

Six Groups of the OXY β -Lactamase Evolved over Millions of Years in *Klebsiella oxytoca*

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The diversity and evolution of the class A OXY β -lactamase from *Klebsiella oxytoca* were investigated and compared to housekeeping gene diversity. The entire *bla*_{OXY} coding region was sequenced in 18 clinical isolates representative of the four *K. oxytoca* β -lactamase gene groups *bla*_{OXY-1} to *bla*_{OXY-4} and of two new groups identified here, *bla*_{OXY-5} (with four isolates with pI 7.2 and one with pI 7.7) and *bla*_{OXY-6} (with four isolates with pI 7.75 and three with pI 8.1). Genes *bla*_{OXY-5} and *bla*_{OXY-6} showed 99.8% within-group nucleotide similarity but differed from each other by 4.2% and from *bla*_{OXY-1}, their closest relative, by 2.5% and 2.9%, respectively. Antimicrobial susceptibility to β -lactams was similar among OXY groups. Nucleotide sequence diversity of the 16S rRNA (1,454 bp), *rpoB* (940 bp), *gyrA* (383 bp), and *gapDH* (573 bp) genes was in agreement with the β -lactamase gene phylogeny. Strains with *bla*_{OXY-1}, *bla*_{OXY-2}, *bla*_{OXY-3}, *bla*_{OXY-4}, and *bla*_{OXY-6} genes formed five phylogenetic groups, named KoI, KoII, KoIII, KoIV, and KoVI, respectively. Isolates harboring *bla*_{OXY-5} appeared to represent an emerging lineage within KoI. We estimated that the *bla*_{OXY} gene has been evolving within *K. oxytoca* for approximately 100 million years, using as calibration the 140-million-year estimation of the *Escherichia coli*-*Salmonella enterica* split. These results show that the *bla*_{OXY} gene has diversified along *K. oxytoca* phylogenetic lines over long periods of time without concomitant evolution of the antimicrobial resistance phenotype.

Klebsiella oxytoca is an important opportunistic pathogen causing serious infections in hospitalized patients, including neonates (16, 19, 28). *K. oxytoca* is naturally resistant to amino- and carboxy-penicillins (21), a phenotype due to the constitutive expression of a chromosomal class A β -lactamase (1), first called K1 (7, 20) or KOXY (26) and now OXY (12). Due to the hyperproduction of the chromosomal β -lactamase, up to 10 to 20% of *K. oxytoca* strains (22) can show high-level resistance to certain expanded-spectrum cephalosporins (ceftriaxone and cefotaxime) and aztreonam (8). Up mutations in the promoter sequence of the genes are responsible for this phenotype (9–11). Overproducers of OXY enzymes are commonly resistant to all combinations of β -lactams with β -lactam inhibitors (21).

Sequence diversity of the *K. oxytoca* chromosomal β -lactamase gene and the existence of discrete groups of OXY enzymes have been described. Fournier et al. (12) found a variant (*bla*_{OXY-2}) of the chromosomal β -lactamase gene that differed from *bla*_{OXY-1} (1) by 12.7% in nucleotide sequence. It was suggested, based on colony hybridization, that the β -lactamase genes of *K. oxytoca* could be classified into two groups (OXY-1 and OXY-2), each representing approximately half of the clinical isolates (12). Recently, Granier et al. (14) identified two additional sequence variants, defined as *bla*_{OXY-3} and *bla*_{OXY-4}, each found in a single strain so far. Gene *bla*_{OXY-3} shares 85 and 84% similarity with *bla*_{OXY-1} and *bla*_{OXY-2}, re-

spectively, whereas the corresponding values for *bla*_{OXY-4} are 95 and 86%, respectively.

The recognition of distinct groups of β -lactamase genes within a pathogenic species is relevant to the epidemiology and control of infections. From an evolutionary point of view, the existence of discrete groups of β -lactamase sequences within *K. oxytoca* could reflect the independent introduction of these variants by several horizontal transfer events from unknown donors. Alternately, these variants could have evolved from a common ancestral β -lactamase gene that was anciently present in *K. oxytoca*. Elements in support of the latter hypothesis include the chromosomal localization of the gene (1, 15) and the correspondence of β -lactamase variation with sequence variation in other regions of the chromosome. It was recently shown that OXY-1 and OXY-2 variant enzyme groups are harbored by two genetic groups of *K. oxytoca* strains, named OXY-1 and OXY-2 (15) and later oxy-1 and oxy-2 (14), that can be distinguished by *rpoB* and 16S rRNA gene sequences and by ERIC-1R PCR (15). In addition, it was suggested that *K. oxytoca* strain SG271, which harbors *bla*_{OXY-3}, represents a new genetic group, named oxy-3 (14). In contrast, it was unclear whether *bla*_{OXY-4}-harboring strain SG266 belongs to genetic group OXY-1 or instead to a new group closely related to OXY-1 (14). Independently, Brisse and Verhoef (4) demonstrated the existence of at least two *K. oxytoca* genetic groups, called KoI and KoII, based on *gyrA* and *parC* gene sequences, random amplified polymorphic DNA analysis, and automated ribotyping. However, the correspondence between the OXY-1 and OXY-2 groups (14, 15) and KoI and KoII groups (4) has not been investigated.

In order to challenge further the hypothesis of an evolution-

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TABLE 1. *K. oxytoca* strains studied, β -lactamase (*bla*) gene sequences, housekeeping gene sequences, and their characteristics^a

No.	Strain name(s)	Geographical source	Clinical source	Phylogenetic group	<i>bla</i> group	<i>bla</i> accession no.	Housekeeping gene accession no.			
							<i>rpoB</i>	<i>gyrA</i>	<i>gapDH</i>	16S rRNA
Study isolates										
1	SB9	Spain	Blood	KoI (f)	<i>bla</i> _{OXY-1}	AJ871864	AJ871801	AJ303591	AJ871820	AJ871855
2	SB71	France	Blood	KoI (f)	<i>bla</i> _{OXY-1}	AJ871865	AJ871802	AJ871839	AJ871821	AJ871856
3	SB136 (ATCC 49131)			KoII (f)	<i>bla</i> _{OXY-2}	AJ871866	AJ871803	AF303608	AJ871822	AJ871857
4	SB175 (ATCC 13182T)			KoII (f)	<i>bla</i> _{OXY-2}	AJ871867	AJ871804	AJ871840	AJ871823	AJ871858
5	SG271		Clinical	KoIII	<i>bla</i> _{OXY-3} (a)	AF491278 (a)	AJ871805	AJ871842	AJ871824	ND ^b
6	SG266		Clinical	KoIV	<i>bla</i> _{OXY-4} (a)	AY077481 (a)	AJ871806	AJ871841	AJ871825	ND
7	SB2908	Italy	Blood	KoI	<i>bla</i> _{OXY-5}	AJ871868	AJ871807	AJ871843	AJ871826	AJ871859
8	SB2909	Italy	Blood	KoI	<i>bla</i> _{OXY-5}	AJ871869	AJ871808	AJ871844	AJ871827	ND
9	SB2910	Italy	Blood	KoI	<i>bla</i> _{OXY-5}	AJ871870	AJ871809	AJ871845	AJ871828	ND
10	SB2933	Switzerland	Blood	KoI	<i>bla</i> _{OXY-5}	AJ871871	AJ871810	AJ871846	AJ871829	ND
11	SB2942	Netherland	Blood	KoI	<i>bla</i> _{OXY-5}	AJ871872	AJ871811	AJ871847	AJ871830	AJ871860
12	SB73	France	Wound	KoVI	<i>bla</i> _{OXY-6}	AJ871873	AJ871812	AJ871848	AJ871831	AJ871861
13	SB75	Germany	Blood	KoVI	<i>bla</i> _{OXY-6}	AJ871874	AJ871813	AJ871849	AJ871832	ND
14	SB324	Germany	Blood	KoVI	<i>bla</i> _{OXY-6}	AJ871875	AJ871814	AJ871850	AJ871833	ND
15	SB352	France	Wound	KoVI	<i>bla</i> _{OXY-8}	AJ871876	AJ871815	AJ871851	AJ871834	ND
16	SB397	South Africa	Blood	KoVI	<i>bla</i> _{OXY-6}	AJ871877	AJ871816	AJ871852	AJ871835	ND
17	SB3037	Ukraine	Wound	KoVI	<i>bla</i> _{OXY-6}	AJ871878	AJ871817	AJ871853	AJ871836	AJ871862
18	SB3051	France	Blood	KoVI	<i>bla</i> _{OXY-6}	AJ871879	AJ871818	AJ871854	AJ871837	AJ871863
<i>K. oxytoca</i> ^c										
19	SL781 (CIP104963)	France		KoI	<i>bla</i> _{OXY-1} (b)	Z30177 (b)	ND	ND	ND	ND
20	SG12	France		KoI	<i>bla</i> _{OXY-1} (a)	AY077482 (a)	ND	ND	ND	ND
21	SG337	France		KoI	<i>bla</i> _{OXY-1} (a)	AY077483 (a)	ND	ND	ND	ND
22	SG49	France		KoI	<i>bla</i> _{OXY-1} (a)	AY077486 (a)	ND	ND	ND	ND
23	KH66	Sweden		KoI	<i>bla</i> _{OXY-1} (c)	Y17715 (c)	ND	ND	ND	ND
24	E23004	Japan		KoI	<i>bla</i> _{OXY-1} (d)	M27459 (d)	ND	ND	ND	ND
25	SG77	France		KoII	<i>bla</i> _{OXY-2} (a)	AY077488 (a)	ND	ND	ND	ND
26	SG43	France		KoII	<i>bla</i> _{OXY-2} (a)	AY077487 (a)	ND	ND	ND	ND
27	SG176	France		KoII	<i>bla</i> _{OXY-2} (a)	AY077485 (a)	ND	ND	ND	ND
28	(CIP106098) SL911	France		KoII	<i>bla</i> _{OXY-2} (b)	Z49084 (b)	ND	ND	ND	ND
29	SC10,436			KoII	<i>bla</i> _{OXY-2} (e)	AY055205 (e)	ND	ND	ND	ND
30	KH11	Sweden		KoII	<i>bla</i> _{OXY-2} (c)	Y17714 (c)	ND	ND	ND	ND
<i>K. pneumoniae</i>										
31	SB132 (ATCC 13883T)			KpI	<i>bla</i> _{SHV} (g)	AJ635407 (g)	AJ871819	AF303606 (f)	AJ875018	ND

^a Phylogenetic groups, *bla* groups, and *bla* accession numbers followed by letter in parentheses were previously determined. References: a, 14; b, 11; c,31; d,1; e,15a; f,4; g,18. Other accession numbers correspond to sequences determined in this work.

^b ND, not determined.

^c Nucleotide sequences included for comparison.

any diversification of OXY variants from a common *K. oxytoca* ancestral enzyme, we investigated the possible congruence of β -lactamase gene phylogeny with the phylogeny derived from three housekeeping genes. In addition, we describe two new β -lactamase groups that we called *bla*_{OXY-5} and *bla*_{OXY-6}.

MATERIALS AND METHODS

Bacterial strains. A total of 182 *K. oxytoca* clinical isolates from a previous study (3) were initially analyzed by PCR using OXY-1- and OXY-2-specific primers (13). We next selected 18 *K. oxytoca* strains for sequencing of several genes (Table 1). Four of these strains were representatives of the previously described KoI and KoII phylogenetic groups (4). Strains SG266, harboring *bla*_{OXY-4}, and SG271, harboring *bla*_{OXY-3} (14), were kindly provided by M.-H. Nicolas-Chanoine. The remaining 12 isolates (Table 1) were selected based on OXY-1 and OXY-2 PCR assays (see Results). Identification was initially performed using a VITEK apparatus (bioMérieux) and the indole test and confirmed for the 18 selected strains based on their *gyrA* sequences (4).

DNA preparation. DNA templates were prepared by suspending a freshly grown colony (approximately 30 mg) in 200 μ l of purified water, heating it at 94°C for 10 min, and submitting the extracts to minicentrifugation at 7,500 \times g for 5 min. Supernatants were stored at -20°C until use.

PCR amplification. In order to amplify the *bla*_{OXY} gene, primers OXY-E and OXY-G (Table 2) were designed based on the alignment of previously published *bla*_{OXY-1} to *bla*_{OXY-4} gene sequences. The amplified portion that permitted us to

determine the sequence of the entire β -lactamase coding region stretched from 189 bp upstream of the start codon to 37 bp downstream of the stop codon. The 50- μ l PCR mixture contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.2 μ M each primer, and 100 μ M each deoxynucleoside triphosphate. Samples were submitted to an initial denaturation step (30 s at 94°C), followed by 35 amplification cycles (94°C, 30 s; 50°C, 30 s; 72°C, 30 s) and a final elongation step of 5 min at 72°C. PCR products were visualized under UV light after agarose gel electrophoresis.

Specific *bla*_{OXY-1} and *bla*_{OXY-2} PCR assays were performed using previously defined (13) primers OXY1-A and OXY1-B and primers OXY2-A and OXY2-B, respectively (Table 2), at an annealing temperature of 50°C.

The nearly complete sequence of the 16S rRNA gene (*rrs*) was obtained using PCR primers Ad and rJ (Table 2). PCR amplification conditions were as described above. Cycling conditions were 4 min at 94°C, followed by 35 cycles (94°C, 1 min; 49°C, 1 min; 72°C, 1 min) and 7 min at 72°C.

Three housekeeping gene portions were amplified by PCR. The sequence of a 940-bp portion of the RNA polymerase beta subunit gene (*rpoB*) was determined using primers VIC2 and VIC3 (Table 2). The sequence of a 383-bp portion of the gyrase subunit A gene (*gyrA*) was determined as previously described (4). The sequence of a 573-bp portion of the glyceraldehyde 3-phosphate dehydrogenase gene (*gapDH*) was determined using primers gapF-173 and gapR-181 (Table 2). PCR amplification conditions were as described for the β -lactamase gene, except that the annealing temperature was 55°C for *rpoB* and 60°C for *gapDH*.

PCR primer pairs were designed to specifically amplify β -lactamase genes of the *bla*_{OXY-5} and *bla*_{OXY-6} groups: OXY5-B, OXY5-C, OXY6-B, and OXY6-C

TABLE 2. Primers, their sequences, and their positions on the gene

Gene and primer	Sequence	Positions ^a
<i>bla</i>		
OXY-E	5'-GGT TTT GGT AAC TGT GAC GGG-3'	-189 to -168
OXY-G	5'-CAG AGT GCA GAG TGT TGC AG-3'	913 to 894
OXY-H	5'-GCG ACA ATA C(G/C)G CGA TGA-3'	397 to 414
OXY1-A	5'-CGT GGC GTA AAA CCG CCC TG-3'	14 to 33
OXY1-B	5'-GTC CGC CAA GGT AGC TAA TC-3'	442 to 423
OXY2-A	5'-AAG GCT GGA GAT TAA CGC AG-3'	285 to 304
OXY2-B	5'-GCC CGC CAA GGT AGC CGA TG-3'	439 to 420
OXY5-B	5'-GAT TAA AAA AGC GGA TTT AGT A-3'	297 to 318
OXY5-C	5'-GCC CGC CAA GGT AC CGA TA-3'	442 to 423
OXY6-B	5'-CGG ATG ATT CGC AAA CCC TT-3'	167 to 186
OXY6-C	5'-CGG GAT GGC TTT CGC TCT GC-3'	274 to 255
<i>rpoB</i>		
VIC2	5'-GGT TAC AAC TTC GAA GAC TC-3'	2469 to 2489
VIC3	5'-GGC GAA ATG GC(A/T) GAG AAC CA-3'	1442 to 1422
<i>gyrA</i>		
gyrA-A	5'-CGC GTA CTA TAC GCC ATG AAC GTA-3'	139 to 163
gyrA-C	5'-ACC GTT GAT CAC TTC GGT CAG G-3'	762 to 741
<i>gapDH</i>		
gapF-173	5'-TGA AAT ATG ACT CCA CTC ACG G-3'	135 to 153
gapR-181	5'-CTT CAG AAG CGG CTT TGA TGG CTT-3'	771 to 747
<i>rns</i>		
Ad	5'-AGA GTT TGA TCM TGG CTC AG-3'	8 to 27
rJ	5'-GGT TAC CTT GTT ACG ACT T-3'	1508 to 1491
D	5'-CAG CAG CCG GCG TAA TAC-3'	518 to 535
E	5'-ATT AGA TAC CCT GGT AGT CC-3'	786 to 805
rE	5'-GGA CTA CCA GGG TAT CTA AT-3'	805 to 786

^a The first position refers to the start codon, except for the *rns* sequences, for which the first position corresponds to the first nucleotide of the sequence available under accession number Y01859 in the GenBank database.

(Table 2). PCR conditions were as described above, using an annealing temperature of 55°C.

Sequence determination. PCR products and sequence reaction products were purified by the ultrafiltration method (Millipore). The Ready Reaction Big Dye Terminator Cycle Sequencing v3.1 kit (Perkin-Elmer) and an ABI 3700 automated capillary DNA sequencer (Perkin-Elmer) were used. Primers used for sequencing were the same as those used for amplification. For the 1,102-bp *bla*_{OXY} gene product amplified with primers OXY-E and OXY-G, the internal sequencing primer OXY-H was used in addition (Table 2). For 16S rRNA gene sequencing, internal primers D, E, and rE (Table 2) were used in addition to the PCR primers.

Phylogenetic analysis. Sequence alignments were obtained using the MegAlign program of the LaserGene package (DNA Star Inc., Madison, Wisconsin). Phylogenetic trees were obtained with PAUP* version 4.0b10 (30), using the neighbor-joining method based on Kimura's two parameter distance. Bootstrap analysis was performed with 1,000 replicates. Trees generated by PAUP* were saved and drawn using TreeEdit v1.0a10 (built by A. Rambaut and M. Charleston in 2001 [http://evolve.zoo.ox.ac.uk]). Numbers of synonymous substitutions per synonymous site (*K_s*) and of nonsynonymous substitutions per nonsynonymous site (*K_a*) were estimated using DNASP version 3.53 (29). Sequences of *rpoB* and *gyrA* used for calibration of the substitution rate were derived from *Salmonella paratyphi* A strain ATCC 9150 and *Escherichia coli* strain K-12.

Determination of MICs. Antimicrobial susceptibility was determined by the dilution method on Müller-Hinton agar according to the recommendations of the French Society for Microbiology (6). The following drugs were used: amoxicillin, ceftazidime, clavulanate, and ticarcillin from GlaxoSmithKline; piperacillin, cephalothin, and cefoxitin from Sigma-Aldrich; cefotaxime from PanPharma; cefepime from Bristol-Myers Squibb; imipenem from Merck Sharp & Dohme-Chibret; aztreonam from Sanofi-Synthelabo; and tazobactam from Wyeth.

Isoelectric focusing. Crude extracts of β-lactamases were obtained by sonication. Isoelectric focusing was performed using a PhastSystem apparatus with PhastGel IEF 3-9 or 5-8 gels (Amersham-Pharmacia Biotech, Freiburg, Germany) and following the manufacturer's recommendations. β-Lactamase activity

was revealed by staining the gel with 0.5 mg/ml of the chromogenic β-lactamase nitrocefin (Oxoid, Basingstoke, England). The β-lactamases used as references were TEM-1 (pI 5.4), TEM-52 (pI 6), SHV-3 (pI 7.0), OXA-30 (pI 7.3), SHV-1 (pI 7.6), OXY-3 (pI 7.7), OXA-35 (pI 8.0), and SHV-5 (pI 8.2).

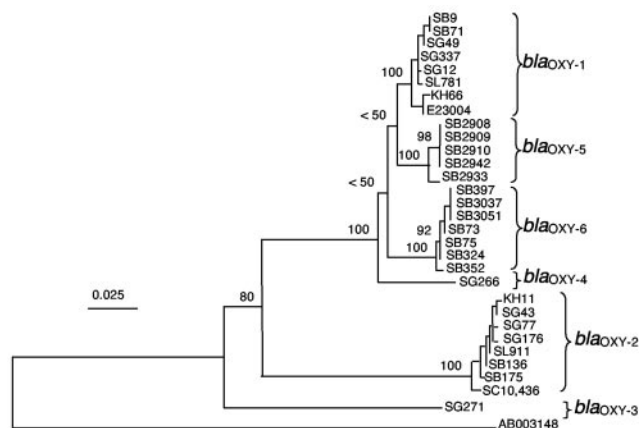


FIG. 1. Phylogeny of the *K. oxytoca* β-lactamase gene. The neighbor-joining tree was rooted using as an outgroup the gene sequence of the SFO-1 β-lactamase (GenBank accession no. AB003148), the closest relative of *bla*_{OXY}. Values at the nodes correspond to bootstrap values obtained after 1,000 replicates. Six major branches of *K. oxytoca* β-lactamase sequences are visible, corresponding to the *bla*_{OXY-1} to *bla*_{OXY-6} β-lactamase gene groups.

TABLE 3. Amino acid sequence variation among OXY-1 to OXY-6 β -lactamase (*bla*) groups

Strain	<i>bla</i> allele ^a	Enzyme ^a	pI	Amino acid at position (Ambler no.):													
				2	4	9 (5)	11 (7)	12 (8)	15 (11)	16 (12)	17 (13)	22 (18)	24 (20)	25 (21)	29 (25)	30 (26)	31 (27)
SL781	<i>bla</i> _{OXY-1-1a}	OXY-1-1	7.5 ^b	L	S	T	L	M	A	A	V	A	G	S	S	A	D
SB71	<i>bla</i> _{OXY-1-2a}	OXY-1-2	7.7														
SB9	<i>bla</i> _{OXY-1-2a}	OXY-1-2	7.7														
SG12	<i>bla</i> _{OXY-1-3a}	OXY-1-3	NA ^c														
SG337	<i>bla</i> _{OXY-1-4a}	OXY-1-4	NA							Del ^d							
SG49	<i>bla</i> _{OXY-1-5a}	OXY-1-5	NA														
KH66	<i>bla</i> _{OXY-1-6a}	OXY-1-6	5.25 ^b														
E23004	<i>bla</i> _{OXY-1-7a}	OXY-1-7	7.4 ^b														
SG271	<i>bla</i> _{OXY-3-1a}	OXY-3-1	6.7 ^b	I	T	S		I		Del	L	C	S		Del	Del	N
SG266	<i>bla</i> _{OXY-4-1a}	OXY-4-1	7.7 ^b			S				Del							
SB2908	<i>bla</i> _{OXY-5-1a}	OXY-5-1	7.2							Del							
SB2909	<i>bla</i> _{OXY-5-1a}	OXY-5-1	7.2							Del							
SB2910	<i>bla</i> _{OXY-5-1a}	OXY-5-1	7.2							Del							
SB2942	<i>bla</i> _{OXY-5-1a}	OXY-5-1	7.2							Del							
SB2933	<i>bla</i> _{OXY-5-2a}	OXY-5-2	7.7							Del							
SB73	<i>bla</i> _{OXY-6-1a}	OXY-6-1	7.75			S											
SB75	<i>bla</i> _{OXY-8-1a}	OXY-6-1	7.75			S											
SB324	<i>bla</i> _{OXY-6-2a}	OXY-6-2	7.75			S				Del							
SB352	<i>bla</i> _{OXY-5-3a}	OXY-6-3	7.75			S											
SB397	<i>bla</i> _{OXY-6-4a}	OXY-6-4	8.1			S											
SB3037	<i>bla</i> _{OXY-6-4a}	OXY-6-4	8.1			S											
SB3051	<i>bla</i> _{OXY-6-4a}	OXY-6-4	8.1			S											
SB136	<i>bla</i> _{OXY-2-1a}	OXY-2-1	NA	I	I	M	L			Del			A			T	
SB175	<i>bla</i> _{OXY-2-2a}	OXY-2-2	NA	I	I	M	L			Del			A			T	
SG77	<i>bla</i> _{OXY-2-3a}	OXY-2-3	NA	I	I	M	L	Del	Del	Del			A			T	
KH11	<i>bla</i> _{OXY-2-4a}	OXY-2-4	NA	I	I	M	L	Del	Del	Del			A			T	
SG43	<i>bla</i> _{OXY-2-5a}	OXY-2-5	NA	I	I	M	L	Del	Del	Del			A			T	
SG176	<i>bla</i> _{OXY-2-6a}	OXY-2-6	NA	I	I	M	L		Del	Del		S	A			T	
SL911	<i>bla</i> _{OXY-2-7a}	OXY-2-7	NA	I	I	M	L		Del	Del			A			T	
SC10,436	<i>bla</i> _{OXY-2-8a}	OXY-2-8	NA	I	I	M	L		Del	Del		S	A			T	

^a Numbered in this study.

^b Value from the literature.

^c NA, not available.

^d Del, deletion.

Nucleotide sequence accession numbers. The β -lactamase, 16S rRNA, *spoB*, *gyrA*, and *gapDH* gene sequences determined in this study have been submitted to the EMBL and GenBank databases and assigned the accession numbers listed in Table 1.

RESULTS

Correspondence between phylogenetic groups KoI and KoII and β -lactamase genes *bla*_{OXY-1} and *bla*_{OXY-2}. In order to establish a possible correspondence between classifications of *K. oxytoca* strains (4) and OXY β -lactamases (12), 183 isolates (182 clinical isolates and 1 from a rice plant) were identified by characterization of the *gyrA* gene and analyzed by PCR using *bla*_{OXY} group-specific primers (13). Based on *gyrA* characterization, 80 isolates could be assigned to KoI, whereas 96 isolates were assigned to KoII and 7 isolates represented a new *gyrA* phylogenetic group (called group KoVI; see below). Of the 80 KoI isolates, 75 were *bla*_{OXY-1} PCR positive and *bla*_{OXY-2} PCR negative. The five remaining isolates were atypical, being negative for both PCR assays. The 96 KoII isolates were *bla*_{OXY-1} PCR negative and *bla*_{OXY-2} PCR positive. The seven KoVI isolates showed the opposite result, being *bla*_{OXY-1} PCR positive and *bla*_{OXY-2} PCR negative. Therefore, we concluded that the OXY-1 and OXY-2 β -lactamase groups corresponded to the KoI and KoII phylogenetic groups, respectively, with the exception of five atypical KoI isolates. In

addition, a new *gyrA* lineage was found, which apparently had a β -lactamase gene corresponding to or related to *bla*_{OXY-1}.

β -Lactamase gene sequencing. The nucleotide sequence of the β -lactamase gene was determined (using PCR primers OXY-E and OXY-G) for the five KoI isolates that were negative for primer OXY1-A and OXY1-B and primer OXY2-A and OXY2-B PCR assays, for the seven KoVI isolates, and for two reference strains each of the KoI and KoII phylogenetic groups (Table 1). Twelve *bla*_{OXY-1} and *bla*_{OXY-2} sequences, as well as the *bla*_{OXY-3} (strain SG271) and *bla*_{OXY-4} (strain SG266) sequences, were retrieved from public databases (Table 1). Considering only the coding region, 23 distinct sequences were found. Besides deletions at codons 11, 12, 25, and 26, there were 207 polymorphic nucleotide sites, of which 66 were variable in only one sequence (singletons) and 141 were variable in at least two sequences (informative sites). The deduced amino acid sequences showed 51 variable amino acid positions. The nucleotide substitutions were approximately three times more frequently synonymous ($n = 158$) than non-synonymous ($n = 51$).

Phylogenetic analysis. The neighbor-joining tree (Fig. 1) obtained for the 30 sequences revealed six branches. The first included the six strains previously described as harboring *bla*_{OXY-1} and the two KoI reference strains SB9 and SB71. The second branch included the six strains previously described as

TABLE 3—Continued

Amino acid at position (Ambler no.):															
32 (28)	33 (29)	34 (30)	38 (34)	39 (35)	43 (39)	44 (40)	54 (50)	56 (52)	58 (54)	61 (56)	75 (72)	90 (87)	91 (88)	92 (89)	101 (98)
A	I	Q	A	D	R	S	N	A	D	T	G	N	P	E	K
T	L				S	T	D	T	N	I		H	K	D	
					H							T		D	
					H										
					H										
					H										
								T							
				N											
				N											
				N											
		H	T						N	I	S		K		N
		H	T						N	I	S		K		N
		H	T						N	I	S		K		N
		H	T						N	I	S		K		N
		H	T						N	I	S		K		N
		H	T						N	I	S		K		N
		H	T						N	I	S		K		N
		H	T						N	I	S		K		N

harboring *bla*_{OXY-2} and two KoII reference strains, SB136 (= ATCC 49131) and SB175 (= ATCC 13182^T). These results firmly demonstrate the correspondence of β-lactamase groups *bla*_{OXY-1} and *bla*_{OXY-2} with phylogenetic groups KoI and KoII, respectively. The third and fourth branches each comprised a single isolate, corresponding to *bla*_{OXY-3} and *bla*_{OXY-4}. The fifth branch was formed by the five atypical KoI isolates, whereas the sixth corresponded to the seven KoVI isolates. We propose to refer to the β-lactamase genes of these two new groups as *bla*_{OXY-5} and *bla*_{OXY-6}, respectively.

The mean nucleotide sequence similarity was clearly higher within all six β-lactamase groups (at least 99.7%) than between any pair of them (see Table 4), as the smallest intergroup difference, observed between the *bla*_{OXY-1} and *bla*_{OXY-5} groups, was 2.5%.

Amino acid sequence diversity of the two new β-lactamase groups, OXY-5 and OXY-6. Among the five isolates harboring *bla*_{OXY-5}, two alleles were distinguished, corresponding to two distinct amino acid sequences, OXY-5-1 and OXY-5-2 (Table 3). These two variants differed by two amino acids, at Ambler positions 39 and 153. Both variants differed from the OXY-1 β-lactamase of strain SL781 by five amino acids, located at Ambler positions 12 (deleted in OXY-5 variants), 100, 138, 140, and 165. None of these changes was specific for OXY-5, as they were also observed in OXY-2, OXY-3, and/or OXY-4 (Table 3).

Among the seven isolates harboring *bla*_{OXY-6}, four alleles were found, corresponding to four distinct amino acid se-

quences, OXY-6-1 to OXY-6-4 (Table 3). These variant enzyme sequences differed by their combination of three amino acid differences at Ambler positions 12 (deleted in strain SB324), 35, and 52, and they presented three common differences compared to the SL781 OXY-1 sequence, at positions 5, 87, and 89. None of these changes was specific for the OXY-6 β-lactamase group, as they were also observed in OXY-3 and/or OXY-4 sequences (Table 3).

The amino acid sequence similarity levels confirmed the distinctness of the two new β-lactamase groups. The highest amino acid sequence similarity of OXY-5 and OXY-6 sequences was observed with OXY-1 (97.5 and 98.3%, respectively) and with OXY-4 (97.2 and 97.7%, respectively), whereas the intragroup value was 99.7% both for OXY-5 and for OXY-6. The most distant amino acid sequences were OXY-2 and OXY-3 (Table 4).

Isoelectric focusing. Analytical isoelectric focusing revealed a single β-lactamase band in all strains. The two distinct OXY-5 amino acid variants differed by their pI values (7.2 for OXY-5-1 and 7.7 for OXY-5-2). In contrast, the four distinct OXY-6 variants exhibited only two distinct pI values, 7.75 (for OXY-6-1, OXY-6-2, and OXY-6-3) and 8.1 (for OXY-6-4). These pI differences are in full agreement with the expected effects of the deduced amino acid changes (Table 3).

Antibiotic susceptibility testing. The MICs of 10 β-lactams and three β-lactam-β-lactam inhibitor combinations were determined (Table 5). The susceptibility level observed for strains harboring *bla*_{OXY-5} and *bla*_{OXY-6} were similar to the

TABLE 3—Continued

Amino acid at position (Ambler no.):													
102 (99)	103 (100)	111 (108)	129 (126)	140 (137)	141 (138)	143 (140)	156 (153)	161 (158)	168 (165)	171 (168)	174 (171)	180 (177)	184 (181)
K	S	I	A	K	M	S	Q	V	T	A	S	K	T
A		V				G	R				T	E	
	A				I	G				M			
	A				I	G				M			
	A				I	G				M			
	A				I	G				M			
	A				I	G	L			M			
A	A		T	L	I	G	H	A		T	T	E	S
A	A		T	L	I	G	R	A		T	T	E	S
A	A		T	L	I	G	R	A		T	T	E	S
A	A		T	L	I	G	R	A		T	T	E	S
A	A		T	L	I	G	R	A		T	T	E	S
A	A		T	L	I	G	R	A		T	T	E	S
A	A		T	L	I	G	R	A		T	T	E	S

values observed for the strains of the OXY-1 and OXY-2 β -lactamase groups. The MICs of amoxicillin, ticarcillin, and piperacillin were slightly lower for strains SG271 (OXY-3) and SG266 (OXY-4). All strains showed resistance against amoxicillin and ticarcillin, which was inhibited in the presence of clavulanate. Resistance to piperacillin was intermediate and fell in the presence of tazobactam. Susceptibility to all other β -lactams tested was observed. The sequence of the promoter region was established for the five bla_{OXY-5} and seven bla_{OXY-6} strains. In agreement with the observed MICs, there were no mutations previously reported to increase the transcription rate.

bla_{OXY-5} - and bla_{OXY-6} -specific PCR assays. The 18 isolates were subjected to amplification with two primer pairs, primers OXY5-B and OXY5-C and primers OXY6-B and OXY6-C. These pairs were designed on regions containing group-specific nucleotide substitutions. OXY5-B and OXY5-C PCR amplification of the expected 127-bp fragment was positive for all isolates harboring the bla_{OXY-5} β -lactamase gene and negative for isolates of all other groups. Conversely, amplification of the 107-bp fragment expected with primers OXY6-B and OXY6-C was positive only with isolates harboring bla_{OXY-6} (data not shown).

Correspondence between β -lactamase and housekeeping gene diversity. In order to compare the phylogeny based on the β -lactamase gene with the evolutionary history of other regions of the *K. oxytoca* chromosome, the nucleotide sequences of portions of the three housekeeping genes *rpoB* (940 nucleo-

tides [nt] sequenced), *gyrA* (383 nt), and *gapDH* (573 nt) were determined in the 18 study isolates. For the three protein coding genes, the alignment revealed no insertion or deletion event, and there was no ambiguous data. The proportion of polymorphic sites was 8.72% (82/940 nt) for *rpoB*, 8.35% (32/383 nt) for *gyrA*, and 6.8% (39/573 nt) for *gapDH*, whereas it was 23% (203/876 nt) for bla_{OXY} (Table 6). The smaller amount of variable sites in the housekeeping genes was mainly due to the rarity of nonsynonymous substitutions (Table 6), indicative of stronger selective pressure against amino acid changes in the three housekeeping genes, relative to the bla_{OXY} gene.

The neighbor-joining trees obtained based on *rpoB*, *gyrA*, and *gapDH* were very similar. Therefore, we concatenated the three genes to estimate more robustly the phylogeny of the strains (Fig. 2). A very close agreement with the phylogeny previously obtained on the basis of the β -lactamase gene (Fig. 1) was observed. Strains with bla_{OXY-2} , bla_{OXY-3} , bla_{OXY-4} , and bla_{OXY-6} genes clustered into four separate branches, each corresponding to their β -lactamase group. These four branches clearly represent distinct phylogenetic groups. For each gene, the nucleotide divergence within groups was much lower than that observed among groups (Table 7). The isolates harboring bla_{OXY-1} and bla_{OXY-5} β -lactamase genes were very close (Fig. 2 and Table 7). Therefore, we consider bla_{OXY-5} -harboring strains as belonging to phylogenetic group KoI (see Discussion). Groups KoII and KoIII appeared to be the most external lineages, as observed with the bla_{OXY} gene.

TABLE 3—Continued

Amino acid at position (Ambler no.):										
194 (190)	200 (197)	230 (227)	233 (230)	240 (237)	256 (255)	261 (260)	262 (261)	270 (269)	275 (274)	278 (277)
R	N	A	A V V	A G G	N	V	L	Q	S	E
			V V	G			P			K
H			V	G				L		D
	D	E	V		D				N	
	D	E	V		D	I			N	
	D	E	V	G	D	I			N	
	D	E	V		D				N	
	D	E	V		D				N	
	D	E	V		D				N	
A		E	V		D				N	

The nearly complete (1,454-bp) 16S rRNA gene sequence was determined for the two KoI and the two KoII reference strains and for two *bla*_{OXY-5}-harboring and three *bla*_{OXY-6}-harboring strains (Table 1). Excluding 12 positions showing within-strain heterogeneity among gene copies (5), the 16S rRNA sequences of *bla*_{OXY-5}- and *bla*_{OXY-6}-harboring strains were completely identical to that of KoI, with the exception of two substitutions for one *bla*_{OXY-6}-harboring strain (SB3051; data not shown).

Ancient presence of the chromosomal β-lactamase gene in *K. oxytoca*. In the absence of bacterial fossils, the molecular clock hypothesis, according to which the rate of synonymous substitution at synonymous sites (*K_s*) along evolutionary lineages is roughly constant over time, is a way to estimate the timing of evolutionary events. In order to estimate the time since divergence of the *K. oxytoca* phylogenetic groups, we

used genes *rpoB* and *gyrA*, both of which showed clock-like behavior. To calibrate the *K_s* values of *rpoB* and *gyrA*, we used two extreme estimations for the time of divergence between *E. coli* and *Salmonella enterica*, 30 and 140 million years (23, 25, 27). The *K_s* of *rpoB* between *E. coli* and *S. enterica* is 0.223, whereas the *K_s* for *gyrA* is 0.367. Table 8 gives the *K_s* values observed among *K. oxytoca* groups. Using the 30-million-year calibration, *rpoB* and *gyrA* *K_s* values indicate a split between KoI and KoII 15 and 11 million years ago, respectively, whereas the two groups with the greatest difference, KoIII and KoIV, would have separated 23 or 18 million years ago, respectively. The divergence of the *bla*_{OXY-5} strains from KoI would be as recent as 3 to 2 million years. Using the 140-million-year calibration, the separation of the KoIII and KoIV groups would be estimated to be as old as 109 or 84 million years, based on *rpoB* and *gyrA*.

TABLE 4. Percent average nucleotide and aminoacid β-lactamase sequence similarity within and among *bla* OXY groups^a

Group (no. of strains)	Avg % similarity (SD)					
	<i>bla</i> _{OXY-1}	<i>bla</i> _{OXY-2}	<i>bla</i> _{OXY-3}	<i>bla</i> _{OXY-4}	<i>bla</i> _{OXY-5}	<i>bla</i> _{OXY-6}
<i>bla</i> _{OXY-1} (8)	99.7 (0.09)/99.4 (0.4)	87.2 (2.3)	86.2 (5)	95.2 (1.7)	97.5 (0.5)	97.1 (0.5)
<i>bla</i> _{OXY-2} (8)	89.5 (0.4)	99.7 (0.1)/99.4 (0.4)	84.9 (5.6)	86.2 (5)	87 (2.9)	86.5 (2.6)
<i>bla</i> _{OXY-3} (1)	90.9 (0.2)	88.6 (0.5)	100 (0)/100 (0)	86.3 (0.0)	85.1 (6.7)	85.6 (5.6)
<i>bla</i> _{OXY-4} (1)	96.8 (0.3)	88.3 (0.3)	90 (0)	100 (0)/100 (0)	95.9 (1.7)	95.6 (0.1)
<i>bla</i> _{OXY-5} (5)	97.5 (0.3)	91.5 (0.3)	90.3 (0)	97.2 (0.4)	99.8 (0.1)/99.7 (0.4)	95.8 (1)
<i>bla</i> _{OXY-6} (7)	98.3 (0.3)	88.7 (0.3)	91.2 (0.3)	97.7 (0.2)	97 (0.2)	99.8 (0.1)/99.7 (0.2)

^a Above diagonal, nucleotide sequence similarity; below diagonal, amino acid sequence similarity; inside diagonal cells: nucleotide/amino acid sequence similarities.

TABLE 5. MICs of 10 β -lactams and three β -lactam- β -lactam inhibitor combinations observed for strains harboring bla_{OXY-1} to bla_{OXY-6} β -lactamase genes

Drug(s)	MIC (μ g/ml)					
	bla_{OXY-1} strains SB9, SB71	bla_{OXY-2} strains SB136, SB175	bla_{OXY-3} strains SG271 ^a	bla_{OXY-4} strains SG266 ^a	bla_{OXY-5} strains SB2908, SB2933	bla_{OXY-6} strains SB73, SB324, SB352, SB397
Amoxicillin	256	512	16	64	256	256
Amoxicillin + 2 μ g/ml of clavulanate	4	4	0.25	0.5	4	2-4
Ticarcillin	128	128	16	64	128	64-128
Ticarcillin + 2 μ g/ml of clavulanate	2	2	0.5	2	2	1-2
Piperacillin	16-32	16-32	1	2	8-32	8-32
Piperacillin + 4 μ g/ml of tazobactam	2	1-2	0.12	0.5	2-4	2-4
Cephalothin	4-8	16	2	2	2-4	4-8
Cefoxitin	4-8	2	0.5	1	2-8	4-8
Cefotaxime	≤ 0.06	$\leq 0.06-0.125$	≤ 0.06	≤ 0.06	≤ 0.06	≤ 0.06
Ceftazidime	0.125-0.25	0.125-0.25	≤ 0.06	≤ 0.06	0.125-0.25	0.25-0.5
Cefepime	≤ 0.06	$\leq 0.06-0.125$	≤ 0.06	≤ 0.06	≤ 0.06	≤ 0.06
Aztreonam	$\leq 0.06-0.25$	0.25	≤ 0.06	≤ 0.06	$\leq 0.06-0.125$	$\leq 0.06-0.25$
Imipenem	0.125	0.125	0.5	0.5	0.125	0.125

^a Values previously published (14).

DISCUSSION

The phylogenetic analysis of all major OXY β -lactamase gene variants exhibited six distinct branches. Although the distinction of branches as new groups rather than new variants within existing groups is partly a matter of arbitrary choice, we decided to consider the two new branches as new groups, as it should stimulate their epidemiological follow-up. In addition, we showed that it was possible to define group-specific PCR primers, which underlines the distinctness of these groups and provides tools for their epidemiological investigation. The individualization of OXY-5 and OXY-6 β -lactamases as two groups, each distinct from OXY-1, is supported by signature amino acids (Table 3), by mean amino acid divergence (Table 4), and by phylogenetic analysis (Fig. 1). The five bla_{OXY-5} -harboring isolates were collected in two Italian cities, in Switzerland, and in The Netherlands, whereas the seven bla_{OXY-6} -harboring isolates came from France, Germany, South Africa, and Ukraine. Both groups therefore appear geographically widespread, although they together represented only 6.5% (12/183) of *K. oxytoca* clinical isolates.

Nucleotide variation at three housekeeping genes and at the 16S rRNA gene was in very close agreement with the phylogeny disclosed for the β -lactamase gene. The results confirm that strains harboring bla_{OXY-1} , bla_{OXY-2} , and bla_{OXY-3} genes fall into three distinct phylogenetic groups, in agreement with the findings of Granier et al. (14, 15). In addition, our data

demonstrate that bla_{OXY-4} -harboring strain SG266 represents still another branch, a result that was not fully established based on shorter portions of the *rpoB* and 16S rRNA gene sequences (14). In contrast to the β -lactamase data, it was not clear if bla_{OXY-5} -harboring strains represent a distinct phylogenetic branch. In fact, although they tend to group together based on housekeeping genes, these strains fell within phylogenetic group KoI. We interpret this apparent discrepancy between the bla and housekeeping genes by hypothesizing a relatively recent emergence from KoI of the evolutionary lineage corresponding to bla_{OXY-5} -harboring strains. Due to a higher rate of nucleotide substitution (Table 6), variation in the β -lactamase gene is expected to reveal lineage splits more quickly than variation at housekeeping genes. Thus, bla_{OXY-5} -harboring strains can be considered as an emerging clone, individualized from KoI only by fast-evolving genetic markers. We propose to refer to the bla_{OXY-5} -harboring strains as the bla_{OXY-5} clone or lineage and to the five phylogenetic groups as KoI to KoIV and KoVI, in continuation of our initial nomenclature (4). These names clearly mark the difference between phylogenetic groups and β -lactamase groups, which should be less confusing than the OXY or oxy group denomination (14, 15).

The phylogenetic agreement between bla and housekeeping genes was not only true for the classification of strains within groups but also appeared to be the case for the hierarchical

TABLE 6. Nucleotide polymorphism observed among the 18 study isolates

Gene	Fragment size (bp)	No. of variable sites (%)			No. of substitutions (%)		
		Singleton	Informative	Total	Synonymous	Nonsynonymous	Total
<i>rpoB</i>	940	34 (3.6)	48 (5.1)	82 (8.7)	82 (8.7)	5 (0.5)	87 (9.3)
<i>gyrA</i>	383	15 (3.9)	17 (4.4)	32 (8.4)	33 (8.6)	1 (0.3)	34 (8.9)
<i>gapDH</i>	573	26 (4.5)	13 (2.3)	39 (6.8)	38 (6.6)	3 (0.5)	41 (7.2)
<i>bla</i>	870, 873, or 876	64 (7.3)	139 (15.9)	203 (23.2)	158 (18.0)	52 (5.9)	210 (24.0)

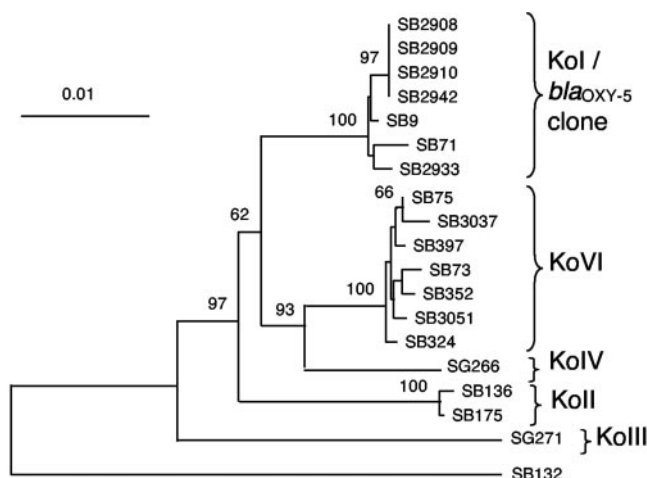


FIG. 2. Phylogeny obtained based on 1,896 nt positions from the three housekeeping genes *rpoB*, *gyrA*, and *gapDH*. The neighbor-joining tree was rooted using *K. pneumoniae* type strain SB132 (= ATCC 13883^T). Values at the nodes correspond to bootstrap values obtained after 1,000 replicates. Five branches of *K. oxytoca* are distinguished, corresponding to phylogenetic groups KoI to KoVI. *bla*_{OXY-5}-harboring strains fall into the KoI branch.

relationships among groups. Because the phylogeny of the β-lactamase gene is concordant with the phylogenies based on housekeeping genes, by far the most likely evolutionary origin of the six OXY β-lactamase groups is diversification from a common ancestor, along with the evolutionary divergence of the phylogenetic groups (17). The ancestry of the set of *K. oxytoca* strains can be conservatively estimated at several tens of millions of years, based on the molecular clock hypothesis. Notably, estimations based on *rpoB* and on *gyrA* were in close agreement. Although the constancy of the nucleotide substitution rate across lineages is a rough statement and should be taken with caution (24), our estimations show beyond doubt an ancient diversification of the OXY groups of β-lactamase, pre-dating by far antibiotic usage in clinical practice. Adding the fact that essentially there is a lack of evolution of the resistance phenotype, the pattern of diversification of the OXY family of β-lactamases appears to conform to the long-term neutral evolutionary scenario that was also described for the evolutionary diversification of the major variants of other families of β-lactamases, such as AmpC or SHV (2, 18).

TABLE 8. Average numbers of synonymous substitutions per synonymous site (K_s values) among the five *K. oxytoca* phylogenetic groups and clone *bla*_{OXY-5}^a

Group or clone	Avg K_s value					
	KoI	KoII	KoIII	KoIV	Clone <i>bla</i> _{OXY-5}	KoVI
KoI		0.114	0.17	0.128	0.02	0.12
KoII	0.135		0.157	0.156	0.111	0.133
KoIII	0.21	0.214		0.175	0.173	0.171
KoIV	0.143	0.126	0.22		0.113	0.117
Clone <i>bla</i> _{OXY-5}	0.022	0.132	0.199	0.143		0.123
KoVI	0.112	0.128	0.188	0.045	0.101	

^a Data computed from gene *rpoB* (above diagonal) and *gyrA* (below diagonal).

TABLE 7. Percent average *rpoB*, *gyrA*, and *gapDH* nucleotide sequence divergence within and among the five *K. oxytoca* phylogenetic groups and clone *bla*_{OXY-5}

Group or clone	Avg % divergence from nucleotide sequence																	
	KoI			KoII			KoIII			KoIV			<i>bla</i> _{OXY-5} clone			KoVI		
	<i>rpoB</i>	<i>gyrA</i>	<i>gapDH</i>	<i>rpoB</i>	<i>gyrA</i>	<i>gapDH</i>	<i>rpoB</i>	<i>gyrA</i>	<i>gapDH</i>	<i>rpoB</i>	<i>gyrA</i>	<i>gapDH</i>	<i>rpoB</i>	<i>gyrA</i>	<i>gapDH</i>	<i>rpoB</i>	<i>gyrA</i>	<i>gapDH</i>
KoI	0.2	0.5	0	3.09	3.2	1.92	4.25	4.96	4.36	3.2	3.65	0.7	0.49	0.52	0.00	3.04	2.68	0.67
KoII				0.11	0.13	0	4.15	5.09	5.4	4.15	3.26	2.44	3	3.13	1.92	3.6	2.68	2.24
KoIII							NA ^a	NA	NA	4.36	5.48	4.88	4.32	4.7	4.36	4.32	4.51	4.51
KoIV							NA	NA	NA	NA	NA	NA	NA	NA	NA	2.96	1.3	0.67
<i>bla</i> _{OXY-5} clone													0.17	0.00	0.06	3.1	2.42	0.67
KoVI																0.4	0.13	0.1

^a NA, not applicable (only one sequence per group).

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