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EBR-1, a Novel Ambler Subclass B1 β-Lactamase from *Empedobacter brevis*

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***Empedobacter brevis* (formerly designated *Flavobacterium breve*) is a gram-negative aerobe involved in nosocomial infections. The Ambler class B β-lactamase gene bla_{EBR-1} was cloned and expressed in *Escherichia coli* from *E. brevis* clinical strain ASS-1, which had reduced susceptibility to expanded-spectrum cephalosporins and carbapenems. Purified β-lactamase EBR-1 hydrolyzed penicillins, cephalosporins, and carbapenems efficiently but not aztreonam. Kinetic parameters of EBR-1 were similar to those of class B enzymes such as BlaB, IND-2, and GOB-1 identified from other *Flavobacteriaceae* species, except for meropenem, which was more hydrolyzed by β-lactamase GOB-1. EBR-1, with a pI of 8.0 and a relative molecular mass of ca. 25 kDa, was classified in functional subgroup 3a, which includes most of the class B β-lactamases. EBR-1, which belongs to molecular subclass B1 of metalloenzymes, shares 58, 57, and 42% amino acid identity with the most closely related β-lactamases, IND-1/IND-2 from *Chryseobacterium indologenes*, CGB-1 from *Chryseobacterium gleum*, and BlaB from *Chryseobacterium meningosepticum*, respectively.**

Flavobacterium breve was included in the original description of the *Flavobacterium* genus in 1923 and was proposed as the type species of the *Flavobacterium* genus by Holmes and Owen in 1979 (13). Its classification was emended in 1994 (36), and *F. breve* was included in the novel species *Empedobacter brevis* belonging to the *Flavobacteriaceae* family (7). This species is the only member of the *Empedobacter* genus. *E. brevis* is a source of meningitis (10). Clinical strains of *E. brevis* have been also reported from blood infections and from other clinical sources (eye swabs, bronchial secretions, and urine) (14, 17). A few publications report the phenotype of resistance to β-lactams of *E. brevis*, *E. brevis* being of intermediate resistance to amoxicillin, ticarcillin, and narrow-spectrum cephalosporins and having a reduced susceptibility (but still being susceptible) to expanded-spectrum cephalosporins and imipenem (14, 17, 22). This species would be less resistant to β-lactams than other flavobacterial species (22).

Several carbapenem-hydrolyzing enzymes have been reported from bacterial species of the *Flavobacteriaceae* family, including BlaB and GOB from *Chryseobacterium meningosepticum*, IND from *Chryseobacterium indologenes*, CGB-1 from *C. gleum*, and a β-lactamase from *Flavobacterium odoratum* (1, 2, 4, 5, 32, 34). They are all Ambler class B enzymes.

We report here a novel chromosome-encoded Ambler class B β-lactamase from another *Flavobacteriaceae* species, *E. brevis*. We compared its activity to those of other metalloenzymes of the *Flavobacteriaceae* family.

MATERIALS AND METHODS

Bacterial strains. *E. brevis* clinical isolate ASS-1, used in this study, was from a rectal swab of an 18-month-old boy hospitalized in the intensive care unit at the Bicêtre hospital (Le-Kremlin-Bicêtre, France) in 1999 for a chemical intoxication. *E. brevis* ASS-1 was identified by standard biochemical techniques and 16S rRNA sequencing (36). *Escherichia coli* DH10B, *E. coli* BL21(DE3), and nalidixic acid-resistant *E. coli* JM109 were used for cloning, subcloning, and conjugation assays, respectively. All strains were stored at −70°C in Trypticase soy (TS) broth (Becton Dickinson, Le Pont-de-Claix, France) supplemented with 15% glycerol until testing.

Antimicrobial agents and MIC determinations. The antimicrobial agents used in this study were obtained in the form of standard laboratory powders and were used immediately after their solubilization. The agents and their sources have been described elsewhere (28). Antibiotic-containing disks (Bio-Rad, Marnes-la-Coquette, France) were used for routine antibiograms (www.sfm.asso.fr).

MICs were determined by an agar dilution technique on Mueller-Hinton plates with an inoculum of 10^4 CFU per spot as previously described (6). The plate contents were incubated at 35°C for 18 h at ambient atmosphere. MICs of β-lactams were determined alone or in combination with a fixed concentration of clavulanic acid (2 μg/ml). MIC results were interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (23).

Cloning experiments and analysis of recombinant plasmids. Genomic DNA from *E. brevis* ASS-1 was extracted as described earlier (15). Cloning experiments were performed with *Sau*3AI-restricted DNA fragments of *E. brevis* ASS-1 into the pBK-CMV plasmid (Stratagene, Amersham Pharmacia Biotech, Orsay, France) as previously described (25). Antibiotic-resistant colonies were selected onto TS agar plates containing amoxicillin (30 μg/ml) and kanamycin (30 μg/ml).

Recombinant plasmid DNAs were obtained from 100-ml TS broth cultures grown overnight in the presence of amoxicillin (30 μg/ml) at 35°C. Plasmid DNAs were recovered by using Qiagen columns (Qiagen, Courtaboeuf, France).

Conjugation assays, plasmid content, and hybridizations. Direct transfer of resistance gene into nalidixic acid-resistant *E. coli* JM109 was attempted by liquid and solid conjugation assays at 35°C (1, 3, 6). Transconjugants were selected on TS agar plates containing nalidixic acid (100 μg/ml) and amoxicillin (30 μg/ml). Extraction of plasmid DNA from *E. brevis* ASS-1 was attempted as previously described (27). Southern hybridization experiments were performed as described earlier (33) by using a 0.8% electrophoresis gel containing either whole-cell or *Hin*dIII- and *Cla*I-restricted DNA of *E. brevis* ASS-1 transferred onto a nylon membrane that was hybridized with a PCR-obtained internal fragment of bla_{EBR-1} with primers EbA (5′-CACTTATTGCA TTGATAGGAAG-3′) and EbB (5′-TTTCGATA TGTCCAGTAGCC-3′). Visualization of hybridizations was made using the enhanced chemiluminescence nonradioactive labeling and detection kit as described by the manufacturer (Amersham Pharmacia Biotech).

DNA sequencing and protein analysis. Both strands of the cloned DNA fragment of recombinant plasmid pEBR-1 were sequenced with an Applied Biosystems sequencer (ABI 377). The nucleotide and deduced protein sequences

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were analyzed with software available over the Internet (www.ncbi.nlm.nih.gov and www.cbs.dtu.dk/services/SignalP/) (24).

Subcloning of the *bla*$_{EBR-1}$ gene in an expression vector. The *bla*$_{EBR-1}$ gene was amplified by PCR with primers EBR-1nde (5′-TTGA CGAGGACATATG AAG AAATTATTTTCAC-3′), which contained an *Nde*I restriction site (underlined), and EBR-1bam (5′-CTATTATATCGGATCCTTTT TTCAATTTTATT TC-3′), which contained a *Bam*HI restriction site (underlined). Amplification was performed as described by the manufacturer (Perkin-Elmer) with an annealing temperature of 53°C for 1 min. After 38 amplification cycles, PCR product was purified, restricted with *Nde*I and *Bam*HI endonucleases, and cloned into expression vector pET-9a (Merck EuroLab, Fontenay-sous-Bois, France) to obtain pET-EBR-1. After purification of plasmid pET-EBR-1, the *Nde*I-*Bam*HI insert was sequenced to rule out any PCR-generated mismatches.

β-Lactamase purification. Cultures of *E. coli* BL21(DE3) harboring recombinant plasmid pET-EBR-1 were grown at 37°C in 2 liters of TS broth containing amoxicillin (20 μg/ml) until an optical density at 600 nm of 1 was reached. The β-lactamase expression was induced for 5 h after addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside, as recommended by the manufacturer (Novagen, Fontenay-sous-Bois, France). Bacterial suspensions were pelleted, resuspended in 40 ml of 30 mM Tris-HCl (pH 9.0), disrupted by sonification (three times at 30 W for 1 min using a Vibra Cell 75022 Phospholyser from Bioblock, Illkirch, France), and centrifuged for 1 h at 48,000 × *g* at 4°C. The suspension was then ultracentrifuged at 100,000 × *g* for 1 h at 4°C, and the supernatant was dialyzed overnight against 30 mM Tris-HCl (pH 9.0).

This extract was loaded onto a preequilibrated Q-Sepharose column (Amersham Pharmacia Biotech). The enzyme recovered in the flowthrough was dialyzed overnight at 4°C against 50 mM phosphate buffer (pH 6.2). The enzyme fraction was then loaded onto a preequilibrated S-Sepharose column (Amersham Pharmacia Biotech). The enzyme was eluted by a 100-ml linear NaCl gradient (0 to 500 mM) in 50 mM phosphate buffer (pH 6.2). The β-lactamase was eluted at 310 mM NaCl. The fractions containing the highest β-lactamase activity were pooled, concentrated, and dialyzed overnight against 50 mM phosphate buffer (pH 7.0) containing 50 μM $ZnCl_2$ and were stored at −80°C.

Biochemical analysis of β-lactamase. Purified β-lactamase was used for kinetic measurements performed at 30°C in 50 mM sodium phosphate (pH 7.0) containing 50 μM $ZnCl_2$. The rates of hydrolysis were determined with the spectrophotometer ULTROSPEC 2000 (Amersham Pharmacia Biotech). The wavelengths and absorption coefficients of β-lactams have been described previously (21).

K_m and k_{cat} values were determined by analyzing the β-lactam hydrolysis under initial rate conditions using the Eadie-Hoffstee linearization of the Michaelis-Menten equation (1). The K_m values were expressed in micromolars, and k_{cat} values were expressed in seconds^{-1}. In the cases of low K_m values, K_i values were determined with benzylpenicillin as the substrate.

Various concentrations of EDTA and clavulanic acid were preincubated with the enzyme for 3 min at 30°C before testing the rate of imipenem (100 μM) hydrolysis. The 50% inhibitory concentrations (IC_{50}s) of these inhibitors were determined as the concentrations that inhibited hydrolysis activity by 50%. Results were expressed in micromolars.

Purified enzyme and nonpurified β-lactamase extracts of a 100-ml culture of *E. brevis* ASS-1 were subjected to isoelectric focusing analysis as previously described (6). The relative molecular mass of the purified β-lactamase and its purity were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed by Coomassie blue staining or by overlaying the gel with 1 mM nitrocefin solution as previously described (6, 20). Specific activities of nonpurified enzymes from cultures of *E. brevis* ASS-1 and *E. coli* BL21(DE3) (pET-EBR-1) and purified enzyme were determined as previously reported with 100 μM imipenem as the substrate (1). One unit of enzyme activity was defined as the activity that hydrolyzed 1 μM imipenem/min; the total protein content was measured with bovine serum albumin as the standard (Bio-Rad DC protein assay kit).

Induction experiments. *E. brevis* ASS-1 was grown at 37°C with or without cefoxitin (0.5 or 1 μg/ml) as the inducer, as reported previously (1). Briefly, overnight cultures were diluted 1:10 and were grown for 1 h and 30 min in a preincubated TS broth on a rotating shaker at 37°C. Then the cultures were grown for an additional 2 h in the presence of the inducer. Hydrolysis measurements were determined with 100 μM imipenem as the substrate.

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. AF416700.

RESULTS AND DISCUSSION

Cloning experiments and preliminary β-lactamase analysis. Imipenem hydrolysis by a culture extract of *E. brevis* ASS-1 suggested the presence of a carbapenem-hydrolyzing-β-lactamase (specific activity, 0.022 U · mg of protein^{-1} with imipenem as the substrate). Cloning of *Sau*3AI-restricted DNA of *E. brevis* into the *Bam*HI site of the pBK-CMV cloning vector gave 30 recombinant *E. coli* DH10B clones growing on ampicillin-containing plates and harboring plasmids with inserts that ranged from 1.7 to 9 kb. Among them, *E. coli* DH10B (pEBR-1) harbored a recombinant plasmid that possessed an 1.7-kb insert and was retained for further analysis.

Isoelectric focusing analysis revealed that *E. coli* DH10B (pEBR-1) produced a β-lactamase activity with a pI of 8.0 (data not shown). Only one β-lactamase activity with an identical pI was detected from culture extracts of *E. brevis* ASS-1. Induction studies with cefoxitin as a β-lactam inducer failed to detect β-lactamase induction with cultures of *E. brevis* ASS-1.

DNA and protein sequence analysis. DNA sequence analysis of the 1,717-bp insert of pEBR-1 identified an open reading frame (ORF) of 708 bp encoding a 236-amino-acid preprotein. A putative cleavage site was found between the Ala-Phe-Gly and Gln-Ile-Lys motifs (Fig. 1). The overall GC content of this ORF was 31.2%, which lies close to the expected range of the G+C ratio of *E. brevis* genes (31 to 33%) (17).

No plasmid was detected in *E. brevis* ASS-1, and direct conjugation assays failed to transfer any β-lactam resistance marker from *E. brevis* ASS-1 to nalidixic acid-resistant *E. coli* JM109. These negative results did not rule out a plasmid location of *bla*$_{EBR-1}$. However, Southern experiments identified *bla*$_{EBR-1}$ at the chromosomal position of migration using whole-cell DNA of *E. brevis* ASS-1 as a template (data not shown). Additionally, using *Hin*dIII and *Cla*I restriction enzymes that did not cut within the *bla*$_{EBR-1}$ sequence, a 1,425-bp fragment of *Hin*dIII- and *Cla*I-restricted DNA of *E. brevis* ASS-1 hybridized with the internal probe for *bla*$_{EBR-1}$ as expected, further underlining the origin of the cloned fragment.

The comparison of the EBR-1 amino acid sequence with those of other class B β-lactamases (1, 2, 4, 5, 8, 16, 18, 20, 26, 29, 31, 32, 37) revealed 58, 57, and 42% amino acid identity with the most closely related β-lactamases IND-1 and IND-2 from *C. indologenes* (2, 5), CGB-1 from *C. gleum* (4), and BlaB from *C. meningosepticum* (1, 32), respectively.

The six conserved amino acids implicated in the Zn^{2+} or/and water molecule binding (His116, His118, Asp120, His196, Cys221, and His220, according to the BBL numbering of metallo-β-lactamases [12, 35, 37, 40]) were identified in the amino acid sequence of EBR-1 (Fig. 1). Additionally, 18 amino acid residues were conserved in metallo-β-lactamases of subclass B1 (30), to which EBR-1 belongs (Fig. 1).

Another ORF was detected in the same orientation and downstream of *bla*$_{EBR-1}$. It encoded a 230-amino-acid protein that did not share significant identity with any amino acid sequence available in the GenBank database.

Susceptibility testing. *E. brevis* ASS-1 was resistant or of intermediate susceptibility to amino- and carboxy-penicillins and to narrow-spectrum cephalosporins, susceptible to expanded-spectrum cephalosporins, and resistant to meropenem (Table 1). MICs of β-lactams were not lowered by the addition

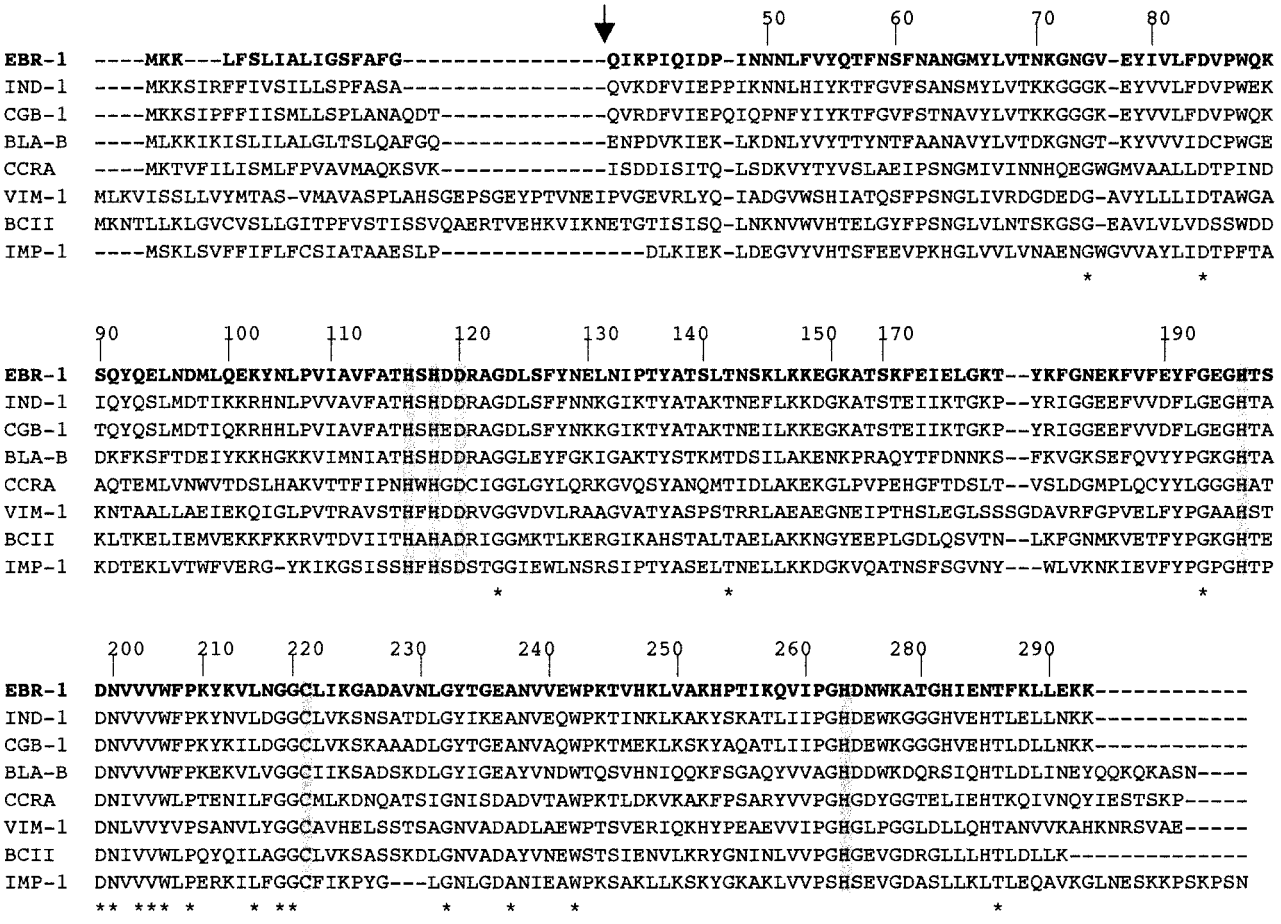

FIG. 1. Comparison of the amino acid sequence of EBR-1 with those of other β-lactamases of subclass B1 of metalloenzymes. The origin of the β-lactamases is as follows: IND-1 from *C. indologenes* 001 (2), CGB-1 from *C. gleum* (4), BlaB from *C. meningosepticum* CIP 6058 (32), CcrA from *Bacteroides fragilis* (31), VIM-1 from *Pseudomonas aeruginosa* VR-143/97 (18), BcII from *Bacillus cereus* 569/H (16), and IMP-1 from *Serratia marcescens* TN9106 (26). The BBL numbering scheme is indicated above the sequences (12). Broken lines indicate gaps introduced in the alignment. The vertical arrow indicates putative cleavage of the peptide leader site for EBR-1. Amino acid residues that may be involved in Zn^{2+} or/and water molecule binding are boxed. Identical amino acids in subclass B1 enzymes are indicated by an asterisk.

of clavulanic acid (Table 1). Once expressed from a recombinant *E. coli* DH10B clone, EBR-1 conferred a similar resistance phenotype to that for BlaB from *C. meningosepticum* expressed in the same *E. coli* DH10B background (1). IND-like β-lactamases conferred a higher degree of resistance to cefotaxime once expressed from *E. coli* DH10B (16 versus 0.5 μg/ml) (5). A slight decreased susceptibility to carbapenems was noticeable for *E. coli* DH10B(pEBR-1), highlighting a potential threat of undetectable dissemination of such metalloenzymes once expressed from *Enterobacteriaceae*. A similar low degree of resistance to carbapenems in *Enterobacteriaceae* has been reported for metalloenzymes of the IND and BlaB series of other flavobacterial species (1, 2, 5, 32).

Biochemical properties of EBR-1. Specific β-lactamase activities of nonpurified extracts from cultures of *E. brevis* ASS-1 and *E. coli* BL21(DE3) (pET-EBR-1) were 0.022 and 0.4 $U \cdot mg\ of\ protein^{-1}$, respectively. This result corresponded to a β-lactamase overexpression in *E. coli* by 18-fold. EBR-1 purification from *E. coli* BL21(DE3) (pET-EBR-1) gave a specific activity of 132 $\mu mol \cdot min^{-1} \cdot mg\ of\ protein^{-1}$ determined with 100 μM imipenem as substrate with a 330-fold purifica-

TABLE 1. MICs of β-lactams for *E. brevis* ASS-1, *E. coli* DH10B(pEBR-1), and reference strain *E. coli* DH10B

β-Lactam(s)	MIC (μg/ml)		
	E. brevis ASS-1	*E. coli* DH10B (pEBR-1)	*E. coli* DH10B
Amoxicillin	64	>512	2
Amoxicillin-CLA[a]	64	>512	2
Ticarcillin	64	>512	2
Piperacillin	2	8	1
Cephalothin	32	32	2
Cefuroxime	32	128	4
Ceftazidime	2	0.5	0.06
Cefotaxime	4	0.5	0.06
Cefepime	0.5	0.12	0.06
Cefpirome	0.25	0.12	0.06
Ceftriaxone	4	0.25	0.06
Cefoxitin	2	8	4
Moxalactam	2	1	0.12
Aztreonam	2	0.12	0.12
Imipenem	8	0.5	0.06
Meropenem	16	0.25	0.06

[a] CLA, clavulanic acid at fixed concentration of 2 μg/ml.

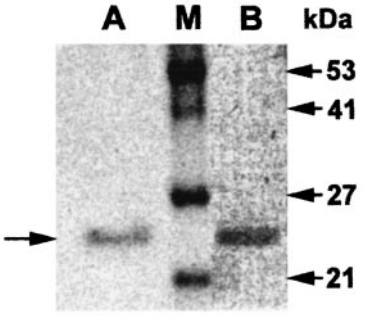

FIG. 2. SDS-PAGE analysis of the purified β-lactamase EBR-1 from a culture of *E. coli* BL21(pET-EBR-1). Lane A, Coomassie blue staining. The horizontal arrow on the left side indicates the migration position of β-lactamase EBR-1. Lane M, size of relative molecular masses expressed in kilodaltons is indicated on the right. Lane B, zymogram of EBR-1.

tion factor. The total quantity of purified β-lactamase was 45 U, with a 12% purification yield. Its purity was estimated to be ca. 90% by SDS-PAGE analysis (Fig. 2).

The mature protein for EBR-1 had a relative molecular mass experimentally determined to be ca. 25 kDa (Fig. 2), which correlated with its calculated molecular mass (24.8 kDa).

Catalytic efficiency values (k_{cat}/K_m) of EBR-1 for penicillins were high, ranging from 760 to 2,500 $s^{-1} \cdot mM^{-1}$ and similar to those obtained for IND-2, BlaB, and GOB-1 (Table 2). Cephalothin, cefuroxime, and ceftriaxone were good substrates for EBR-1 due to high affinity for these substrates (low K_m values). The hydrolysis spectrum of EBR-1 encompassed cefotaxime, ceftazidime, and cefpirome despite low catalytic efficiencies.

TABLE 2. Kinetic parameters of EBR-1 from *E. coli* BL21(DE3) compared with those of IND-2 from *C. indologenes* CIP 101026 (5), BlaB from *C. meningosepticum* CIP 6058 (32), and GOB-1 from *C. meningosepticum* PINT (1)[d]

β-Lactam	EBR-1 parameters			k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	IND-2	BlaB	GOB-1
Benzylpenicillin	115	47	2,500	4,600	8,800	1,900
Amoxicillin	640	690	900	700	—[a]	350
Ticarcillin	226	300	750	—	—	520
Piperacillin	410	490	850	2,000	—	1,700
Cephalothin	6	31	200	1,200	—	700
Cephaloridine	2	440	5	—	480	—
Cefuroxime	0.5	1[b]	350	—	—	1,000
Ceftriaxone	3	7.5[b]	400	—	—	—
Ceftazidime	>2.5	>1,000	2	5	—	800
Cefotaxime	1	50	20	900	220	850
Cefepime	<0.01	ND[c]	ND	<0.001	—	200
Cefpirome	0.5	370	2	—	—	—
Cefoxitin	0.2	140	2	50	250	250
Moxalactam	2	620	2	—	—	130
Imipenem	190	782	250	600	950	700
Meropenem	>190	>2,000	100	200	—	5,500
Aztreonam	<0.1	ND	ND	ND	ND	ND

[a] —, not available.
[b] In cases of low K_m values, K_i values were determined with benzylpenicillin as the substrate.
[c] ND, not determinable.
[d] Data are means of three independent measurements. Standard deviations were within 15%.

As for all metallo-β-lactamases, the monobactam aztreonam was not hydrolyzed by EBR-1 (9). It was like BlaB and IND-2 β-lactamases from *C. meningosepticum* and *C. indologenes* (5, 32), respectively, but unlike the plasmid-mediated VIM- and IMP-type β-lactamases (19, 29). EBR-1 had high k_{cat} values for imipenem with a high catalytic efficiency (k_{cat}/K_m; 250 $s^{-1} \cdot mM^{-1}$). These kinetic parameters showed that EBR-1 was a member of functional group 3a of the Bush classification for metallo-β-lactamases (9). This group includes most of the class B β-lactamases responsible for intrinsic or acquired resistance to carbapenems. As a zinc-dependent protein, EBR-1 activity was inhibited by EDTA (IC_{50} = 70 μM) but not by class A β-lactamase inhibitors, such as clavulanate (IC_{50} > 1 mM).

Conclusion. In addition to GOB, BlaB, IND, and CGB of other flavobacterial species, we report a novel metalloenzyme from the same flavobacterial reservoir, which constitutes the most important source of known class B β-lactamase genes. Identification of EBR-1 adds to the diversity of class B enzymes of this reservoir. Class B enzymes are the only β-lactamases produced by *C. indologenes* and *E. brevis*. Most of the natural producers of class B enzymes also produce class A β-lactamases, such as *C. meningosepticum* (6), *C. gleum* (3), *Stenotrophomonas maltophilia* (38), or class D β-lactamases, such as *Legionella gormanii* (11) and *Aeromonas* spp. (39). The reason why several environmental gram-negative aerobes produce multiple β-lactamases with complementary or partially overlapping substrate profiles remains to be determined.

EBR-1 is distantly related to the plasmid-mediated class B β-lactamases increasingly reported worldwide in clinically significant gram-negative rods (19). Finally, identification of the wide-spectrum hydrolysis profile of EBR-1 underlines that therapies that include β-lactam antibiotics for treating clinical infections due to *Flavobacteriaceae* should be precautionary.

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