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Genetic and Biochemical Characterization of a Chromosome-Encoded Carbapenem-Hydrolyzing Ambler Class D β-Lactamase from *Shewanella algae*

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A chromosome-encoded β-lactamase gene from *Shewanella algae* clinical isolate KB-1 was cloned and expressed in *Escherichia coli*. It encoded the Ambler class D enzyme OXA-55, sharing less than 55% identity with any other oxacillinases. Although conferring a narrow-spectrum β-lactam resistance phenotype, OXA-55 had carbapenem-hydrolyzing activity that mirrored the reduced susceptibility to imipenem observed in *S. algae* KB-1. Very similar oxacillinases were found in other *S. algae* isolates.

Bacteria of the genus *Shewanella* are gram-negative bacilli, facultatively anaerobic, belonging to the family *Alteromonadaceae* (25), that are widely distributed in marine and freshwater environments (23). *Shewanella algae* is a bacterial species recently identified (23, 24) that is closely related to *Shewanella putrefaciens* (formerly *Pseudomonas putrefaciens*) (25). Both *S. putrefaciens* and *S. algae* species are rare human pathogens (12, 15, 25).

Recently, we have characterized β-lactamase OXA-54 from *S. oneidensis* and we have shown that this Ambler class D enzyme hydrolyzed imipenem significantly (19). Three *S. algae* isolates were obtained from clinical samples during the last 2 years from our hospital, and the aim of the present study was to determine their β-lactamase content.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. algae* clinical isolate KB-1 was isolated from an arm wound of a 10-year-old girl hospitalized at the Bicêtre hospital in 2001 (Kremlin-Bicêtre, France). *S. algae* KB-2 and KB-3 were from a peritoneum aspiration and from a deep pus sample of the flank, respectively, from patients also hospitalized at Bicêtre hospital. These isolates were identified by the API 32GN system (bioMérieux, Marcy l'Etoile, France) and by sequencing of 16S rRNA genes using primers 16S 8-27 (5′-AGAGTTTGATCHTGGYTYAGA-3′; H is A, T, or C and Y is C or T) and 16S 1512-1491 (5′-ACGGYTACCTTGT TACGACTTC-3′; Y is C or T) (9, 11, 23).

S. putrefaciens CIP 8040 (Institut Pasteur strain collection, Paris, France) was used as reference strain. *Escherichia coli* reference strain DH10B, and plasmid pBK-CMV (Stratagene, Amsterdam, The Netherlands) were used for cloning experiments, whereas rifampin-resistant *E. coli* JM109 was used for conjugation experiments (3).

Antimicrobial agents and MIC determinations. The agents and their sources have been referenced elsewhere (17). MICs were determined by an agar dilution technique on Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Paris, France) with an inoculum of 10^4 CFU per spot and were interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (14).

Plasmid DNA content and conjugation. Plasmid DNA extraction of *S. algae* KB-1 was performed according to different methods as previously described (18). Direct transfer of β-lactam resistance markers into rifampin-resistant *E. coli* JM109 was attempted by liquid and solid mating-out assays and by electroporation of a putative plasmid DNA suspension of *S. algae* KB-1 into *E. coli* DH10B. Transconjugants and electroporants were selected on Trypticase soy (TS) agar plates containing rifampin (50 μg/ml) plus amoxicillin (50 μg/ml) or amoxicillin only, respectively.

Cloning experiments. Whole-cell DNAs of *S. algae* KB-1, KB-2, and KB-3 were extracted as previously described (17). Cloning experiments were performed with Sau3AI-digested DNA of *S. algae* KB-1 and BamHI-restricted plasmid pBK-CMV, followed by expression of recombinant plasmids in *E. coli* DH10B as described previously (18). Transformants were selected on TS agar containing amoxicillin (100 μg/ml) and kanamycin (30 μg/ml). Antibiograms obtained by disk diffusion were performed with *E. coli* DH10B harboring recombinant plasmids, and sizes of the plasmid inserts were determined by restriction analysis (22). A recombinant plasmid, pS-1, obtained from cloning of Sau3AI-restricted fragments of *S. algae* KB-1, was retained for further analyses.

DNA sequencing and protein analysis. Both strands of the cloned DNA fragment of recombinant plasmid pS-1 were sequenced with an Applied Biosystems sequencer (ABI 377). The nucleotide and deduced protein sequences were analyzed with software available over the internet at the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov). PCR experiments were performed as previously described (20) using specific primers OXA-55/1 (5′-CATCTACCTTTAAAATTCCC-3′) and OXA-55/2 (5′-AGCTGTTCCTGCTTGAGCAC-3′) for the bla_{OXA-55} gene to amplify and subsequently to sequence bla_{OXA-55}-type genes from *S. algae* isolates KB-2 and KB-3.

IEF analysis. Isoelectric focusing (IEF) analysis was performed with an Ampholine polyacrylamide gel (pH 3.5 to 9.5) (Amersham Pharmacia Biosciences, Orsay, France), as described previously (18) with culture extracts of *S. algae* KB-1, KB-2, and KB-3, *S. putrefaciens* CIP 8040T, and *E. coli* DH10B harboring recombinant plasmid pS-1.

β-Lactamase purification. Culture of *E. coli* DH10B(pS-1) that produced OXA-55 was grown overnight at 37°C in 4 liters of TS broth containing 50 μg of ticarcillin per ml and 30 μg of kanamycin per ml. The protein extracts obtained were purified as described previously (20). Briefly, culture extracts were subjected to several purification steps, including ion-exchange chromatography with a 2- by 5-cm Q-Sepharose column (Amersham Pharmacia Biotech, Orsay, France) using a 30 mM Tris-HCl buffer (pH 8) followed by a 2- by 4-cm S-Sepharose column (Amersham Pharmacia Biotech) using a 20 mM BisTris buffer (pH 6.5). Elution of the β-lactamase was performed with a K_2SO_4 gradient (0 to 500 mM) in order to prevent potential activity inhibition by NaCl. The fractions containing the highest β-lactamase activity, determined by the nitrocefin test (Oxoid, Dardilly, France), were concentrated using Centrisart-C30 spin

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TABLE 1. MICs of β-lactams for *S. algae* KB-1, KB-2, and KB-3; *S. putrefaciens* CIP 8040; and *E. coli* DH10B

β-Lactam(s)[a]	MIC (μg/ml)				
	S. algae KB-1, KB-2, and KB-3	*S. putrefaciens* CIP 8040	*E. coli*[b]		
			DH10B(pS-1)	DH10B(pSon)[c]	DH10B
Amoxicillin	16–32	32	128	>512	4
Amoxicillin + CLA	8–16	32	128	>512	4
Ticarcillin	2–4	4	512	>512	4
Ticarcillin + CLA	2–4	4	256	>512	4
Piperacillin	1	1	4	32	2
Piperacillin + TZB	1	1	4	32	2
Cephalothin	256–512	64	4	4	4
Cefuroxime	4	2	4	4	4
Cefoxitin	16	4	1	4	4
Ceftazidime	0.25	0.25	0.12	0.12	0.06
Cefotaxime	0.12	0.06	0.06	0.12	0.06
Cefepime	0.12	0.06	0.06	0.06	0.06
Cefpirome	0.06	0.06	0.06	0.25	0.06
Moxalactam	2	2	0.12	2	0.06
Aztreonam	0.25	0.25	0.06	0.06	0.12
Imipenem	4	1	0.25	1	0.06
Meropenem	0.25	0.06	0.06	0.12	0.06

[a] CLA, clavulanic acid at a fixed concentration of 2 μg/ml; TZB, tazobactam at a fixed concentration of 4 μg/ml.
[b] Recombinant plasmid pS-1 expressed β-lactamase OXA-55 from *S. algae* KB-1, whereas plasmid pSon expressed β-lactamase OXA-54 from *S. oneidensis* MR-1.
[c] Data are from reference 19.

columns (Sartorius, Göttingen, Germany) and dialyzed against 100 mM phosphate buffer (pH 7.0).

Kinetic studies. Purified β-lactamase was used for kinetic measurements performed at 30°C in 100 mM sodium phosphate (pH 7.0) (17). The k_{cat} and K_m values were determined by analyzing β-lactam hydrolysis under initial rate conditions with a UV spectrophotometer, as previously described (18). The 50% inhibitory concentrations (IC_{50}) of clavulanic acid, tazobactam, sulbactam, and NaCl were determined (18). Various concentrations of these inhibitors were preincubated with purified enzyme for 3 min at 30°C to determine the concentrations that decreased the rate of hydrolysis of 100 μmol of cephalothin by 50%. Specific activities of protein extracts and purified β-lactamase from culture of *E. coli* DH10B(pS-1) were determined as described previously (2) with 100 μM cephalothin or imipenem as substrate. The protein purification rate was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE analysis. The protein content was measured by the Bio-Rad DC protein assay.

Nucleotide sequence accession number. The nucleotide sequence and deduced β-lactamase amino acid sequence reported in this work have been assigned to the GenBank and EMBL databases under accession no. AY343493.

RESULTS AND DISCUSSION

Strain identification, susceptibility testing, and IEF analysis. Identification of clinical isolates KB-1, KB-2, and KB-3 with the API 32GN system resulted in identification of *S. putrefaciens*, but this biochemical method could not distinguish between *S. putrefaciens* and *S. algae* (25). Thus, 16S rRNA genes were amplified from isolates KB-1, KB-2, and KB-3 and results were compared to the sequence of 16S rRNA gene of *S. putrefaciens* CIP 8040 (8). Indeed, isolates KB-1, KB-2, and KB-3 belonged to the species *S. algae*. The sequence identity of 16S rRNA genes of these three isolates was 98%, whereas they shared 92% identity with the sequence of the 16S rRNA gene of *S. putrefaciens* CIP 8040T.

MICs of β-lactams for *S. algae* KB-1 showed that it was resistant to cephalothin; had reduced susceptibility to amoxicillin, cefoxitin, cefuroxime, and imipenem; and was susceptible to the other β-lactams (Table 1). Addition of clavulanic acid and tazobactam did not significantly decrease MICs of β-lactams (Table 1). *S. algae* KB-2 and KB-3 had a similar resistance pattern (Table 1), whereas MICs of all β-lactams for *S. putrefaciens* CIP 8040 were lower than those for *S. algae* strains.

β-Lactamase extracts of *S. algae* KB-1, KB-2, and KB-3 submitted to IEF analysis gave one β-lactamase band with a pI of 8.6 (data not shown).

Cloning and sequence analysis of β-lactamase genes. Clonings gave recombinant *E. coli* DH10B(pS-1) that displayed an inhibitor-resistant β-lactamase phenotype that hydrolyzed imipenem significantly (data not shown).

DNA sequence analysis of the 1,965-bp insert of pS-1 revealed an open reading frame of 870 bp that encoded a 289-amino-acid protein (OXA-55). The G+C content of this open reading frame was 54%, which was within the range of G+C content of genes of *S. algae* species (23) (Fig. 1). Downstream of the bla_{OXA-55} gene, a 375-bp sequence corresponding to the 3′-end part of a gene encoding a putative LysR-type transcriptional regulator was found. This sequence shared 72% nucleotide identity with the sequence located downstream of bla_{OXA-54} of *S. oneidensis* (19).

Genetic location of β-lactamase gene. Plasmid extraction of *S. algae* KB-1 as well as transformation and conjugation experiments failed, suggesting a chromosomal location of this gene that was strengthened by the presence of similar surrounding DNA sequences to those surrounding the β-lactamase gene identified in *S. oneidensis*.

Sequence analysis of β-lactamase OXA-55. The 289-amino-acid protein OXA-55 shared some identity with several Ambler class D enzymes. β-Lactamase OXA-55 had 55% amino acid identity with OXA-54 from *S. oneidensis* (19), 41% with OXA-10, 39% with OXA-23 and -27 from *A. baumannii*, and 33% with the cluster OXA-24, -25, -26, and -40 from *A. baumannii* (1, 4, 5, 10, 11, 16).

Two conserved motifs, S-T-F-K and K-T-G, were identified

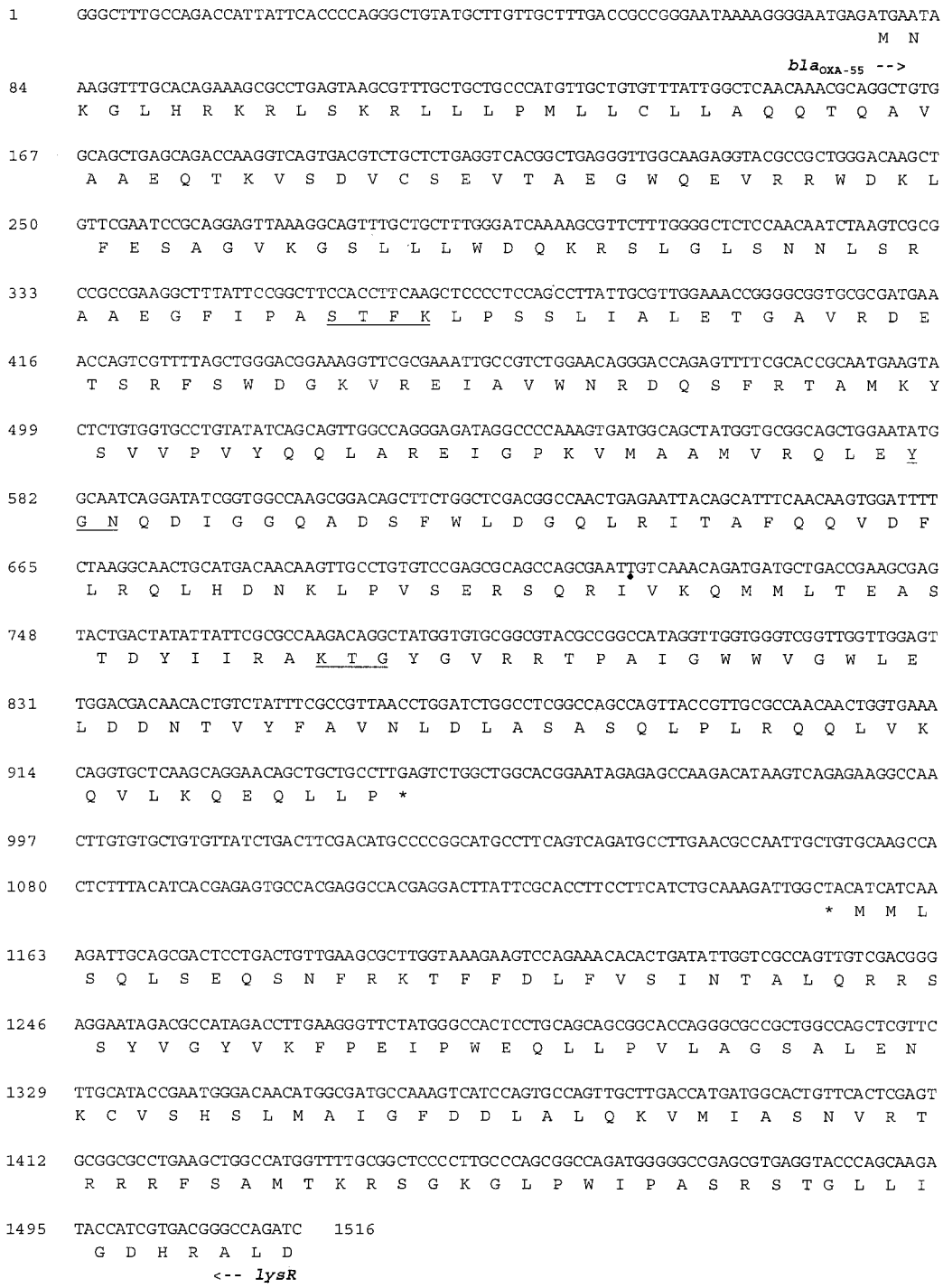

FIG. 1. Nucleotide sequence of a 1,516-bp fragment of recombinant plasmid pS-1 containing the bla_{OXA-55} gene and the 3′-end part of a gene encoding a putative LysR-type transcriptional regulator. The deduced amino acid sequence is designated in the single-letter code below the nucleotide sequence, and conserved residues of serine-based β-lactamases are underlined. The stars indicate stop codons.

at class D β-lactamase (DBL) positions 70 to 73 and 216 to 218, respectively (7). These motifs are involved in the function of the serine active site of oxacillinases (6, 13). The typical motif YGN of oxacillinases was found at DBL positions 144 to 146, as observed for OXA-54, whereas the oxacillinases with carbapenem-hydrolyzing properties identified in *A. baumannii* (OXA-23 to -27 and OXA-40) possessed an FGN motif at this position (1, 5, 10, 11, 16). However, as we have found previously, the phenylalanine residue at position 144 is not critical for imipenem hydrolysis of those oxacillinases (11).

A detailed analysis of the amino acid sequence of OXA-55 did

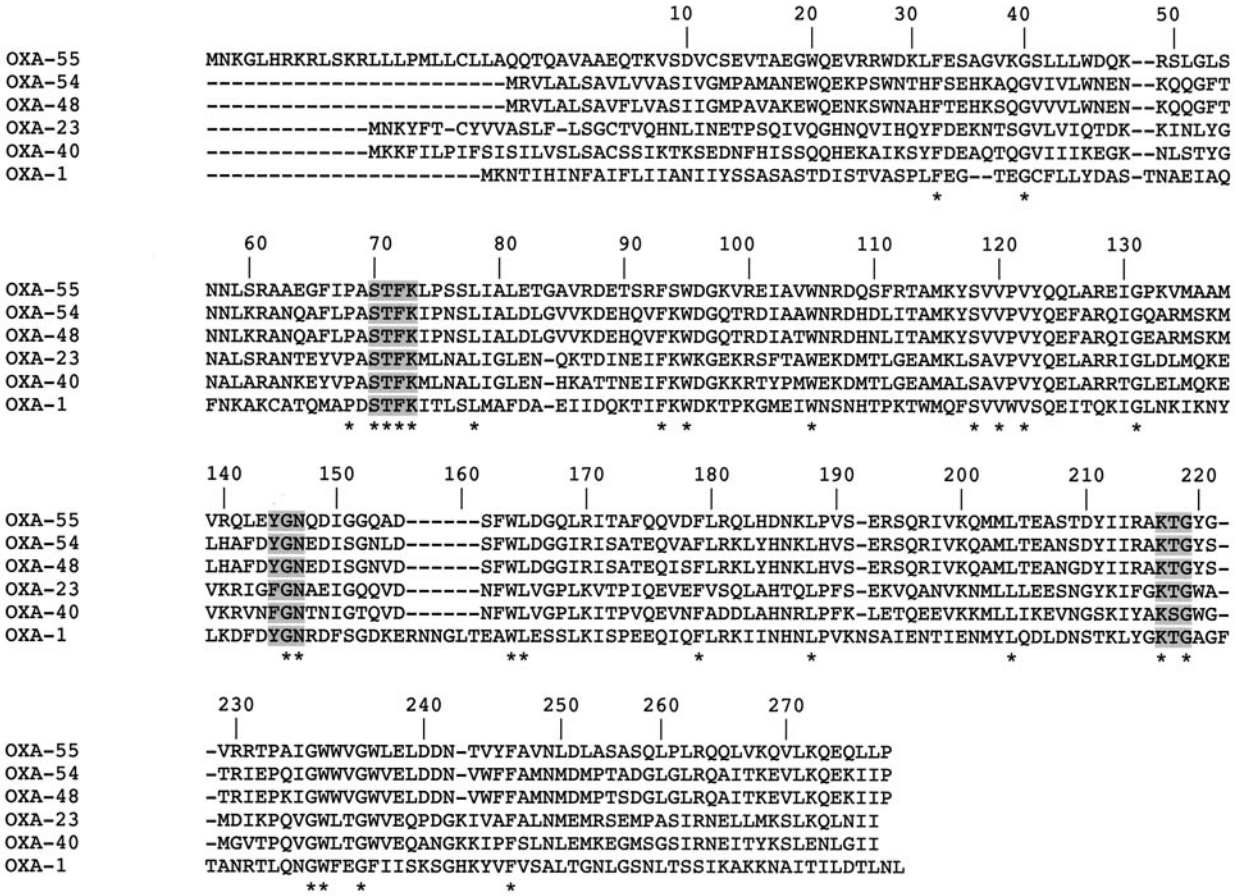

FIG. 2. Comparison of the amino acid sequence of OXA-55 to those of oxacillinases OXA-54 from *S. oneidensis* MR-1 (19), OXA-48 from *K. pneumoniae* 11978 (21), OXA-23 from *A. baumannii* 6B92 (10), OXA-40 from *A. baumannii* CLA-1 (11), and OXA-1 from *E. coli* K10-35 (13). The shaded boxes and stars indicate conserved and highly conserved residues among oxacillinases, and stars alone indicate highly conserved residues. Numbering of β-lactamases is according to the DBL system (7).

not identify any specific amino acid residue that could explain the carbapenem-hydrolyzing property of this enzyme (Fig. 2).

PCR experiments using primers annealing the bla_{OXA-55} gene allowed us to identify two bla_{OXA-55}-type β-lactamase genes from *S. algae* isolates KB-2 and KB-3. The sequence of these β-lactamases shared at least 98% identity with that of *S. algae* KB-1 (data not shown).

The very likely chromosomal location of bla_{OXA-55} and the presence of almost identical genes in other *S. algae* isolates argue for OXA-55-type enzymes being naturally produced by *S. algae*.

Susceptibility testing of recombinant strains. MICs of β-lactams for *E. coli* DH10B(pS-1) showed that it was resistant to amoxicillin and ticarcillin and had a reduced susceptibility to imipenem (Table 1). A similar low level of resistance to carbapenems has been reported for other carbapenem-hydrolyzing enzymes once expressed in *E. coli* (3, 5, 10, 11, 20). Clavulanic acid and tazobactam did not restore β-lactam activities significantly when OXA-55 was expressed (Table 1).

Kinetic analysis of OXA-55. After purification from culture extracts of *E. coli* DH10B(pS-1), the specific activity of β-lactamase OXA-55 against cephalothin was determined to be 0.5 U/mg of protein and its purification factor was 30-fold. Protein purity was estimated to be >95% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (data not shown). β-Lactamase OXA-55 had a narrow hydrolytic spectrum, including penicillins and narrow-spectrum cephalosporins (Table 2). Hydrolysis of oxacillin and cloxacillin was detected as observed for most oxacillinases (13) but not for other carbapenem-hydrolyzing oxacillinases (1, 4, 5, 10, 11, 16).

The overall catalytic activity of OXA-55 is less robust than that of other carbapenem-hydrolyzing oxacillinases, including OXA-54 from *S. oneidensis* (1, 5, 10, 11, 16, 19). Nevertheless, the ability of OXA-55 to hydrolyze imipenem was similar to that of OXA-54 and OXA-40, with relative k_{cat}/K_m values compared to benzylpenicillin of 3, 10, and 7%, respectively (Table 2). Hydrolysis of imipenem by OXA-55 was low: its catalytic activity (5 $mM^{-1} \cdot s^{-1}$) was lower than that reported for OXA-40 from *Acinetobacter baumannii* CLA-1 (15 $mM^{-1} \cdot s^{-1}$) (11). Hydrolysis of meropenem was detected, although at a lower level than that of imipenem.

Activity inhibition measured by determination of IC_{50}s showed that OXA-55 was weakly inhibited by clavulanic acid

TABLE 2. Kinetic parameters and relative k_{cat}/K_m values of purified β-lactamase OXA-55 from *S. algae* KB-1, OXA-54 from *S. oneidensis* MR-1, and OXA-40 from *A. baumannii* CLA-1[a]

Substrate	OXA-55				OXA-54 (relative k_{cat}/K_m)[b]	OXA-40 (relative k_{cat}/K_m)[c]
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	Relative k_{cat}/K_m		
Benzylpenicillin	4	25	160	100	100	100
Ampicillin	8	500	15	10	6	9
Ticarcillin	1	20	50	31	6	9
Piperacillin	3	110	30	17	4	23
Cephalothin	0.6	70	10	5	0.5	23
Cephaloridine	10	750	15	9	1.5	2
Cefotaxime	ND	—	—	—	0.4	—
Ceftazidime	2	4,700	0.5	0.25	—	5
Cefepime	ND	—	—	—	0.1	—
Cefpirome	0.1	670	0.1	0.06	2	—
Oxacillin	5	390	10	6	23	1
Cloxacillin	0.5	190	3	1.5	—	—
Aztreonam	ND	—	—	—	—	—
Imipenem	0.1	20	5	3	10	7
Meropenem	0.05	500	0.1	0.06	0.02	—

[a] Data are means of three independent experiments. Standard deviations were within 10% of the means. Relative values were calculated according to the value obtained for benzylpenicillin, which was set at 100. ND, no detectable hydrolysis (< 0.01 s^{-1}). —, not determinable.
[b] Data are from reference 19.
[c] Data are from reference 11.

(700 μM), tazobactam (12 μM), and sulbactam (300 μM), like most oxacillinases (13).

OXA-55 activity was inhibited by NaCl (IC_{50}, 48 mM), like most oxacillinases except those possessing an FGN motif at DBL positions 144 to 146—i.e., several carbapenem-hydrolyzing oxacillinases (1, 4, 5, 10, 11, 16).

The β-lactamase OXA-55 from *S. algae* may constitute as well a reservoir for oxacillinase genes able to spread through a mobilization process like those of *S. oneidensis*. Indeed, the plasmid-mediated carbapenem-hydrolyzing β-lactamase OXA-48, which is almost identical to the chromosome-encoded β-lactamase of *S. oneidensis*, has been identified very recently in a *Klebsiella pneumoniae* clinical isolate (21).

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