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Sfh-I, a Subclass B2 Metallo-β-Lactamase from a *Serratia fonticola* Environmental Isolate

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An environmental isolate of *Serratia fonticola* resistant to carbapenems was shown to contain a genetic determinant encoding a metallo-β-lactamase of the subclass B2. The Sfh-I enzyme exhibits some divergence from the previously characterized enzymes of this subclass. This is the first example of a naturally occurring metallo-β-lactamase in *Enterobacteriaceae*.

Metallo-β-lactamases (MBLs) are enzymes that show carbapenemase activity (3) and require a zinc cofactor (16). The environmental microbiota is presumed to constitute an important reservoir of these harmful genetic determinants. A survey study concerning the bacterial populations of untreated drinking waters in the region of Trás-os-Montes (northeastern Portugal) revealed the presence of bacterial isolates resistant to carbapenems. One isolate had a high level of resistance to imipenem, with a MIC of 32 μg/ml. For identification at the species level, the 16S rRNA gene was amplified with universal primers (Table 1) and sequenced. The sequence clearly assigns the strain to the species *Serratia fonticola*. This result agrees with the identification obtained with the API-32GN System (BioMérieux, Marcy l'Etoile, France). The strain was designated *S. fonticola* UTAD54. Determination of the MICs of antibiotics with E-test gradient strips (AB Biodisk, Solna, Sweden) indicated resistance to penicillins, cephalothin, and cefuroxime, antibiotics to which other strains of the same species are susceptible (Table 2).

A shotgun cloning approach was used to assess the genetic determinant of imipenem resistance. For that purpose, the total DNA of *S. fonticola* UTAD54 was partially digested with *Sau*3AI and the resulting fragments were ligated to the *Escherichia coli* vector pBGS19$^+$ (13) linearized with *Bam*HI. After transformation on *E. coli* XL2 Blue (Stratagene, La Jolla, Calif.), the resulting genomic library was amplified by cultivation in the presence of kanamycin and stored as several aliquots. Small volumes of the genomic library were plated on LA medium supplemented with imipenem at a concentration of 3 μg/ml. One clone containing a recombinant plasmid carrying an insert of 5 kb was chosen for further characterization. The recombinant plasmid was named pSF14. The susceptibility of the recombinant clone to several antibiotics was tested (Table 2). In contrast to untransformed *E. coli*, the recombinant strain showed resistance to carbapenems. *E. coli* XL2 Blue(pSF14) exhibited decreased in vitro susceptibility to several β-lactams in comparison with XL2 Blue. The relative decrease in susceptibility was greater with penicillins and carbapenems than with cephalosporins. Susceptibility to aztreonam was not affected. *S. fonticola* UTAD54 exhibited low overall susceptibility to β-lactams (Table 2).

With a primer-walking strategy, the complete nucleotide sequence of both strands of the insert contained in pSF14 was determined. Analysis of the nucleotide sequence revealed the presence of an open reading frame contained in a short region of approximately 1 kb, with a moles percent G+C content of 42.5%. This value is outside the range of the moles percent G+C content attributed to the genome of *S. fonticola* (48.8 to 52.5%) (6). The open reading frame encodes a polypeptide of

TABLE 1. Oligonucleotide primers used in this study

PCR target and primer	Sequence 5′-3′	Reference
16S rRNA gene		
rD1	AAG GAG GTG ATC CAG CC	17
fD1	AGA GTT TGA TCC TGG CTC AG	17
BSF517/17	GCC AGC AGC CGC GGT AA	18
Class I integrons		
Int 1F	GGC ATC CAA GCA GCA AG	7
Int 1B	AAG CAG ACT TGA CCT GA	7
fonA-like genes		
FON_f	GAT CGA TAC CGC CGA TAA TTC GC	This study
FON_r	ACG GCG ATA TCG TTA GTG GTA CC	This study
Sfh-I-encoding gene		
SFH-I_f	GAT CTC GAG ATG GCT TCT GAA AAA AAC TTA ACG	This study
SFH-I_r	GAT GAA TTC TTA CTT AGG CGC CTT CTC AAG CAG	This study

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TABLE 2. MICs of antibiotics for *S. fonticola* LMG7887 (type strain), *S. fonticola* CIP103580, *S. fonticola* UTAD54, *E. coli* XL2 Blue(pSF14), and *E. coli* XL2 Blue (reference strain)

Antibiotic(s)[a]	MIC (μg/ml)				
	S. fonticola LMG7882^T	*S. fonticola* CIP103580	*S. fonticola* UTAD54	*E. coli* XL2 Blue(pSF14)	*E. coli* XL2 Blue
Amoxicillin	12	64	>256	4	4
Amoxicillin-CLA	0.75	1.5	12	3	3
Piperacillin	1.0	2.0	32	0.75	0.75
Piperacillin-TZB	0.19	1.0	0.38	0.5	0.5
Ticarcillin-CLA	1.9	1.5	1.5	1.5	0.75
Mecillinam	ND[b]	ND	>256	0.75	0.75
Cephalothin	24	ND	>256	24	6
Cefoxitin	6	6	6	4	4
Cefuroxime	16	48	>256	2	2
Cefotaxime	0.125	0.50	2	0.064	0.064
Ceftazidime	0.023	0.125	0.064	0.19	0.19
Cefepime	0.016	0.023	0.032	0.032	0.032
Cefpirome	0.016	ND	0.032	0.016	0.016
Imipenem	0.25	0.38	>32	>32	0.125
Meropenem	0.016	0.047	>32	>32	0.008
Aztreonam	0.094	0.25	1.5	0.19	0.19

[a] CLA, clavulanic acid; TZB, tazobactam.
[b] ND, not done.

253 amino acids. A BLAST (1) search showed that the deduced amino acid sequence displays similarity to β-lactamases belonging to molecular class B (2). The highest similarity score was obtained with the CphA enzyme of *A. hydrophila* (8). This new MBL was named Sfh-I, and its genetic determinant was named $bla_{\text{Sfh-I}}$.

The deduced amino-terminal sequence of the protein exhibits features typical of prokaryotic signal peptides targeting protein secretion into the periplasmic space via the general secretory pathway. According to known patterns (10), the most likely cleavage site is located between the amino acids at positions 21 and 22, which are alanine and serine, respectively. The theoretical molecular masses of the complete and mature forms of the Sfh-I protein are 28.50 and 26.13 kDa, respectively.

As shown in Fig. 1, the amino acid sequence of Sfh-I could be aligned with the sequences of the subclass B2 enzymes without introducing major gaps. The exception is a short insertion sequence of four amino acids (Glu-Gln-Gly-Glu) located at position 252 according to the BBL numbering scheme (5). The Sfh-I sequence retains four invariant residues shared by the other class B β-lactamases (His-118, Asp-120, His-196,

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ImiS   MMKGWIKCGL AGAVVLMASF WGGSVRAAGM SLTQVSGPVY VVEDNYYVQE NSMVYFGAKG  60
CphA   MMKGWMKCGL AGAVVLMASF WGGSVRAAGM SLTQVSGPVY VVEDNYYVQE NSMVYFGAKG  60
Sfh-I  -MN--IKY-L FTAVTFLLIA CESMASEKNL TLTHFKGPLY IVEDKEYVQE NSMVYIGTDG  56
        *   .*  *   **  .             . .**   **.* .***  **** ***** *  *

BBL n°                                    116 120
ImiS   VTVVGATWTP DTARELHKLI KRVSRKPVLE VINTNYHTDR AGGNAYWKSI GAKVISTRQT 120
CphA   VTVVGATWTP DTARELHKLI KRVSRKPVLE VINTNYHTDR AGGNAYWKSI GAKVVSTRQT 120
Sfh-I  ITIIGATWTP ETAETLYKEI RKVSPLPINE VINTNYHTDR AGGNAYWKTL GAKIVATQMT 116
       .*..******  **  * * *  ***  *. * ********** ********.* ***.. *  *
                                       z z z

BBL n°                                                              196
ImiS   RDLMKSDWAE IVAFTRKGLP EYPDLPLVLP NVVHEGDFTL QEGKLRAFYL GPAHSPDGIF 180
CphA   RDLMKSDWAE IVAFTRKGLP EYPDLPLVLP NVVHDGDFTL QEGKVRAFYA GPAHTPDGIF 180
Sfh-I  YDLQKSQWGS IVNFTRQGNN KYPNLEKSLP DTVFPGDFNL QNGSIRAMYL GEAHTKDGIF 176
        ** ** *   ** *** *    **.*   ** . *  *** * * * .** *  * **. ****
                                                            z

BBL n°              221                             252          263
                                                    abcd
ImiS   VYFPDQQVLY GNCILKEKLG NLSFADVKAY PQTLERLKAM K----LPIKT VVGGHDSPLH 236
CphA   VYFPDEQVLY GNCILKEKLG NLSFADVKAY PQTLERLKAM K----LPIKT VIGGHDSPLH 236
Sfh-I  VYFPAERVLY GNCILKENLG NMSFANRTEY PKTLEKLKGL IEQGELKVDS IIAGHDTPIH 234
       **** . *** ******* ** ******   * * ****** .      * . . .. ***.***
                   z                                                 z

ImiS   GPELIDHYEA LIKAASQS 254
CphA   GPELIDHYEA LIKAAPQS 254
Sfh-I  DVGLIDHYLT LLEKAPK- 253
          *****   *.  *
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FIG. 1. Alignment of the amino acid sequences of MBLs included in subclass B2. Residues conserved in the three enzymes are indicated by asterisks; dots indicate functionally homologous residues. The BBL numbering scheme is used for residues conserved in MBLs. Amino acids presumptively involved in binding to zinc are indicated by the letter z.

TABLE 3. Pairwise percent amino acid identities between Sfh-I and other class B β-lactamases

β-Lactamase (accession no.)	% Identity between amino acid sequences[a]											
	IND-1[b]	BlaB	CcrA	Bc-II	VIM-1	IMP-1	SPM-1	ImiS	CphA	Sfh-I	L1	CAU-1
BlaB (X96858)	43.1											
CcrA (M63556)	28.5	25.3										
Bc-II (M11189)	33.5	32.9	29.7									
VIM-1 (Y18050)	25.5	23.3	27.3	33.1								
IMP-1 (S71932)	28.0	26.9	28.9	27.2	22.6							
SPM-1 (CAD37801)	25.1	23.7	18.5	22.2	20.3	28.5						
ImiS (Y10415)	21.8	21.3	18.9	21.0	20.3	18.3	22.8					
CphA (X57102)	22.2	21.3	20.5	21.8	20.7	18.7	23.6	96.5				
Sfh-I	23.4	20.5	19.3	18.3	16.9	17.9	15.4	52.0	53.5			
L1 (X75074)	10.9	11.2	10.8	12.5	14.7	12.2	10.9	11.0	12.2	12.6		
CAU-1 (AJ308331)	14.2	11.6	12.4	13.2	13.5	13.4	12.7	14.6	15.0	14.6	28.3	
THIN-B (AJ250876)	29.1	10.8	14.1	13.6	15.0	13.8	10.5	13.4	13.8	11.5	32.1	29.4

[a] Identity values are expressed as percentages calculated with the complete amino acid sequences, including the signal peptides. The values were derived by dividing the number of perfect matches by the length of the shorter sequence, excluding gaps, by a multiple-alignment method.
[b] The accession number of IND-1 is AF099139.

and His-263 [BBL numbering]). The motif His-X-His-X-Asp (positions 116 to 120 [BBL consensus]), present in the MBLs belonging to subclasses B1 and B3, is changed to the related sequence Asn-Tyr-His-Thr-Asp, a distinctive feature of subclass 2 MBLs. This motif is presumed to be involved in the coordination of the two zinc ions found in the active site of these enzymes. The amino acids Asp-120, Cys-221, and His-263 (consensus BBL), which are presumably involved in the binding of the second zinc ion in the enzymes of subclass B2, are conserved in Sfh-I.

Pairwise alignment (14) of the sequence of Sfh-I with those of other MBLs confirmed the closest relationship of the new enzyme to the subclass B2 enzymes. In fact, Sfh-I displays 53.8% identity of residues with CphA from *A. hydrophila* and 52.2% identity with ImiS from *Aeromonas veronii* (Table 3). Identity to subclass 1 enzymes is between 17.9% (IMP-1) and 23.4% (IND-1). Comparison to the newly characterized MBL SPM-1 from *Pseudomonas aeruginosa* (15) revealed an identity of 15.4%. Lower identity percentages are found when comparisons are made with subclass B3 enzymes: the range is 11.5% (THIN-B) to 14.6% (CAU-1). These results are consistent with the classification of Sfh-I as a member of subclass B2.

Specific oligonucleotide primers were designed that amplify the $bla_{\text{Sfh-I}}$ gene of strain UTAD54 (Table 1). With DNA from *S. fonticola* strains LMG 7882$^{\text{T}}$, DSM 9663, and CIP 103850, no amplification products were obtained. The amplicon from *S. fonticola* UTAD54 labeled with digoxigenin (Roche Molecular Biochemicals, Indianapolis, Ind.) was used to probe Southern blots of digested and undigested DNAs isolated from the same strains. This approach did not detect $bla_{\text{Sfh-I}}$-like sequences in strains LMG 7882$^{\text{T}}$, DSM 9663, and CIP 103850 (data not shown). The probe clearly hybridizes to the band representing the undigested chromosomal DNA of UTAD54. With different extraction methods, no DNA of plasmid origin was detectable on ethidium bromide-stained agarose gels after electrophoresis, suggesting that $bla_{\text{Sfh-I}}$ is a chromosomal gene. The presence of integrons was also checked with a primer set designed to anneal to the 5′ and 3′ conserved sequences of flanking regions containing integrated gene cassettes (Table 1). Amplifications by PCR did not produce DNA fragments (data not shown).

The high MICs of antibiotics to which the recombinant *E. coli* XL2 Blue(pSF14) is susceptible (e.g., amoxicillin and narrow- and expanded-spectrum cephalosporins) indicated the existence of additional β-lactam resistance mechanisms in UTAD54. The presence of class A β-lactamases in a clinical isolate of *S. fonticola* was previously reported (11); later, a gene encoding a β-lactamase 96% homologous to that one (SFO-1; GenBank accession number AB003148) was detected in *Enterobacter cloacae* (9). Also, six nucleotide sequences have been deposited in the GenBank database (accession numbers AJ251239 to -44) that represent six variants of the *fonA* gene for a class A β-lactamase present in different *S. fonticola* strains. The *fonA1* variant is from the type strain. Specific primers based on the nucleotide sequences of those β-lactamases were designed (Table 1). Amplification by PCR resulted in a DNA fragment with the expected size (approximately 550 bp) in all of the *S. fonticola* strains studied, including UTAD54. The MBL-producing bacteria are known to possess other β-lactamase-encoding determinants, and that is also the case with *S. fonticola* UTAD54.

The environmental microbiota can constitute an important reservoir of genetic determinants of antibiotic resistance. Recently, MBLs were detected in bacteria that are not of clinical origin, as is the case with THIN-B from *Janthinobacterium lividum* (12) and CAU-1 from *Caulobacter crescentus* (4). In this work, we have identified a putative MBL-encoding gene in an environmental isolate that is a member of the family *Enterobacteriaceae*.

Nucleotide sequence accession numbers. The nucleotide sequence of the 16S rRNA gene of *S. fonticola* UTAD54 was deposited in the GenBank database and assigned accession number AY236502. The $bla_{\text{Sfh-I}}$ nucleotide sequence was deposited in the GenBank database and assigned accession number AF197943.

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