the *bla*_{OXY-2} gene, resulted from the deletion of the first three or the last three nucleotides of the palindromic sequence GCCG, starting at position 69.

Overall, a single *bla*_{OXY} gene sequence was observed for the *bla*_{OXY-1} genes harboured by our four clinical isolates, whereas five single *bla*_{OXY} gene sequences were observed for

Table 1. GenBank accession numbers

<i>K. oxytoca</i> strain	<i>bla</i> _{OXY}	<i>rpoB</i>	16S rDNA
ATCC 13182 ^T	AF473577	U77442*	AF129440*
SL781 (reference strain)	Z30177*	AF469462	AF469361
SL911 (reference strain)	Z49084*	AF469466	AF469359
SG4	AY077489	Identical to U77442	AF469360
SG12	AY077482	AF469463	AF469362
SG43	AY077487	ND	ND
SG49	AY077486	AF480166	Identical to AF469362
SG62	Identical to Z30177	Identical to AF469467	Identical to AF469360
SG74	Identical to AF473577	ND	ND
SG77	AY077488	AF469467	Identical to AF129440
SG81	Identical to AF473577	Identical to AF469467	Identical to AF129440
SG176	AY077485	ND	ND
SG337	AY077483	AF469465	Identical to AF469362
SG344	AY077484	AF469464	Identical to AF469362

*Previously published sequences.

ND, Not determined.

Table 2. Nucleotide sequence of *bla*_{OXY-1} genes of *Klebsiella oxytoca* clinical isolates in comparison with that of reference strain SL781

Strain	Hospital (year)	Nucleotide position* (codon†)							Reference
		−8	+1‡	68–76§ (11–13)	658 (208)	723 (230)	744 (237)	788 (252)	
SL781		G	A	GCC GCC GTT (Ala Ala Val)	G (Val)	C (Ala)	C (Ala)	G (Pro)	Fournier & Roy (1997)
SG12	1 (1997)	A					G (Gly)		This study
SG49	2 (1991)	A			A (Val)	T (Val)	G (Gly)		This study
SG337	5 (1996)	A		GCC---GTT (Ala Val)		T (Val)			This study
SG344	6 (1998)	A			A (Val)	T (Val)	G (Gly)	C (Pro)	This study

*Fournier *et al.* (1995).†Ambler *et al.* (1991).

‡Transcriptional start site.

§-, Deletion.

Table 3. Nucleotide sequence of *bla*_{OXY-2} genes of *Klebsiella oxytoca* clinical isolates in comparison with that of reference strain SL911

Strain	Hospital (year)	Nucleotide position* (codon†)												Reference	
		−12	−8	+1‡	66–74§ (11–13)			93 (20)	139 (35)	602 (190)	621 (197)	728 (233)	789 (255)		804 (260)
SL911		G	G	C	GCC	GCC	GTT (Ala Ala Val)	G (Gly)	A(Asp)	A (Leu)	G (Asp)	C (Gly)	G (Asp)	G (Val)	Fournier & Roy (1997)
SG43	2 (1991)	T			GCC---GTT (Ala Val)										This study
SG69	2 (1998)	T			GCC---GTT (Ala Val)										This study
SG77	3 (1994)		A		GCC---GTT (Ala Val)										This study
SG9	1 (1984)		A		GCC---GTT (Ala Val)										This study
SG74	1 (1999)									G (Leu)				A (Ile)	This study
SG81	1 (1999)									G (Leu)				A (Ile)	This study; Granier <i>et al.</i> (2002b)
SG4	1 (1998)		A							G (Leu)	A (Asn)	A (Gly)			This study
SG62	4 (1998)		A						C (Ala)	G (Leu)	A (Asn)		A (Asn)		This study
SG176	1 (2000)							A (Ser)							This study

*Fournier *et al.* (1995).†Ambler *et al.* (1991).

‡Transcriptional start site.

§-, Deletion.

the *bla*_{OXY-2} genes harboured by our nine clinical isolates (Tables 2 and 3).

Table 4 shows that the mean percentage of *bla*_{OXY} gene identity observed within each group of *bla*_{OXY} genes was >99 %, whereas it was lower (about 87 %) between the two groups of *bla*_{OXY} genes.

16S rRNA and *rpoB* gene sequences

The sequence of the 16S rRNA and *rpoB* genes was determined for five strains harbouring the *bla*_{OXY-1} gene (the four clinical isolates and reference strain SL781) and for five strains harbouring the *bla*_{OXY-2} gene, including four clinical isolates (SG77, SG81, SG4 and SG62) and reference strain SL911.

The sequence of the 16S rDNA fragment studied (387 bp) was identical for the five strains harbouring the *bla*_{OXY-1} gene, except for reference strain SL781, in which a single nucleotide substitution was observed. The sequence of the corresponding 16S rDNA fragment in the strains possessing the *bla*_{OXY-2} gene was also largely conserved, but four different substitutions were observed, each in a single strain. Comparison of the 16S rDNA consensus sequences of the two types of strains, available as Supplementary Fig. A in IJSEM Online, showed that the two sequences differed from each other by seven nucleotide substitutions, located close to each other in a small fragment from positions 435 to 458 according to the numbering of the *E. coli* 16S rDNA sequence.

By using the *rpoB* fragment (512 bp), which is relevant to the differentiation of enterobacterial species, we observed that the nucleotide sequence was identical for three of the five strains harbouring the *bla*_{OXY-1} gene, whereas the two remaining strains differed from the three others (as well as from each other) by one substitution. The same feature was observed in the five strains possessing the *bla*_{OXY-2} gene, leading to an identical nucleotide sequence for three strains and two different nucleotide substitutions distinguishing the two remaining strains from each other as well as from the other three strains. As indicated in Fig. B (available as supplementary data in IJSEM Online), the *rpoB* consensus

sequence of the strains with the *bla*_{OXY-1} gene differed from that of the strains with the *bla*_{OXY-2} gene by 17 nucleotide substitutions, distributed all along the 512 bp fragment.

Table 4 shows that the mean percentage of gene identity for pairs of strains was very high (>99 %) for both the *rpoB* and 16S rRNA genes within each group of strains, but lower between the two groups of strains: <97 % for the *rpoB* gene and approximately 97.5 % for the 16S rRNA gene.

Phylogenetic relationships among *K. oxytoca* and other genera in the *Enterobacteriaceae*

The phylogenetic trees resulting from the separate analyses of *rpoB* and 16S rDNA datasets are not shown here. Combination of the datasets globally produced a similar topology, with better robustness and resolution. The combined analysis was computed on 980 molecular characters, of which 61 were parsimony-informative. This led to three trees (length=392 steps, Ciex=0.6250, Hiex=0.3750). The ten unrelated *K. oxytoca* strains were monophyletic and separated into two clades, corresponding to the strains with the *bla*_{OXY-1} gene and the strains with the *bla*_{OXY-2} gene. This result was supported by high bootstrap values of 97 and 99 %, respectively. The strict consensus of the three trees is shown in Fig. 1 with the bootstrap values.

Arbitrarily amplified DNA polymorphism

As shown in Fig. 2, although the arbitrarily amplified DNA profiles of the ten unlinked strains differed from each other, we can observe two clearly distinct profile types, each related to one of the two types of strains defined by the OXY β -lactamase harboured. Thus, two intense bands of approximately 1400 and 1600 bp are always present in strains harbouring the *bla*_{OXY-1} gene and never in the strains with the *bla*_{OXY-2} gene; likewise, one or two intense bands of approximately 500 bp are always present in strains possessing the *bla*_{OXY-2} gene and never in those with the *bla*_{OXY-1} gene.

Table 4. Mean percentage of *bla*_{OXY}, *rpoB* and 16S rRNA gene identity within and between the groups of strains with *bla*_{OXY-1} and *bla*_{OXY-2} genes

The *bla*_{OXY-1} group includes strains SG12, SG49, SG337, SG344 and SL781; the *bla*_{OXY-2} group includes strains SG4, SG62, SG77, SG81 and SL911.

Comparison of strains	Mean percentage identity $\pm$ SE		
	<i>bla</i> _{OXY}	<i>rpoB</i>	16S rRNA
<i>bla</i> _{OXY-1} vs <i>bla</i> _{OXY-1}	99.659 $\pm$ 0.202	99.646 $\pm$ 0.153	99.684 $\pm$ 0.408
<i>bla</i> _{OXY-2} vs <i>bla</i> _{OXY-2}	99.651 $\pm$ 0.260	99.841 $\pm$ 0.124	99.528 $\pm$ 0.408
<i>bla</i> _{OXY-1} vs <i>bla</i> _{OXY-2}	86.857 $\pm$ 0.403	96.562 $\pm$ 0.241	97.801 $\pm$ 0.215

Fig. 1. Phylogenetic tree derived from combined analysis of the 16S rDNA and *rpoB* sequences of five *Klebsiella oxytoca* strains harbouring the *bla*_{OXY-1} gene (SG344, SL781, SG337, SG12 and SG49) and five harbouring the *bla*_{OXY-2} gene (SL911, SG81, SG77, SG62 and SG4), *Escherichia coli* ATCC 25922^T, *Raoultella ornithinolytica* ATCC 31898^T, *Raoultella planticola* ATCC 33531^T and *Klebsiella pneumoniae* ATCC 13883^T. The tree was determined by the parsimony method with *Rickettsia conorii* ATCC VR-141^T as the outgroup. Numbers within the dendrogram indicate the bootstrap values of the branching order (only values of 70 and above are shown).

DISCUSSION

*bla*_{OXY} promoter regions

Ten of the thirteen clinical isolates of *K. oxytoca* had been collected over a 17-year period in six French hospitals, because of their resistance spectrum that extended to ceftriaxone, cefotaxime and aztreonam. Such an extension was previously demonstrated to be due to the over-production of β -lactamase in relation to up-mutations (Fournier *et al.*, 1995). Effectively, we found the two previously determined types of substitutions located in the -10 region of the promoter of the gene encoding the OXY-1 and OXY-2 β -lactamase of the ten clinical isolates (Fournier *et al.*, 1996a). For the three remaining strains, which were phenotypically susceptible to third-generation cephalosporins and aztreonam, no substitution was found in the promoter region.

β -Lactamase diversity within the OXY-1 and OXY-2 groups

Among the OXY-1 and OXY-2 β -lactamases found in our clinical isolates, some differences were observed in the primary gene sequence of the β -lactamase in each group.

Fig. 2. Agarose gel electrophoresis of arbitrarily amplified DNA polymorphism of *Klebsiella oxytoca* isolates. Strains SG12, SG49, SG337, SG344 and reference strain SL781 (harbouring the *bla*_{OXY-1} gene) are presented in lanes 1–5, respectively. Strains SG4, SG62, SG77, SG81 and reference strain SL911 (harbouring the *bla*_{OXY-2} gene) are presented in lanes 7–11, respectively. Lane 6 corresponds to the molecular size marker (100 bp ladder, Amersham Biosciences) with the most intense band corresponding to 800 bp. Arrows indicate the principal fragments which allow us to distinguish the two types of *K. oxytoca* strains.

Regarding the β -lactamases of the OXY-1 group, the two amino acid substitutions observed in our strains, Ala237Gly and Ala230Val, have already been described and shown to have no effect on hydrolytic activity (Fournier & Roy, 1997). However, they have never been described alone (Ala237Gly in strain SG12), nor combined with each other without other amino acid substitutions (strains SG49 and SG344), nor combined with the new genetic feature displayed by the OXY-1 β -lactamase of strain SG337. Indeed, the *bla*_{OXY-1} gene of this strain displayed a deletion of three nucleotides leading to the deletion of the last of the four Ala residues located 12 codons downstream from the starting Met, i.e.

located in the signal peptide. By taking into account the published protein sequences of OXY-1 β -lactamase and those presented here, eight OXY-1 β -lactamases have been molecularly characterized to date (Fournier & Roy, 1997; Wu *et al.*, 1999).

Concerning the OXY-2 β -lactamases, those produced by strains SG4 and SG62 corresponded to two of the four published by Fournier *et al.* (1997). Although they were not epidemiologically related, four of our clinical isolates had an identical OXY-2 β -lactamase, characterized by the deletion of an Ala residue belonging to the Ala-Ala-Ala motif located in the signal peptide. By consulting the literature, we found that this deletion is present in two previously published OXY-2 β -lactamases, without mention by the authors (Kimura *et al.*, 1996; Wu *et al.*, 1999). Another modification was found in the signal peptide sequence, namely Gly20Ser (strain SG176), which was also published previously, but was associated with other amino acid substitutions (Granier *et al.*, 2002a; Sirot *et al.*, 1998). Finally, strains SG74 and SG81 harboured an OXY-2 β -lactamase with a novel amino acid substitution, Val260Ile. As both strains SG74 and SG81 were susceptible to third-generation cephalosporins and aztreonam, substitution Val260Ile does not appear to modify the hydrolytic activity of the enzyme.

Overall, by considering the published OXY-2 β -lactamases and those presented here, nine OXY-2 β -lactamases have been characterized to date. Comparison of the sequence of published *bla*_{OXY} genes with those found in our clinical isolates confirmed that the percentage of gene identity was extremely high (>99%) within each *bla*_{OXY} gene group, and significantly lower, about 87%, between the two *bla*_{OXY} gene groups.

Two clades in the *K. oxytoca* taxon

By studying three chromosomal genes (*bla*_{OXY}, 16S rDNA and *rpoB*) of ten unrelated *K. oxytoca* strains, we were able to define two clades in the *K. oxytoca* taxon. This finding is in accordance with the study of Brisse *et al.* (2001), which had shown, using other genes (namely *gyrA* and *parC*) and different typing methods (RAPD and ribotyping), that six clinical isolates of *K. oxytoca* and reference strain *K. oxytoca* NCTC 49131 were classified into two clusters. The new relevant point in our study is the demonstration that this genetic delineation was also obtained from the chromosomal β -lactamase gene of *K. oxytoca*, which does not belong to the housekeeping gene family. To our knowledge, it is the first demonstration of a chromosomal β -lactamase that is able to divide a taxon into clades. This finding suggests that chromosomal *bla* genes, which are extremely common in the *Enterobacteriaceae*, are affected by an evolutionary process, as well as housekeeping genes. Therefore, evolution of the *bla* gene might also reflect species evolution.

The second significant point of our study is that the 16S rRNA gene, although it has a low evolutionary rate, was also

able to define the two clades in the *K. oxytoca* taxon (Palys *et al.*, 1997). Moreover, another discriminatory gene recently used in parallel with the 16S rRNA gene to establish the phylogenetic relationships of enterobacterial species, namely the *rpoB* gene, was also able to delineate the two clades (Mollet *et al.*, 1997).

Regarding *K. oxytoca* strain ATCC 13182^T, which was used by Mollet *et al.* (1997) and Drancourt *et al.* (2001) to establish the phylogenetic relationships between enterobacterial species, we deduced from the comparison of its 16S rDNA and *rpoB* sequences (GenBank accession numbers AF129440 and U77442, respectively) with the 16S rDNA and *rpoB* consensus sequences from this study, that its β -lactamase belongs to the OXY-2 β -lactamase group. We confirmed this classification by sequencing the *bla*_{OXY} gene and we found that the OXY-2 β -lactamase of this strain differs from the reference sequence (strain SL911) by the substitution Val260Ile (data not shown).

This result means that species identification and determination of the OXY β -lactamase category can be achieved by a single PCR, and sequencing of either the 16S rRNA gene or *rpoB* gene. To state that PCR of the *bla*_{OXY} gene can also be used to identify the species as well as the category of β -lactamase, we have to verify that the primers used are not able to amplify any other *bla* gene.

Considering the fact that each *K. oxytoca* isolate is classified into one of the two clades regardless of the gene sequence used (*bla*_{OXY}, 16S rDNA, *rpoB*) and that the two clades were delineated with bootstrap values as high as those that delineate the two *Raoultella* species studied, we suggest that *K. oxytoca* should be divided into two genetic groups, group oxy-1 represented by *K. oxytoca* strain SL781 (=CIP 104963) and group oxy-2 by *K. oxytoca* strain SL911 (=CIP 106098).

The last point resulting from our study is the fact that the two genetic groups of *K. oxytoca* can be distinguished by using the ERIC-1R PCR method, as the isolates of these two groups display distinct profiles, each with characteristic bands. Such a procedure, if it is confirmed on a larger collection of *K. oxytoca* isolates, could be carried out in molecular epidemiological studies and in laboratories that do not yet have access to the gene amplification and sequencing methods, which are both time-consuming and expensive (Olive & Bean, 1999).

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