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Biochemical Characterization of CPS-1, a Subclass B3 Metallo-β-Lactamase from a *Chryseobacterium piscium* Soil Isolate

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CPS-1 is a subclass B3 metallo-β-lactamase from a *Chryseobacterium piscium* isolate collected from soil, showing 68% amino acid identity to the GOB-1 enzyme. CPS-1 was overproduced in *Escherichia coli* Rosetta (DE3), purified by chromatography, and biochemically characterized. This enzyme exhibits a broad-spectrum substrate profile, including penicillins, cephalosporins, and carbapenems, which overall resembles those of L1, GOB-1, and acquired subclass B3 enzymes AIM-1 and SMB-1.

Metallo-β-lactamases (MBLs) are among the most clinically relevant β-lactamases because of their broad-spectrum activity against most β-lactams, including carbapenems, and lack of susceptibility to β-lactamase inhibitors available for clinical use (e.g., clavulanate, sulbactam, tazobactam, and avibactam) (1). MBLs require a metal cofactor for β-lactam hydrolysis and are inhibited by EDTA (2, 3). They are classified functionally as group 3 (2) and structurally as class B (4) enzymes. According to structural classification, they are further divided into subclass B1, B2, and B3 enzymes (3, 4). MBL-encoding genes were first identified as resident chromosomal resistance determinants in environmental bacteria and, hence, not considered a public health threat (5). However, MBL-encoding genes associated with mobile genetic elements have subsequently emerged among major Gram-negative pathogens (6, 7), posing a significant problem in the treatment of Gram-negative infections (8).

Genus *Chryseobacterium* comprises species living in the environment that can occasionally behave as opportunistic pathogens (9). Some species of this genus, such as *Chryseobacterium gleum* and *Chryseobacterium indologenes*, have been shown to produce MBLs as resident enzymes. For instance, *C. gleum* produces CGB-1, a subclass B1 MBL presenting low affinity for carbapenems (10), while *C. indologenes* produces IND-type (IND-1 to IND-15) subclass B1 MBLs exhibiting heterogeneous structural and biochemical properties (11, 12).

We recently discovered CPS-1 (GenBank accession number AJP77054.1), a new subclass B3 MBL from a *Chryseobacterium piscium* strain (Stok-1) isolated from soil in Warwickshire, United Kingdom (13). In this article, we report the structural features and biochemical properties of CPS-1 compared to those of previously described MBLs and of putative MBLs encoded by genomes of *Chryseobacterium* species available in the Integrated Microbial Genomes database.

CPS-1 shared the highest amino acid (aa) identity with putative MBLs detected in *Chryseobacterium caeni* (81%) (here referred to as CPS-2; GenBank accession number WP_027382699.1) and *Chryseobacterium formosense* (80%) (here referred to as CPS-3; GenBank accession number KFF00120.1) and with the GOB-1 MBL from *Elizabethkingia meningoseptica*, formerly *Chryseobacterium meningosepticum* (68%) (14). CPS-1 appeared to be more distantly related to other subclass B3 enzymes, including FEZ-1 (35% aa identity) from *Legionella* (*Fluoribacter*) *gormanii* (15), BJP-1 (31% aa identity) from *Bradyrhizobium japonicum* (16), and L1 (25% aa identity) from *Stenotrophomonas maltophilia* (17), although it could be aligned with these enzymes without introducing major gaps (Fig. 1). Compared to GOB-1, 92-aa substitutions were detected in the CPS-1 enzyme, including Glu165Lys, His228Lys, and Met221Leu (BBL numbering scheme) (4). Amino acid residues spanning positions 156 to 166 (loop 1) and 220 to 230 (loop 2) are considered to cover the active site groove of subclass B3 enzymes (17, 18). Position 221 is critical for MBL structure and catalysis (19), and the Ser221Met substitution observed in GOB enzymes with respect to nearly all other subclass B3 enzymes has been shown to contribute to enzyme stability due to the hydrophobic nature of Met (19, 20). We hypothesize a similar role for the Leu residue at position 221 in CPS-1, being a Leu hydrophobic amino acid. Similar to CPS-1, CPS-2 and CPS-3 also displayed Met and Leu, respectively, at position 221, indicating that both substitutions can occur among CPS-like enzymes.

The bla_{CPS-1} open reading frame (ORF) was amplified from *C. piscium* Stok-1 genomic DNA with primers containing NdeI (CPS-1F, 5′-GCGCATATGAGAAACCTGACACTTTT-3′) and BamHI (CPS-1R, 5′-CGGGATCCTTATTTTTTCGCTGAATCTT-3′) restriction sites (underlined). The NdeI-BamHI-digested bla_{CPS-1} ORF was cloned into the corresponding sites in the pET-9a expression vector (Merck Millipore, Germany) to produce the recombinant plasmid pET-CPS-1. The cloned insert was subjected to confirmatory sequencing (Macrogen, Republic of Korea) to exclude the presence of mutations introduced during the PCR. *Escherichia coli* Rosetta (DE3) cells (Merck Millipore, Germany) were transformed with pET-CPS-1 by electroporation

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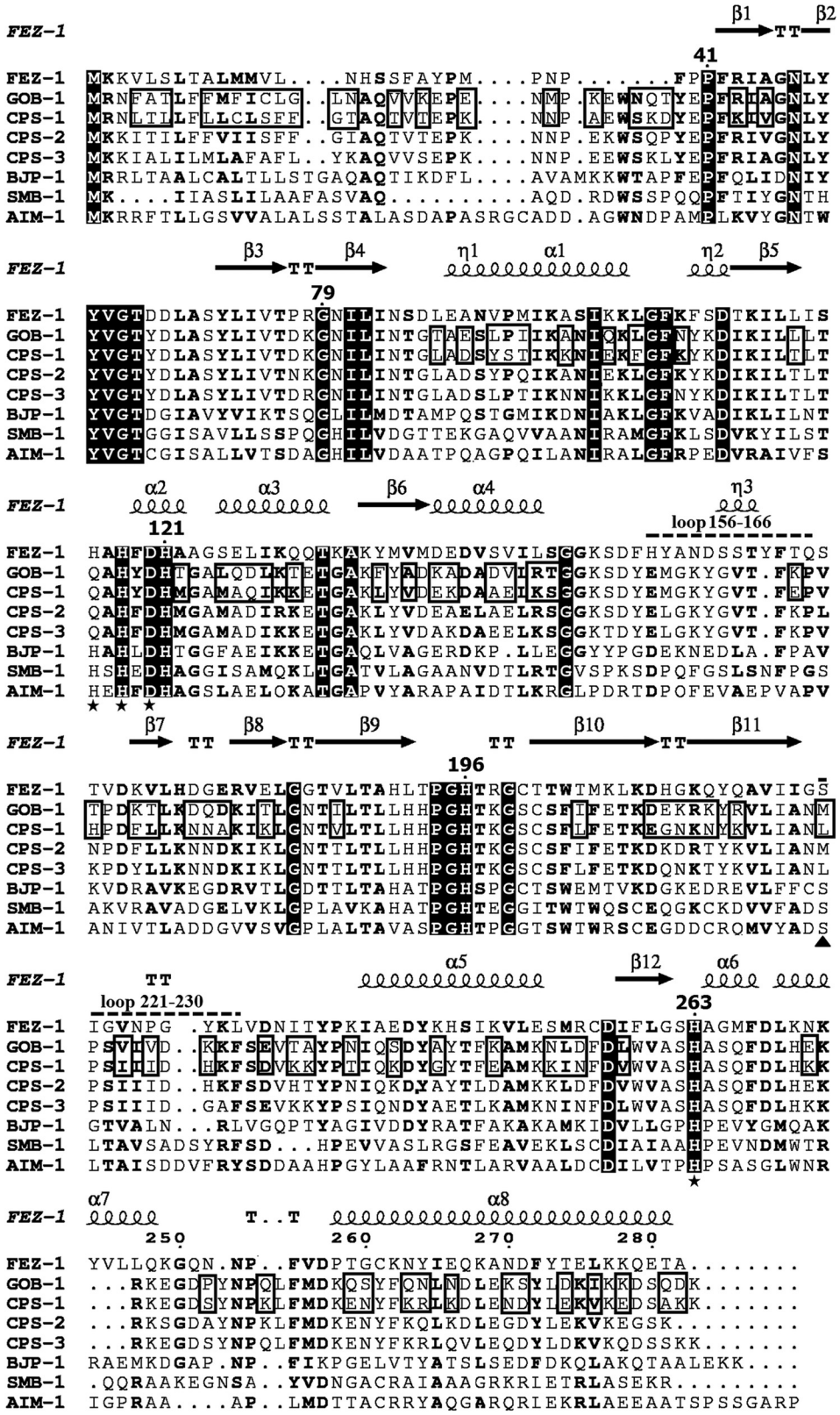

FIG 1 Amino acid alignment of CPS-1 (GenBank accession number AJP77054.1), CPS-2 (GenBank accession number WP_027382699.1), CPS-3 (GenBank accession number KFF00120.1), GOB-1 (GenBank accession number AAF04458), BJP-1 (NP_772870), AIM-1 (GenBank accession number AM998375), and SMB-1 (GenBank accession number AB636283) with the secondary structure of FEZ-1 (GenBank accession number CAB96921). Stars, metal binding residues; triangle, position 221; boxes, residue differences between CPS-1 and GOB-1; broken lines, loops spanning the active site groove of subclass B3 MBLs. The figure was made by using ESPrint (29).

TABLE 1 Kinetic parameters of purified CPS-1 enzyme for the hydrolysis of different β-lactams, in comparison with those reported in the scientific literature for other subclass B3 metallo-β-lactamases (MBLs)

β-Lactam substrate	CPS-1			k_{cat}/K_m ($M^1 \cdot s^{-1}$) for subclass B3 metallo-β-lactamase[a]:					
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^1 \cdot s^{-1}$)	GOB-1	FEZ-1	BJP-1	L1	AIM-1	SMB-1
Benzylpenicillin	1,200 ± 34	195 ± 16	6.2×10^6	1.87×10^6	1.1×10^5	1.3×10^5	5.5×10^6	2.6×10^7	-[b]
Ampicillin	3,000 ± 86	393 ± 26	7.6×10^6	3.5×10^5	1.1×10^4	1.9×10^4	1.9×10^6	1.4×10^6	2.4×10^6
Ticarcillin	>700	>580	1.2×10^6	5.2×10^5	1.3×10^4	ND	9×10^5	-	-
Temocillin	>8	>670	1.9×10^4	-	1.3×10^4	ND	-	-	-
Cephalothin	63 ± 2	50 ± 6	1.3×10^6	6.7×10^5	2.5×10^6	5.8×10^5	1.5×10^6	1.4×10^7	1.9×10^6
Cefoxitin[c]	20 ± 0.8	5 ± 0.3	4.0×10^6	2.5×10^5	2.7×10^5	7.1×10^4	6.7×10^5	5.7×10^6	1.5×10^6
Cefuroxime	46 ± 1	33 ± 3	1.4×10^6	9.8×10^5	6.6×10^6	5×10^5	4.1×10^5	9.9×10^6	1.4×10^6
Ceftriaxone	170 ± 5	116 ± 9	1.5×10^6	-	-	-	-	-	-
Ceftazidime	>80	>400	2.0×10^5	7.6×10^5	4.0×10^3	4.3×10^3	1.8×10^5	4.9×10^4	7.7×10^4
Cefotaxime	130 ± 10	76 ± 12	1.7×10^6	8.5×10^5	$>2.4 \times 10^6$	1.4×10^5	8.8×10^5	1.2×10^7	8.9×10^5
Cefepime	15 ± 1	184 ± 32	8.2×10^4	2.0×10^5	6.0×10^3	2.0×10^2	2.5×10^4	1.6×10^5	3.6×10^3
Imipenem	150 ± 7	26 ± 4	5.8×10^6	6.6×10^5	2.0×10^5	6.0×10^4	7.3×10^5	1.7×10^7	3.9×10^6
Meropenem	180 ± 7	51 ± 5	3.5×10^6	5.3×10^6	5.0×10^5	8.3×10^5	4.5×10^6	6.8×10^6	4.2×10^6
Ertapenem	62 ± 2	72 ± 7	8.6×10^5	-	-	-	-	-	-
Doripenem	300 ± 8	45 ± 3	6.7×10^6	-	-	-	-	-	-
Aztreonam	<0.08	ND	ND	ND	ND	ND	-	ND	ND

[a] GOB-1, FEZ-1, BJP-1, and L1 are resident MBLs produced by *Elizabethkingia meningoseptica* (14), *Legionella gormanii* (15), *Bradyrhizobium japonicum* (16), and *Stenotrophomonas maltophilia* (23, 24), respectively. AIM-1 and SMB-1 are acquired subclass B3 metallo-β-lactamases produced by *Pseudomonas aeruginosa* (25) and *Serratia marcescens* (26) clinical isolates, respectively. ND, data not determined.

[b] -, data not available.

[c] K_m was determined as an inhibition constant (K_i) by using 145 μM imipenem as reporter substrate.

(2.5 kV, 200 Ω, 25 μF; Bio-Rad Gene Pulser II). To produce CPS-1 enzyme, *E. coli* Rosetta (DE3) (pET-CPS-1) was grown in 1 liter of ZYP-5052 medium at 37°C for 8 h. Harvested cells (centrifugation at 8,000 × *g* for 45 min at 4°C) were resuspended in 50 ml of 10 mM HEPES buffer containing 50 μM $ZnSO_4$ (pH 7.5) and lysed by sonication (Labsonic L sonicator, B. Braun, Germany). The cleared lysate obtained by centrifuging the lysed cells at 130,000 × *g* for 50 min was loaded on a CM Sepharose fast flow column (GE Healthcare, Sweden) preequilibrated with 10 mM HEPES buffer containing 50 μM $ZnSO_4$ (pH 7.5). Proteins were eluted in 10 mM HEPES buffer containing 50 μM $ZnSO_4$ (pH 7.5) and 0.15 M NaCl. The β-lactamase activity was monitored spectrophotometrically using 150 μM imipenem (Fresenius Kabi, Italy) as the substrate, as described previously (7). Fractions showing high specific activities were pooled and concentrated 30-fold by ultrafiltration (Merck Millipore). The concentrated sample was then loaded on a Superdex 75 prep-grade column (GE Healthcare, Sweden) preequilibrated with 10 mM HEPES buffer containing 50 μM Zn SO_4 and 150 mM NaCl. Proteins were eluted in the same solution as that used for preequilibration. CPS-1 concentration was determined by the Bradford protein assay (Bio-Rad Laboratories, Germany), and purity was estimated after electrophoresis on sodium dodecyl sulfate-polyacrylamide gel (Life Technologies, CA, USA). The purification procedure yielded ~5 mg of >95% pure enzyme per liter of culture. The purified CPS-1 preparation was subjected to electron spray-ionization mass spectrometry (ESI-MS), which revealed the presence of a main protein species with a molecular mass equal to 31,216 Da, in excellent agreement with the CPS-1 theoretical mass obtained after the cleavage of an 18-residue NH_2-terminal signal peptide (theoretical mass, 31,213.6 Da). Kinetic parameters for the hydrolysis of β-lactam substrates were determined spectrophotometrically in 50 mM HEPES buffer containing 50 μM $ZnSO_4$ and 20 μg/ml bovine serum albumin (BSA) (pH 7.5) at 30°C by Hanes-Wolff linearization of the Michaelis-Menten equation. The values for changes in the extinction coefficients of the substrates used were described by Laraki et al. (21). The K_m for cefoxitin was determined as the inhibition constant as previously described by using 145 μM imipenem as a reporter substrate (22).

CPS-1 exhibited broad-spectrum activity toward different classes of β-lactam antibiotics, with a catalytic efficiency (k_{cat}/K_m) of $>10^6$ $M^1 \cdot s^{-1}$ for penicillins (ampicillin, benzylpenicillin, ticarcillin), cephalothin, some oxyimino-cephalosporins (cefuroxime, ceftriaxone, cefotaxime), cephamycins (cefoxitin), and carbapenems (imipenem, meropenem, doripenem) (Table 1). Substrate turnover rates (k_{cat}) and K_m values were generally higher for penicillins than for cephalosporins and carbapenems. Among the tested β-lactams, CPS-1 exhibited better recognition of cefoxitin (low K_m) (Table 1). The k_{cat}/K_m ratio for ceftazidime was 10-fold lower than that for other oxyimino-cephalosporins. Cefepime also represented a poorer substrate for CPS-1, similar to observations for other subclass B3 MBLs, such as L1, THIN-B, FEZ-1, and BJP-1 (15, 16, 23), while GOB-1 hydrolyzed cefepime better than CPS-1 (14). CPS-1 catalytic efficiency for temocillin was comparable to that of FEZ-1 (15), whereas this substrate is not recognized by BJP-1 (16), and data are not available for GOB-1, THIN-B, AIM-1, and SMB-1. Aztreonam was not hydrolyzed by CPS-1, in line with the notion that monobactams are not MBL substrates (24). Despite the overall high sequence similarity between CPS-1 and GOB-1, catalytic efficiencies of CPS-1 for penicillins (except for benzylpenicillin), cephalosporins (except for ceftazidime and cefepime), and imipenem were higher by an order of magnitude than those of GOB-1. Interestingly, CPS-1 showed comparable catalytic efficiencies for most cephalosporins, carbapenems, and penicillins, thus differing from the most closely related subclass B3 enzymes that generally display preferences for a cer-

tain type of β-lactam substrate. For example, GOB-1 hydrolyzes meropenem better than imipenem (14), FEZ-1 hydrolyzes cephalosporins better than penicillins (15), and BJP-1 prefers narrow-spectrum cephalosporins over penicillins (16). A broad-spectrum substrate profile is a feature characteristic of the acquired subclass B3 MBLs known to date, namely, AIM-1 detected in *Pseudomonas aeruginosa* isolates (25) and SMB-1 detected in a *Serratia marcescens* isolate (26). In these enzymes, recognition of β-lactam substrates is likely mediated by the presence of Gln157 in loop 2 (18, 27). AIM-1 hydrolyzes benzylpenicillin, most cephalosporins (cephalothin, cefotaxime, cefuroxime), and imipenem with a catalytic efficiency 1 order of magnitude higher than that of CPS-1, while SMB-1 has catalytic efficiencies comparable to that of CPS-1 for most substrates except for ceftazidime and cefepime, which are hydrolyzed less efficiently by SMB-1 than by CPS-1 (26). The high catalytic efficiency of CPS-1 for ampicillin, cefoxitin, and ceftazidime may account for the high MIC values observed in recombinant *E. coli* TOP10 expressing bla_{CPS-1} from the pZE21MCS vector (64, 64, and 4 μg/ml, respectively) (13). However, the MICs of cefotaxime and meropenem were low (0.5 and 0.094 μg/ml, respectively) despite the high catalytic efficiency observed for these substrates. Apparent discordance between catalytic efficiency and MIC values of different β-lactams has been observed for other subclass B3 MBLs in *E. coli* laboratory strains (28).

In conclusion, CPS-1 is a new member of subclass B3 MBLs with broad substrate specificity, as it is able to efficiently hydrolyze penicillins, cephalosporins, and carbapenems of clinical importance. The broad-spectrum profile of CPS-1 resembles the catalytic efficiencies of AIM-1 and SMB-1, even though CPS-1 is distantly related to these subclass B3 MBLs based on sequence homology.

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