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Novel Class A β-Lactamase Sed-1 from *Citrobacter sedlakii*: Genetic Diversity of β-Lactamases within the *Citrobacter* Genus

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***Citrobacter sedlakii* 2596, a clinical strain resistant to aminopenicillins, carboxypenicillins, and early cephalosporins such as cephalothin, but remaining susceptible to acylureidopenicillins, carbapenems, and later cephalosporins such as cefotaxime, was isolated from the bile of a patient treated with β-lactam and quinolone antibiotics. The isolate produced an inducible class A β-lactamase of pI 8.6, named Sed-1, which was purified. Characterized by a molecular mass of 30 kDa, Sed-1 preferentially hydrolyzed benzylpenicillin, cephalothin, and cloxacillin. The corresponding gene, bla_{Sed-1}, was cloned and sequenced. Its deduced amino acid sequence shared more than 60% identity with the chromosome-encoded β-lactamases from *Citrobacter koseri* (formerly *C. diversus*) (84%), *Klebsiella oxytoca* (74%), *Serratia fonticola* (67%), and *Proteus vulgaris* (63%) and 71% identity with the plasmid-mediated enzyme MEN-1. A gene coding for a LysR transcriptional regulator was found upstream from bla_{Sed-1}. This regulator, named SedR, displayed 90% identity with the AmpR sequence of the chromosomal β-lactamase from *C. koseri* and 63 and 50% identity with the AmpR sequences of *P. vulgaris* and *Enterobacter cloacae*, respectively. By using DNA-DNA hybridization, a bla_{Sed-1}-like gene was identified in two reference strains, *C. sedlakii* (CIP-105037) and *Citrobacter rodentium* (CIP-104675), but not in the 18 strains of *C. koseri* studied. Two DNA fragments were amplified and sequenced from the reference strains of *C. sedlakii* CIP-105037 and *C. rodentium* CIP-104675 using two primers specific for bla_{Sed-1}. They shared 98 and 80% identity with bla_{Sed-1}, respectively, confirming the diversity of the chromosomally encoded class A β-lactamases found in *Citrobacter*.**

The genus *Citrobacter*, which was defined in 1932, initially encompassed seven species including *Citrobacter freundii* (type species) and *Citrobacter koseri* (formerly termed *Citrobacter diversus* or *Levinea malonatica*) (60). In 1993, Brenner et al. identified eight new DNA hybridization groups genetically distinct from *C. freundii* and *C. koseri* (13). These additional genomospecies included *C. sedlakii* (genomospecies 8) and *C. rodentium* (genomospecies 9, a bacterial pathogen of rodents).

In the *Citrobacter* genus, resistance to β-lactam antibiotics is mainly mediated by production of chromosomally encoded β-lactamases. *C. freundii* produces an inducible Ambler class C β-lactamase (60). In *C. koseri* (formerly *C. diversus*), which is naturally resistant to aminopenicillins and carboxypenicillins, resistance to β-lactams is mediated by a chromosome-encoded class A β-lactamase (4). The cloning and sequencing of the corresponding gene, which has been termed bla_{CdiA}, revealed a high degree of similarity (75%) between CdiA and the class A β-lactamase from *Klebsiella oxytoca* (33). Previous experiments carried out by DNA amplification with primers specific for bla_{CdiA} showed that this gene is not ubiquitous in *C. koseri* (32). Accordingly, different chromosome-encoded β-lactamases with distinct isoelectric points (pIs) varying from 4.8 to 9.5 have been identified in *C. koseri*, but the corresponding genes have not been characterized at the genetic level (27, 31, 41, 42, 47, 53, 55). The LysR-type transcriptional regulator (LTTR) protein CdiR, divergently transcribed from CdiA, has also been characterized. The AmpR regulator protein most closely related to CdiR is the *Proteus vulgaris* CumR protein, with an amino acid identity of 66%, whereas only 46% identity was found with the AmpR protein from *C. freundii*, strengthening the idea of a wide genetic diversity of the genes determining resistance to β-lactam antibiotics in the *Citrobacter* genus.

C. sedlakii has been rarely described since its first identification in 1993 (1, 2, 13, 23, 43). Strains isolated from human stools, blood, and wounds were studied by Brenner et al. (13), and a report of neonatal meningitis and brain abscess involving a strain resistant to ampicillin (MIC, 16 μg/ml), cefazolin (MIC, 16 μg/ml), and cefuroxime (MIC, 16 μg/ml), was made in 1997 by Dyer et al. (23), but the mechanism of resistance to β-lactams in these strains has not been investigated.

In the present study, we report the cloning and sequencing of the gene coding for the class A β-lactamase from *C. sedlakii*. The biochemical characteristics of this enzyme, named Sed-1, were studied. The transcriptional regulator associated with bla_{Sed-1} was also cloned and sequenced. In order to address the issue of the genetic diversity of the β-lactamases found within the *Citrobacter* genus, DNA-DNA hybridization, high-stringency PCR, and DNA sequencing were used to study the distribution of bla_{Sed-1} in 21 isolates of *Citrobacter* spp.

MATERIALS AND METHODS

Bacterial strains and plasmids. The clinical strain 2596 of *C. sedlakii* was isolated in 1997 from the bile of a patient treated with β-lactam and quinolone

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TABLE 1. Nucleotide sequences of primers

Primer	Nucleotide sequence (5′ → 3′)	Genes	Annealing temp (°C)	Reference
AmpC div sens	AACGAGGTCGTCAGACG	bla_{CdiA}	49	33
AmpC div A sens	CAGAATATCTTTACGCCC			
Class A	AGCGAYAAYACGGCGATG	Class A[a]	55	8
Class A rev	TGCKCCGGTYTTATCGCC			
SHV rev	GCGTTGCCAGTGCTCGATCAG	bla_{SHV}	62	6
SHV bis	ATGCGTTATATTCGCCTGTGTATT			
TEM H	TGAGATCGAAGGGCCGTT	bla_{TEM}	52	54
TEM rev	GGTCTGACAGTTACCAAT			
OXA C	AGGTGCCATGAAAACATT	bla_{OXA} group 1[b]	45	
OXA C bis	TTAGCCACCAATGATGAA			
OXA A	AAGGAAAAGTTAATGGCA	bla_{OXA} group 2[b]	44	
OXA A bis	TTTATCGCGCAGCGTCCG			
OXA E	AGGACTTGGGACATCGAT	bla_{OXA18}	47	48
OXA E bis	TGACTGGTCAGAAGTTTT			
OXA B	AAGGAGGCTTCCTTGATAA	bla_{OXA20}	49	38
OXA B bis	TTGGGTGGCAAAGCATTG			
OXA C	As indicated above	bla_{OXA13}	49	37
OXA O bis	TTATGTGCTTAGTGCATC			
SmeA	CGGTCCTGA GGGGATGAC	bla_{Sme-1}	53	39
SmeB	CGTGATGCTTCCGCAATA			
A1	GGAATTCCTWTGCTGCGCBCTGCTGCT	bla_{AmpC}[c]	60	34
A2	CGGGATCCCTGCCAGTTTTGATAAAA			
B1	GGAATTCCTCAFCGAGCAGACSCTGTT	bla_{AmpC}[c]	60	34
B2	CGGGATCCCCCCGCACMTKAYRTAGOTGTGG			
CsA S	GCGCTGATTAATACCGC	bla_{Sed-1}	49	Present study
CsA AS	GCATCCTGCTGTGGCTGT			
CUM 1	GGTCGTTTAGGTTAAAAC	bla_{CUM}	48	22
CUM 1 bis	CCAGTGTTTTGGTAAACC			
OXY 1	GTTCGTGGCGTAAAAC	bla_{OXY}	49	25
OXY 1 rev	TAACACCTCTTTGCGGCT			
RNA-S	AGAGTTTGATCCTGGYTCAG	16S RNA	56	61
RNA-AS	CTTTACGCCCARTAAWTCCG			

[a] Degenerated oligonucleotide primer designed from the consensus sequence of five class A β-lactamase genes (bla_{CdiA}, bla_{OXY}, bla_{TOHO-1}, bla_{MEN}, and bla_{SHV}).
[b] Group 1 included oxacillinases OXA-5, -7, -11, -14, -16, and -17 and OXA-10 or PSE-2 (16, 18, 19, 21, 28, 29, 52). Group 2 included oxacillinases OXA-2, -3, -15, and -21 (17, 20, 50, 57).
[c] Degenerated oligonucleotide primers were designed from the consensus sequence of the *ampC* genes of *E. coli, E. cloacae*, and *C. freundii* for A1 and A2 and from MOX-1, FOX-1, and *ampC* of *S. marcescens* for B1 and B2.

antibiotics. The strain was identified as *C. sedlakii* by biochemical tests using BIOTYPE 100 (Biomérieux) and the programs RECOGNIZER, ADANSON, and DENDOGRAPH of the Taxotron package (Institut Pasteur Taxolab). The identification was confirmed by amplifying and determining the nucleotide sequence of a PCR-amplified DNA fragment of 568 bp corresponding to the 16S RNA of the strain (the sequences of the primers are given in Table 1). The other bacterial strains and plasmids used in this work are listed in Table 2.

Antibiotics, media, and susceptibility testing. The antibiotics were obtained from the following suppliers: ampicillin, oxacillin, and aztreonam from Bristol-Myers Squibb, Paris, France; chloramphenicol, cefuroxime, and cephalothin from Sigma Chemical Co., St. Louis, Mo.; cefoxitin and imipenem from Merck Sharp & Dohme, Chibret, France; cefotaxime, cefpirome, and rifampin from Hoescht-Marion-Roussel, Paris, France; ceftazidime and nitrocefin from Glaxo, Nanterre, France; ticarcillin, amoxicillin, and clavulanate, from SmithKline Beecham, Paris, France; piperacillin from Lederle, Paris, France; and benzylpenicillin from Sarbach, Suresnes, France.

MICs were determined on Mueller-Hinton (MH) agar by dilution technique (24) with a Steers multiple inoculator and an inoculum of 10^4 CFU per spot. The plates were incubated at 37°C for 18 h. Brain heart infusion (BHI), Luria-Bertani (LB), and MH media were from Gibco-BRL.

Mating-out assays and plasmid content analysis. Transfer of of β-lactam resistance to *E. coli* K-12 was attempted by liquid and solid mating-out assays. The recipient and donor cells were mixed into a ratio of 1:1 or 4:1 and were incubated in BHI with moderate shaking at 37°C for 3 h. After incubation, 200 μl of each mixture was plated out on a Millipore filter disk onto BHI plates and incubated for 18 h at 37°C. Transconjugants were selected on LB agar containing ampicillin (50 or 100 μg/ml) and rifampin (50 or 100 μg/ml). *C. sedlakii* 2596 was examined for its plasmid DNA content by the procedures of Birnboim (9) and Takahashi and Nagano (56).

DNA amplification. DNA amplification of β-lactamase genes was carried out with the various specific primers (Eurogentec, Seraing, Belgium) listed in Table 1. The DNA amplifications were performed on 100-μl samples containing DNA (5 μl), deoxynucleoside triphosphate (250 μM), primers (0.4 μM concentrations each), *Taq* DNA polymerase (1 U), and its buffer. The following cycles were used: 10 min of denaturation at 94°C (1 cycle); 1 min of denaturation at 94°C, 1 min of annealing (see temperatures in Table 1), and 1 min of polymerization at 72°C (35 cycles), followed by 10 min of extension at 72°C. The amplified products were analyzed by electrophoresis of 5-μl aliquots on 1% agarose gels.

Nucleic acid techniques and sequence analysis. Genomic DNA from *C. sedlakii* 2596 was extracted as described previously (49). For cloning experiments, the extracted DNA was partially digested with *Sau*3AI. The fragments were ligated into the dephosphorylated vector pBC SK$^+$ previously digested with *Bam*HI. The ligations were done at 4°C for 16 h with 100 ng of chromosomal DNA, 200 ng of digested plasmid vector pBC SK$^+$, and 1 U of T4 DNA ligase (Amersham). After purification and concentration with the High Pure PCR product purification kit (Boehringer Mannheim), the ligation mixture was transformed by electroporation into *Escherichia coli* Top10. Transformants resistant to β-lactam antibiotics were selected on LB agar plates supplemented with 50 μg of amoxicillin/ml. Recombinant plasmid DNA was extracted using the rapid procedure of Birnboim (9) from 5-ml aliquots of overnight cultures grown at 37°C in BHI in the presence of amoxicillin (50 μg/ml) and chloramphenicol (50 μg/ml).

The inserted DNA fragment was sequenced on both strands by primer walking using the *Taq* DyeDeoxy Terminator cycle sequencing kit (Perkin Elmer) and an Applied Biosystems sequencer (PRISM 377). Sequence analysis was performed with the software available on the National Center for Biotechnology Information website. The program ORF Finder was used to determine all the putative open reading frames (ORFs), which were analyzed using the BLASTP program

TABLE 2. Bacterial strains and plasmids used

Strain or plasmid	Genotype or phenotype	Reference or source
Strains		
C. sedlakii 2596[a]	Clinical isolate resistant to β-lactams	Present study
E. coli Top10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC)ϕ80*lac*Z ΔM15Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (StrR) *endA1 nupG*	
E. coli K12	Rifampin	
C. koseri CKB	ESBL[b]	Clinical isolate
C. koseri CKC	ESBL	Clinical isolate
C. koseri CKD	ESBL	Clinical isolate
C. koseri CKE	ESBL	Clinical isolate
C. koseri CKG	ESBL	Clinical isolate
C. koseri CK1	Wild type	Clinical isolate
C. koseri CK2	Wild type	Clinical isolate
C. koseri CK3	Wild type	Clinical isolate
C. koseri CK4	Wild type	Clinical isolate
C. koseri CK5	Wild type	Clinical isolate
C. koseri CK7	Wild type	Clinical isolate
C. koseri CK10	Wild type	Clinical isolate
C. koseri CIP-82.87	Wild type (reference strain)	CIP
C. koseri CIP-76.3	Wild type (reference strain)	CIP
C. koseri CIP-72.8	Wild type (reference strain)	CIP
C. koseri CIP-72.14	Penicillinase	CIP
C. diversus CIP-82.94	Wild type (reference strain)	CIP
L. malonatica CIP-82.88	Wild type (reference strain)	CIP
C. sedlakii CIP-105037	Wild type (reference strain)	CIP
C. rodentium CIP-104675	Wild type (reference strain)	CIP
Plasmids		
pBC SK$^+$	Chloramphenicol	Stratagene
pBC 2596	pBC SK$^+$ + 2.78-kb *Sau*3AI fragment from genomic DNA of *C. sedlakii* 2596	Present study

[a] The strain was deposited in the CIP under the number CIP-106793.
[b] Phenotype corresponding to penicillin and oxyminocephalosporin resistance by production of ESBLs.

(3). The Sed-1 primary structure was analyzed with the software SignalP and ProtParam, which are available on the ExPASY tools website (Swiss Institute of Bioinformatics [5]).

For the hybridization experiments, genomic DNA was extracted from *Citrobacter* strains as described previously (49), except that phenol-chloroform was replaced by *N*-cetyl-*N,N,N*-trimethylammonium bromide (Merck). DNA was denatured by heating at 100°C for 10 min, and 5 μl of each sample was hybridized onto a nylon membrane (Hybond-N$^+$; Amersham). The DNA was cross-linked on the membrane by 5 min of UV exposure. The hybridization was performed according to the manufacturer's instructions (ECL kit; Amersham) using as a probe an internal fragment of 668 bp obtained by PCR from the $bla_{\text{Sed-1}}$ gene.

Preparation of crude extracts and isoelectrofocusing. Exponentially growing cells were harvested and resuspended in 600 μl of 50 mM phosphate sodium buffer (pH 7.0). The suspensions were disrupted by sonication and the crude extracts were used for β-lactamase detection. Isoelectric focusing was performed with an LKB Multiphor apparatus using polyacrylamide gel plates (Pharmacia Biotech, Saint Quentin en Yvelines, France) at pH 3.5 to 9.5 Gels were focused at 30 W for 90 min at 10°C. β-lactamase activity was revealed by staining the gel with the chromogenic β-lactam nitrocefin (40).

β-Lactamase purification and kinetic assays. *C. sedlakii* 2596 was grown overnight at 37°C in 6 liters of BHI broth supplemented with ampicillin (50 μg/ml) and cefoxitin (2 μg/ml) in order to induce β-lactamase production. After centrifugation at 5,000 × *g* for 10 min at 4°C, the bacterial pellet (30 g) was resuspended in 120 ml of 50 mM Tris (pH 8.0). Bacterial cells were lysed by ultrasonic treatment and the suspension was clarified by centrifugation at 38,000 × *g* at 4°C. The nucleic acids contained in the supernatant were precipitated by adding spermine (0.2 M) at 4°C, followed by centrifugation for 10 min at 12,000 × *g* and 60 min at 48,000 × *g*. The supernatant was then dialyzed overnight against 3 liters of 50 mM Tris (pH 8.0). After an additional centrifugation at 12,000 × *g* for 30 min, the supernatant was applied onto a 2.5- by 10-cm Q-Sepharose Fast Flow column (Pharmacia Co. Ltd., Uppsala, Sweden) previously equilibrated with the dialysis buffer. β-lactamase activity was detected in the unabsorbed fraction with the chromogenic cephalosporin nitrocefin (40). The active fractions were pooled, dialyzed overnight at 4°C against 2 liters of 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 8.0]), and loaded onto a 2.5- by 10-cm S-Sepharose cation exchange column (Pharmacia Co. Ltd.) previously equilibrated with the dialysis buffer. The protein was eluted by a linear gradient of 0 to 1 M NaCl in 40 mM HEPES (pH 8.0). Active fractions were pooled, dialyzed overnight at 4°C against 1 liter of 40 mM Tris (pH 9.0), and then loaded on a Mono Q anion exchange column previously equilibrated with the dialysis buffer. The enzyme was eluted by a linear gradient of 0 to 1 M NaCl in 40 mM Tris (pH 9.0). The active fractions were pooled and dialyzed overnight at 4°C against 1 liter of 40 mM HEPES (pH 8.0) and loaded on a Mono S cation exchange column equilibrated with the dialysis buffer. The β-lactamase activity was eluted in the nonadsorbed fraction. Enzyme purity was assessed by electrophoresis on sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels. The intensity of the β-lactamase band was measured using a computerized densitometer (Densylab; Bioprobe) from a gel stained with Coomassie blue. The enzyme concentration was determined in reference to a standard bovine serum albumin scale analyzed under the same conditions. β-lactamase activity was renatured by soaking an SDS-polyacrylamide gel in 100 mM Tris-HCl (pH 7.0) for 1 h and was detected by overlaying the gel with nitrocefin (1,000 μg/ml). N-terminal sequences were determined after protein purification using an Applied Biosystems sequencer. The purified protein was stored in 50% glycerol at −20°C.

Kinetic measurements and inhibition of β-lactamase activity. The kinetic parameters K_m and k_{cat} were determined spectrophotometrically at 35°C in 50 mM phosphate buffer (pH 7.0) using an Uvikon 940 spectrophotometer. The absorption coefficients used were those previously described (11). Kinetic parameters were determined by fitting the Henri-Michaelis-Menten equation to the experimental data by using the regression analysis program LEONORA written by Cornish-Bowden (15). The values of k_{cat} and K_m were estimated using a nonlinear least-squares regression method with dynamic weights (15).

Enzyme inhibition was studied with benzylpenicillin (100 μM) as the substrate. The different inhibitors, at various concentrations, were preincubated with the enzyme for 5 min at 35°C prior to addition of the substrate. The concentration of inhibitor required to inhibit 50% of the β-lactamase activity (IC_{50}) was determined graphically for clavulanic acid, sulbactam, and cefoxitin.

Nucleotide sequence accession numbers. The nucleotide sequences described in this report have been deposited in GenBank under accession numbers AF321607 and AF321608.

TABLE 3. MICs of β-lactam antibiotics for various strains studied

β-Lactam	MIC (μg/ml)				
	C. sedlakii 2596	*E. coli* Top10 (pBC2596)	*E. coli* Top10[a]	*C. sedlakii* CIP-105037[b]	*C. koseri* CK1[c]
Amoxicillin	>512	>512	2	>512	64
Amoxicillin + CLA[d]	8	512	2	8	1
Ticarcillin	>512	>512	1	512	64
Piperacillin	8	128	2	2	8
Cephalothin	256	>512	1	256	2
Cephalothin + CLA	4	1	1	4	ND[f]
Cefuroxime	64	>512	4	32	4
Cefuroxime + FOX[e]	256	ND	ND	256	ND
Cefoxitin	4	2	2	4	2
Cefotaxime	0.25	2	<0.125	<0.125	<0.125
Aztreonam	2	16	<0.125	1	<0.125

[a] Reference strain.
[b] Reference strain from CIP.
[c] A wild-type strain.
[d] CLA, clavulanic acid at a fixed concentration of 4 μg/ml.
[e] FOX, cefoxitin at a fixed concentration of 2 μg/ml.
[f] ND, not determined.

RESULTS

Antibiotic susceptibility. Analysis of *C. sedlakii* 2596 by the conventional disk susceptibility assay indicated that the strain produced an inducible β-lactamase inhibited by clavulanic acid. Indeed, a synergy was detected between clavulanic acid on one hand and aztreonam, cefuroxime, or cefotaxime on the other hand, while an antagonism phenomenon was visible between cefoxitin or imipenem and extended-spectrum cephalosporins like cefotaxime or cefepime (data not shown). Determination of the MICs of β-lactams for strain 2596 showed that it was resistant to penicillins and to early cephalosporins such as cephalothin and cefuroxime, but it remained susceptible to acylureidopenicillins such as piperacillin and to later β-lactams such as cefoxitin, cefotaxime, and aztreonam (Table 3). The MICs of amoxicillin and cephalothin were significantly lowered by clavulanic acid, while that of cefuroxime was increased fourfold when cefoxitin was added at a concentration of 2 μg/ml. Such a phenotype closely resembled that of *C. sedlakii* CIP-105037, a reference strain from the Collection of the Institut Pasteur (CIP), but it was significantly different from that of the wild-type strain CK1 of *C. koseri* (Table 3).

Transfer of resistance and cloning of the *bla* gene from *C. sedlakii* 2596. No plasmid was detectable in *C. sedlakii* 2596, suggesting that the β-lactamase gene was located on the chromosome. Accordingly, repeated mating-out experiments failed to transfer the β-lactam resistance into *E. coli*. The negative DNA amplification tests obtained with 15 pairs of primers designed to amplify the most frequent class A, C, and D β-lactamases (listed in Table 1) suggested that *C. sedlakii* 2596 harbored a β-lactamase gene characterized by a nucleotide sequence substantially different from that of the most commonly encountered *bla* genes.

The genomic DNA from *C. sedlakii* 2596 was partially digested with the restriction endonuclease *Sau*3AI and was ligated to the *Bam*HI site of pBC SK$^+$ vector. After electroporation into *E. coli* Top10, recombinant clones were selected on plates containing amoxicillin (50 μg/ml). Analysis by disk susceptibility assay of *E. coli* Top10 harboring the recombinant plasmid pBC 2596 revealed an antagonism between cefoxitin and extended-spectrum cephalosporins, as was observed for *C. sedlakii* 2596 (data not shown). Overall, the β-lactam MICs determined for *E. coli*(pBC 2596) were higher than those for *C. sedlakii* 2596, but the resistance profiles of the two strains were similar (Table 3).

Sequence analysis of $bla_{\text{Sed-1}}$ and bla_{SedR}. Restriction analysis of the recombinant plasmid pBC 2596 showed the presence of a 2.78-kb DNA insert. The sequencing of this fragment revealed two ORFs in opposite orientations, SedR and Sed-1 (Fig. 1). The nucleotide sequence of $bla_{\text{Sed-1}}$, which is 888 bp long, displayed 82% identity with the chromosomal gene coding for the CdiA β-lactamase from *C. koseri* and 80% identity with the *bla* gene from *K. oxytoca*. The *bla* Sed-1 gene was found to encode a 31.9-kDa protein comprising 295 amino acid residues. Analysis of the amino acid sequence with the program SignalP suggested that the cleavage site in the precursor protein is located between the first alanine and glutamine residues in the amino acid sequence LH**AQ**ATSDVQQ (Fig. 1). This putative site corresponds well to the N-terminal sequence determined experimentally for the mature β-lactamase purified from *C. sedlakii* 2596, which was QATSDVQQVQ KKLAALEKQ. The pI and molecular mass values of 8.86 and 28.6 kDa, respectively, which were predicted from the sequence of the deduced mature protein Sed-1, were in agreement with the values measured by isoelectrofocusing (8.6) and by SDS-PAGE analysis (30 kDa).

The degree of amino acid sequence identity found between Sed-1 and the TEM and SHV enzymes was rather low (about 40%), while the protein had high identity with the chromosome-encoded β-lactamases of *C. koseri* (84%), *K. oxytoca* (74%), *Serratia fonticola* (67%), *P. vulgaris* (63%), and the plasmid-mediated enzymes MEN-1 (71%) and TOHO-1 (70%). A multiple amino acid sequence alignment of these class A β-lactamases is shown in Fig. 2. Most of the residues known to be involved in the catalytic mechanism and in substrate binding are conserved in Sed-1, including the consensus sequences ^{70}SXXK^{73}, ^{130}SDN^{132}, ^{234}KTG^{236}, and the highly conserved residue Glu-166 (based on the numbering system of Ambler et al. [4]) (Fig. 2). Other important conserved residues

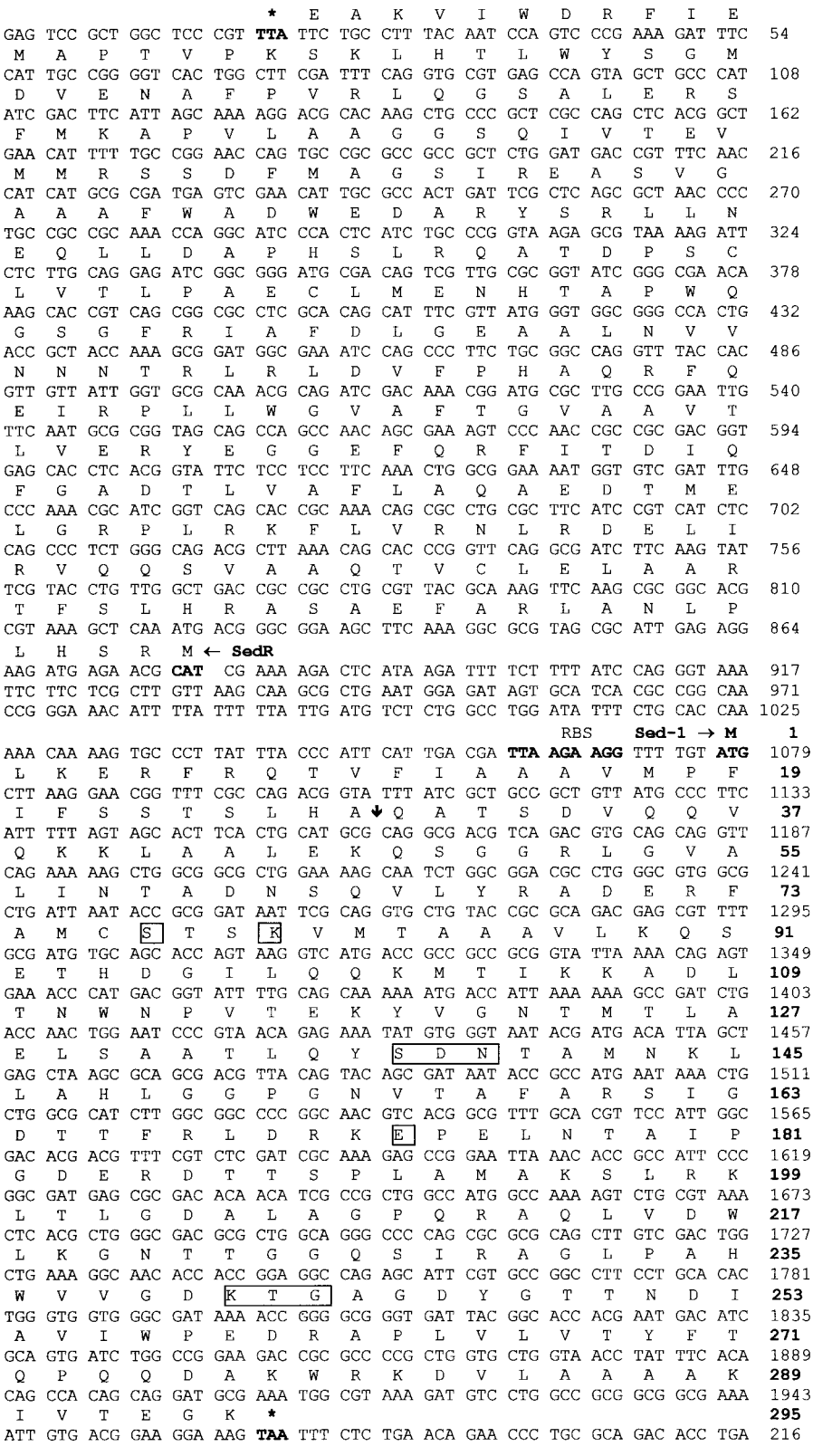

FIG. 1. Nucleotide sequences of $bla_{\text{Sed-1}}$ and bla_{SedR} from *E. coli*(pBC2596). The deduced amino acid sequence is indicated in single-letter code above the nucleotide sequence. RBS indicates a potential ribosome-binding site. The Sed-1 signal peptide extends from amino acid 1 to 28, and the postulated cleavage site is indicated by a vertical arrow. The residues conserved in the active site of serine β-lactamases are boxed. Two numbering schemes are indicated: the one in boldface is for the Sed-1 amino acid sequence, and the other one is for the Sed-1 and SedR nucleotide sequences.

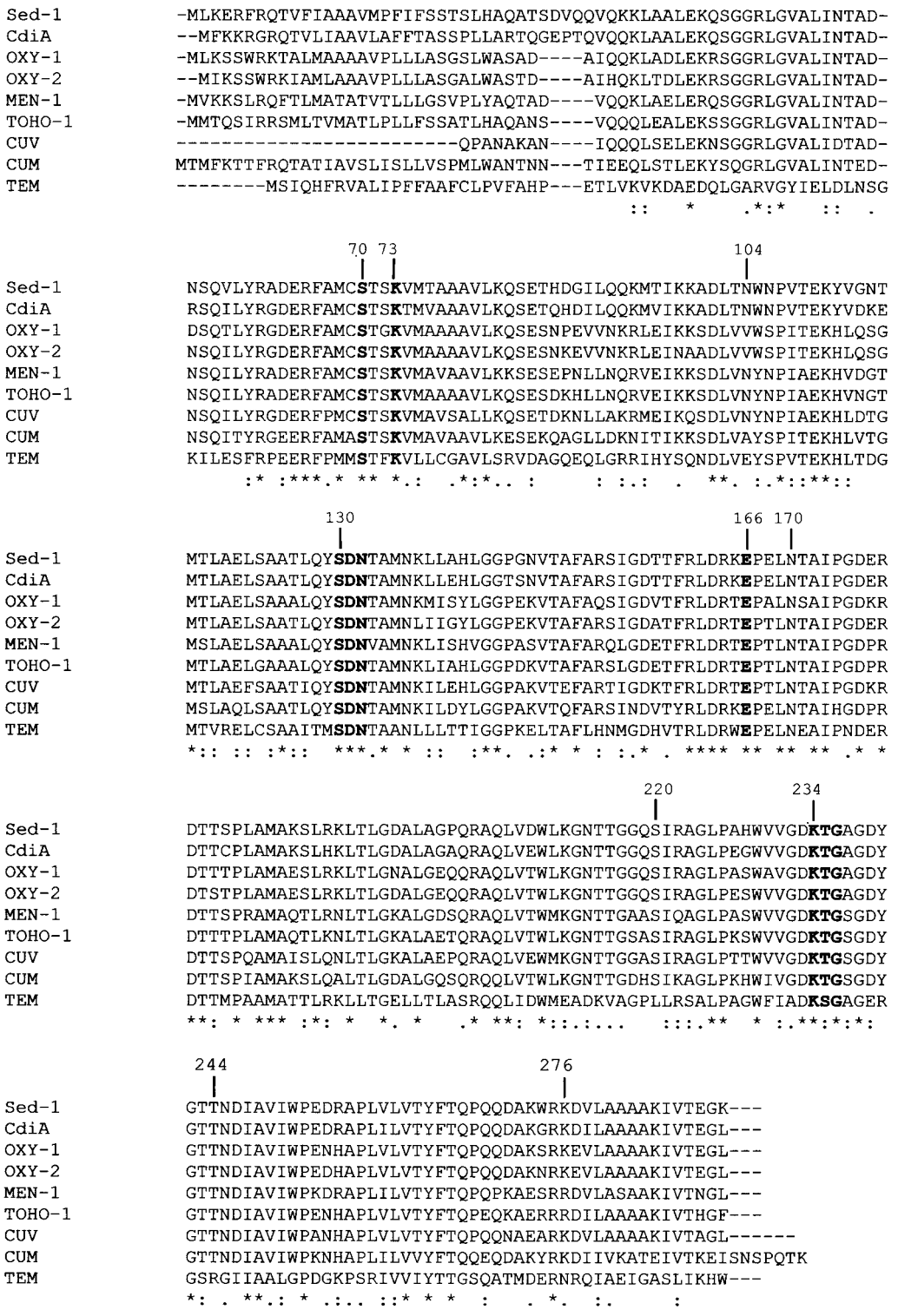

FIG. 2. Multiple alignment of amino acid sequences for nine class A β-lactamases. Dashes indicate gaps inserted in the alignment; asterisks indicate identical residues. Numbering is according to Ambler (R. P. Ambler et al., Letter, Biochem. J. 276:269–270, 1991). Boldface residues are the highly conserved residues involved in the catalytic site of the protein. The β-lactamases included in the alignment are Sed-1 from *C. sedlakii* (present study), CdiA from *C. diversus* (32), OXY-1 and OXY-2 from *K. oxytoca* (25), MEN-1 from *E. coli* (8), TOHO-1 from *E. coli* (30), CUV from *S. fonticola* (44), CUM from *P. vulgaris* (22), and TEM from *E. coli* (54).

were identified in Sed-1, such as Arg-164, Asn-170, and Asp-179, which are located in the Ω-loop structure. It must be noted here that the sequence of this catalytically important loop is highly conserved in Sed-1, compared to the class A β-lactamases presented in Fig. 2. Regarding the other positions known to contribute significantly to the catalytic activity of class A β-lactamases, we found in Sed-1 a cysteine in position 69, an asparagine in position 104, and an alanine and a glycine in positions 237 and 238, respectively. No arginine was identified in Sed-1 in either position 220 or position 244, but a basic residue (a Lys for Sed-1) is present in position 276, a feature which is shared by the seven class A β-lactamases related to the chromosomal enzyme of *K. oxytoca* shown in Fig. 2 (OXY-1, OXY-2, CdiA, MEN-1, TOHO-1, CUV, and CUM).

The bla_{SedR} gene found upstream from bla_{Sed-1} is an 861-bp ORF divergently transcribed from bla_{Sed-1} which encodes a 32-kDa protein comprising 286 amino acid residues. This protein displayed an identity of 90% with CdiR, the transcriptional regulator of the class A β-lactamase CdiA from *C. koseri*, and 68% identity with CumR from *P. vulgaris*. The identity found with the transcriptional regulator AmpR of class C β-lactamases was lower (47% with the *C. freundii* AmpR protein). As shown in Fig. 3, the identity is mainly located at the level of the N-terminal part of the sequences, where the helix-turn-helix motif required for binding to the *ampR-ampA* intercistronic region is found. Regarding the 20 residues involved in this motif, 14 are strictly conserved among the five AmpR sequences of class A β-lactamases shown in Fig. 3.

Purification and kinetic study of the β-lactamase Sed-1. After four purification steps, two bands of 30 kDa (major band) and 26 kDa (minor band) were observed on SDS-PAGE analysis of the enzyme preparation. By soaking the polyacrylamide gel in Tris-HCl (pH 7.0), the β-lactamase activity could be renatured and was associated with the 30-kDa band. Isoelectric focusing performed from the partially purified enzyme confirmed the pI of 8.6 initially found for Sed-1 from *C. sedlakii* 2596.

As shown in Table 4, the highest catalytic activities were measured for benzylpenicillin, ampicillin, cefpirome, cephalothin, and cloxacillin (k_{cat} values between 300 and 165 s^{-1}). Nevertheless, the high K_m values found for ampicillin (565 μM) and cefpirome (1,000 μM) reduced the corresponding catalytic efficiencies of these two drugs (k_{cat}/K_M = 425 and 215 $s^{-1} \cdot mM^{-1}$, respectively) when compared to those of benzylpenicillin (6,650 $s^{-1} \cdot mM^{-1}$), cephalothin (6,000 $s^{-1} \cdot mM^{-1}$), and cloxacillin (1,270 $s^{-1} \cdot mM^{-1}$). Cefuroxime had a relatively low k_{cat} (65 s^{-1}) but a high apparent affinity (K_M = 20 μM), resulting in a catalytic efficiency (k_{cat}/K_M = 3,250 $s^{-1} \cdot mM^{-1}$) higher than that observed for cloxacillin. Ticarcillin, piperacillin, oxyimino-cephalosporins, and aztreonam were hydrolyzed with a lower catalytic efficiency (2 to 625 $s^{-1} \cdot mM^{-1}$; Table 4). Imipenem and cefoxitin hydrolysis was not detectable. The IC_{50}s determined with benzylpenicillin as a substrate showed that Sed-1 was well inhibited by clavulanic acid but poorly inhibited by sulbactam (0.065 and 2.5 μM, respectively). These IC_{50}s are comparable to the values of 0.09 μM (for clavulanic acid) and 6.1 μM (for sulbactam) obtained for the TEM-1 β-lactamase (14). Sed-1 is also weakly inhibited by cefoxitin, with an IC_{50} of 16 μM.

β-Lactamase diversity within the *Citrobacter* genus. Twenty strains representing three species (*C. koseri, C. sedlakii*, and *C. rodentium*) within the *Citrobacter* genus were studied. The two reference strains, *C. sedlakii* CIP-105037 and *C. rodentium* CIP-104675, were from the CIP. For *C. koseri*, 12 clinical isolates were studied in addition to three CIP reference strains. All the strains were identified using Api 20E galleries, the identification being confirmed by PCR and sequencing of the hypervariable region in the 16S rRNA. In order to characterize the β-lactamases present in each isolate, the pIs of the β-lactamases produced by each strain were determined by isoelectrofocusing. Genetic characterizations by PCR with primers specific to bla_{TEM}, bla_{CdiA}, and bla_{Sed-1} were also carried out. The results are shown in Table 5.

The clinical strain 2596 and the reference strain CIP-105037 of *C. sedlakii* displayed very similar phenotypes: they were resistant to aminopenicillins, carboxypenicillins, and early cephalosporins but remained susceptible to piperacillin (Table 3). An antagonism between imipenem and extended-spectrum cephalosporins could be observed for both strains, indicating inducible β-lactamase production in *C. sedlakii*. The hybridization experiment carried out with a bla_{Sed-1}-specific probe gave a positive result for both strains. Accordingly, sequencing of the DNA products amplified with primers specific for bla_{Sed-1} showed that *C. sedlakii* CIP-105037 harbored a β-lactamase gene, the sequence of which is very similar to that of bla_{Sed-1} (98% identity for 640 bp sequenced). The differences found at the amino acid level between the two β-lactamases accounted for the difference in pI values observed for *C. sedlakii* 2596 (8.6) and for *C. sedlakii* CIP-105037 (8.7).

The *C. rodentium* CIP-104675 strain produced a β-lactamase characterized by a pI value of 8.7 and yielded a positive hybridization signal with the internal probe specific for bla_{Sed-1}. The DNA fragment of 640 bp obtained by PCR from *C. rodentium* CIP-104675 was sequenced and was found to share 80% identity with bla_{Sed-1}.

Regarding *C. koseri*, three different phenotypes of resistance to β-lactams (wild type, penicillinase, and extended-spectrum β-lactamase [ESBL] phenotypes) could be identified among the 17 strains included in the present study. As shown in Table 5, very heterogeneous pI values were found for these different strains of *C. koseri* which produced different combinations of noninducible β-lactamases, each strain being characterized by one, two, or three distinct pI values. PCR amplification and sequence analysis were made in an attempt to identify the corresponding β-lactamase genes. Amplification with the primers specific for bla_{TEM} confirmed the presence of this gene in the six strains showing a penicillinase or an ESBL phenotype. By contrast, the amplifications with the bla_{CdiA} and bla_{Sed-1} primers remained negative in all the strains tested, and none of the *C. koseri* strains hybridized with the bla_{Sed-1} internal probe.

DISCUSSION

C. sedlakii 2596, which produces the chromosomally encoded class A β-lactamase Sed-1, was resistant to aminopenicillins, carboxypenicillins, and early cephalosporins but not to acylureidopenicillins such as piperacillin. This resistance profile can be readily explained by the hydrolytic properties of Sed-1 (high catalytic efficiency against benzylpenicillin, clox-

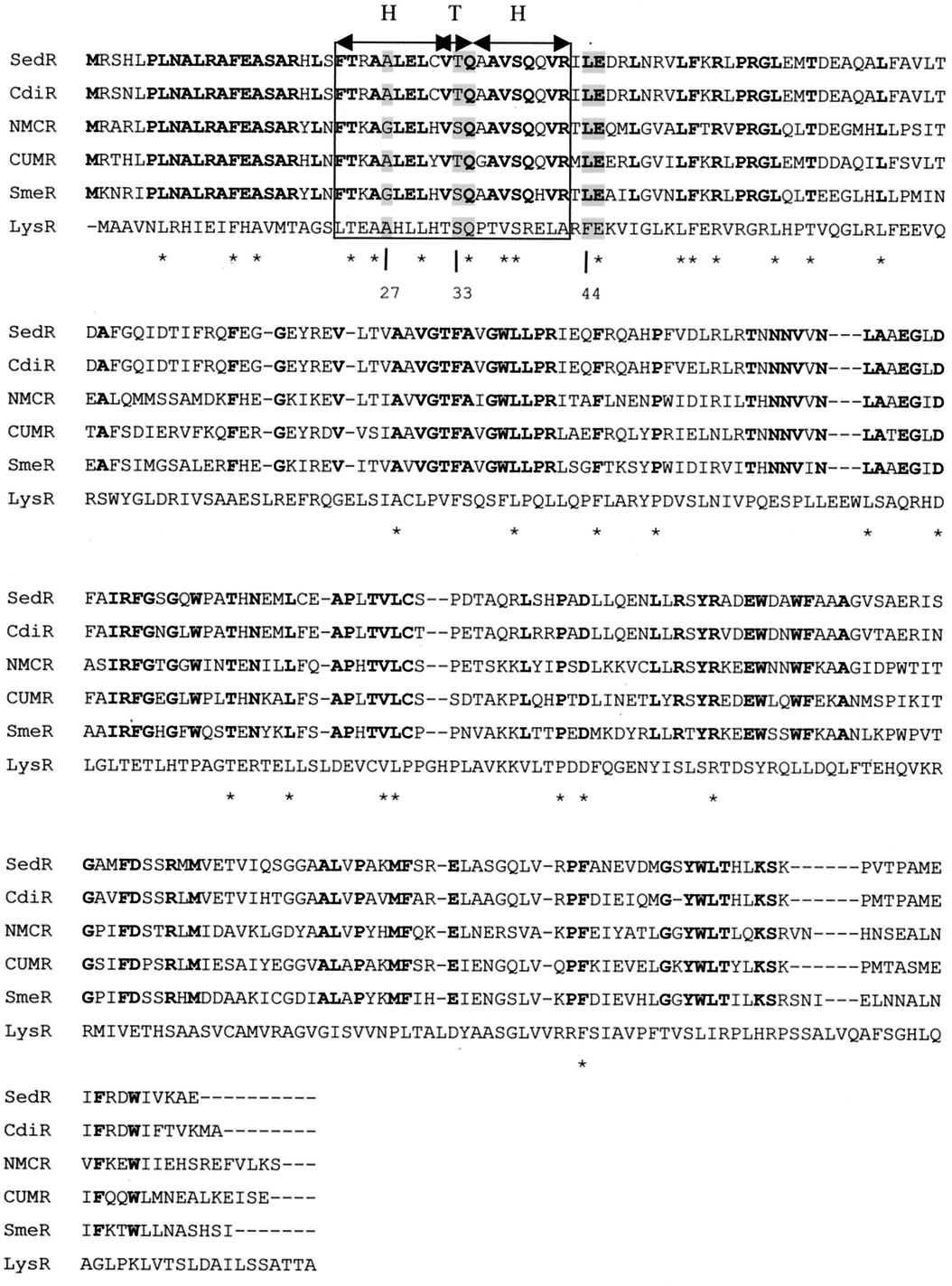

FIG. 3. Alignment of LysR-type proteins. The AmpR sequences involved in the class A β-lactamase regulation systems were from *C. diversus* (CdiR), *E. cloacae* (NMCR), *P. vulgaris* (CumR), and *Serratia marcescens* (SmeR). LysR, the type regulator protein, was from *E. coli*. The predicted helix-turn-helix domain is boxed. Residues identical in SedR and the AmpR family sequences are indicated in boldface, whereas those identical in SedR and the LysR family sequences are indicated with asterisks. The residues highly conserved in LTTRs are shaded in gray (the amino acid numbering is indicated below the multiple alignment).

acillin, and early cephalosporins such as cephalothin and cefuroxime, but low efficiency for piperacillin), which closely resemble those of the cefuroximases, such as the chromosome-encoded β-lactamase CUM from *P. vulgaris* or the plasmid-encoded β-lactamases FEC-1, FPM-1, and FUR from *E. coli, Proteus mirabilis*, and *Klebsiella pneumoniae*, respectively (36, 45, 58, 59). Cefotaxime, cefpirome, and aztreonam were also hydrolyzed by Sed-1 but with low catalytic efficiencies and moderate apparent affinities (Table 4). Clavulanic acid and sulbactam inhibited the β-lactamase activity of Sed-1 with

TABLE 4. Kinetic parameters of various β-lactam antibiotics for the Sed-1 β-lactamase from *C. sedlakii* 2596

Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	IC_{50} (μM)
Benzylpenicillin	300 ± 3	45 ± 2	6,650 ± 230	
Ampicillin	240 ± 20	565 ± 75	425 ± 30	
Ticarcillin	25 ± 1	40 ± 4	625 ± 50	
Piperacillin	20 ± 0.05	165 ± 1	120 ± 0.04	
Cephalothin	180 ± 0.3	30 ± 2	6,000 ± 30	
Cefoxitin	<0.01	ND[b]	ND	16
Cefuroxime	65 ± 5	20 ± 0.3	3,250 ± 100	
Cefotaxime	80 ± 5	170 ± 20	470 ± 40	
Ceftazidime	5 ± 0.1	2,380 ± 200	2 ± 0.1	
Aztreonam	30 ± 3	390 ± 80	75 ± 8	
Cefpirome	215 ± 15	1,000 ± 100	215 ± 15	
Oxacillin	85 ± 8	340 ± 60	255 ± 20	
Cloxacillin	165 ± 8	130 ± 10	1,270 ± 40	
Imipenem	<0.01	ND	ND	
Clavulanic acid				0.065
Sulbactam				2.5

[a] Mean ± standard deviation values are indicated.
[b] ND, not determined.

IC_{50}s similar to those encountered among class A β-lactamases highly susceptible to these inhibitors, such as TEM-1.

With the aim of determining whether the unusual substrate profile of Sed-1, including its significant activities with cefuroxime, cefotaxime, and cefpirome, could be related to the presence of specific amino acid residues in the sequence of the enzyme, we compared its amino acid sequence with those from other class A β-lactamases. A high percentage of identity was found between Sed-1 and the chromosome-encoded β-lactamases of *C. koseri* (84%), *K. oxytoca* (74%), *S. fonticola* (67%), *P. vulgaris* (63%), and the plasmid-mediated enzymes MEN-1 or CTX-M (71%) and TOHO-1 (70%), suggesting that these class A enzymes may be derived from a common ancestor. All the conserved residues considered to be important for catalysis in class A β-lactamases (^{70}SXXK^{73}, ^{130}SDN^{132}, ^{234}KTG^{236}, and E^{166}) were found in Sed-1, as was the Ω-loop, an important structural element including the amino acid residues 161 to 179 in class A enzymes.

Regarding positions 104, 164, 179, 205, 237, 238, and 240, which are involved in the extended substrate specificity of the mutants of the class A β-lactamases TEM and SHV (35), it is striking that an Asn was found at position 104 in Sed-1. Indeed, according to Petit et al. (46), residue 104 contributes to the precise positioning of the ^{130}SDN^{132} loop, which is a crucial structural and catalytic element for the binding and hydrolysis of β-lactams. Accordingly, amino acid modifications are found at this position in a large number of ESBLs derived from TEM-1 and TEM-2. Moreover, an asparagine residue is also found at position 104 in the nine CTX-M variants described to date and also in related enzymes such as TOHO-1 from *E. coli* and CUV from *S. fonticola* (Fig. 2), which, like Sed-1, are enzymes that efficiently hydrolyze cefuroxime and cefotaxime

TABLE 5. Isoelectric points and results of the hybridization and amplification experiments carried out on the different *Citrobacter* isolates

Strain	Phenotype	Isoelectric point(s)	PCR result			Hybridization with bla_{Sed-1} probe
			bla_{TEM}	bla_{CdiA}	bla_{Sed-1}	
C. sedlakii 2596	Wild type	8.6	−	−	+	+
C. sedlakii CIP105037	Wild type	8.7	−	−	+	+
C. rodentium CIP104675	Wild type	8.7	−	−	+	+
C. koseri CKB	ESBL	6.8, 6.1, 4.8	+	−	−	−
C. koseri CKC	ESBL	6.1, 5.7, 4.8	+	−	−	−
C. koseri CKD	ESBL	6.8, 6.1, 4.8	+	−	−	−
C. koseri CKE	ESBL	6.8, 6.1, 4.8	+	−	−	−
C. koseri CKG	ESBL	6.1	+	−	−	−
C. koseri CIP-72.14	Penicillinase	5.4	+	−	−	−
C. koseri CK1	Wild type	7, 4.7	−	−	−	−
C. koseri CK2	Wild type	8.3	−	−	−	−
C. koseri CK3	Wild type	5.6, 5.4	−	−	−	−
C. koseri CK4	Wild type	5	−	−	−	−
C. koseri CK5	Wild type	7	−	−	−	−
C. koseri CK7	Wild type	5	−	−	−	−
C. koseri CK10	Wild type	8.2, 5.6, 5.3	−	−	−	−
C. koseri CIP-82.87	Wild type	5.7	−	−	−	−
C. koseri CIP-76.3	Wild type	5	−	−	−	−
C. koseri CIP-82.94	Wild type	7.4	−	−	−	−
L. malonatica CIP82.88	Wild type	8.1, 5, 4.5	−	−	−	−

but not ceftazidime (8, 10, 12, 26). Note here that the residue found at position ABL 237 in Sed-1 is an alanine, whereas a serine has been found at this position in CUV, CUM, MEN-1 (CTX-M), and TOHO-1. In TEM-type ESBLs, as in other class A enzymes with an extended spectrum of activity, the presence of a serine residue in position 237 has been clearly shown to increase the level of β-lactamase activity against expanded-spectrum cephalosporins. Consequently, the presence of Ala 237 in Sed-1 could explain the relatively low level of activity the enzyme displays against cefotaxime, compared to the activities reported for the related enzymes MEN-1 (CTX-M), CUV, CUM, and TOHO-1, which all confer a high level of resistance to this drug (7, 22, 30, 44).

Other amino acid residues of interest, which are not strictly conserved in class A βlactamases, were found in Sed-1. Among them, there is a cysteine at position 69 in Sed-1, which neighbors the active-site serine found at position 70. Cys 69 is present in all the β-lactamases belonging to the *K. oxytoca* subgroup (Fig. 2), so that this residue could have a significant role in the substrate profile of these enzymes. Also note that there is no arginine residue at positions ABL 220 or 244 in Sed-1. Matagne et al. (35) have suggested that a basic residue is found in position ABL 276 when Arg is absent at positions 220 and 244. This is the case in Sed-1, which presents a Lysine at position 276 that is highly conserved in the cefuroximases group and perfectly aligned with the corresponding arginine residue found in MEN-1 and TOHO-1 (Fig. 2).

The negative results obtained from the conjugation and plasmid extraction experiments strongly suggested a chromosomal location for the $bla_{\text{Sed-1}}$ gene. The presence, upstream from $bla_{\text{Sed-1}}$, of a gene coding for a transcriptional regulator belonging to the LysR family reinforces this hypothesis. In *C. sedlakii*, the regulator protein SedR induces the production of Sed-1 when the bacteria are grown in the presence of an inducer β-lactam antibiotic such as cefoxitin and imipenem, as illustrated by the increase of the MIC of cefuroxime observed in the presence of cefoxitin for *C. sedlakii* 2596 (Table 3). Regarding its amino acid sequence, SedR is more related to the regulators of the class A β-lactamases from *C. koseri* and *P. vulgaris* (90% identity with CdiR and 68% identity with CumR) than to the regulators of the class C β-lactamases (47% identity with the *C. freundii* AmpR protein), suggesting that the relationships among LysR proteins are related to the type of β-lactamase they regulate. The similarities are the highest around the N-terminal region, where the consensus sequence for the helix-turn-helix motif is found. In this region, the residues conserved among the LTTRs which are Ala(Gly)-27, Ser(Thr)-33, Gln-34, Pro-35, Phe(Leu)-44, and Glu-45 (51), are all present in SedR, except for Pro-35.

β-Lactamase distribution within the *Citrobacter* genus is complex. *C. freundii* produces an inducible chromosome-encoded class C β-lactamase (62), whereas *C. sedlakii, C. rodentium*, and *C. koseri* produce class A β-lactamases (27, 41, 47, also, the present study). On the basis of the sequencing of the DNA fragments amplified in this study, *C. sedlakii* CIP-105037 and *C. rodentium* CIP-104675 harbor β-lactamase genes sharing 98 and 80% of identity to $bla_{\text{Sed-1}}$, respectively. The expression of $bla_{\text{Sed-1}}$, and also that of the closely related gene found in *C. sedlakii* CIP-105037, is inducible, whereas that of the $bla_{\text{Sed-1}}$-like gene found in *C. rodentium* CIP-104675 seems to be constitutive. Regarding *C. koseri*, DNA amplification experiments with $bla_{\text{Sed-1}}$-specific primers showed that this β-lactamase gene was not present in the 17 *C. koseri* isolates tested in this study. Moreover, the results obtained by PCR were confirmed by dot-blot hybridization with a $bla_{\text{Sed-1}}$-specific probe, strongly supporting the idea that this gene is not present in *C. koseri*. More surprisingly, no DNA amplification could be obtained using the set of primers designed to amplify specifically the bla_{CdiA} gene initially reported in the strain ULA27 of *C. koseri*, suggesting that the latter gene is not ubiquitous in this species or, alternatively, that the strain used for the initial characterization of bla_{CdiA} was not a *C. koseri* strain. Such a hypothesis is confirmed by the fact that the constitutive resistance phenotypes observed for the clinical isolates of *C. koseri* included in the present study were all clearly different from the inducible resistance phenotype initially reported for the strain ULA27 of *C. koseri*, which produces CdiA under control of the regulator protein CdiR (33). Moreover, we have characterized 13 distinct isoelectric point values among the 17 *C. koseri* strains studied, with one to three different pI values detected per strain. The strains with a wild-type phenotype had pI values varying from 4.7 to 8.2. Such values are in agreement with those found in the literature, which vary from 4.8 to 9.5 (27, 47). For the strains displaying an ESBL profile, three pI values of 6.8, 6.1, and 4.8 were found, one corresponding to the pI value of a TEM variant, the identification of which was confirmed by PCR with bla_{TEM} primers. Therefore, it is likely that the β-lactamase genes found in *C. koseri* encompass a series of genes that have significantly diverged, as previously described by Jones et al. (32), and which are characterized by a nucleotide sequence different from that of $bla_{\text{Sed-1}}$ and bla_{CdiA}. Further studies on the genetic characterization of these genes must be done to elucidate the origin of the *bla* genes in *C. koseri*.

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