

# Genetic and Biochemical Characterization of CGB-1, an Ambler Class B Carbapenem-Hydrolyzing $\beta$ -Lactamase from *Chryseobacterium gleum*

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*Chryseobacterium gleum* (previously included in the *Flavobacterium* IIb species) is a gram-negative aerobe that is a source of nosocomial infections. An Ambler class B  $\beta$ -lactamase gene was cloned and expressed in *Escherichia coli* from reference strain *C. gleum* CIP 103039 that had reduced susceptibility to expanded-spectrum cephalosporins and carbapenems. The purified  $\beta$ -lactamase, CGB-1, with a pI value of 8.6 and a determined relative molecular mass of ca. 26 kDa, hydrolyzed penicillins; narrow- and expanded-spectrum cephalosporins; and carbapenems. CGB-1 was a novel member of the molecular subclass B1 of metallo-enzymes. It had 83 and 42% amino acid identity with IND-1 from *Chryseobacterium indologenes* and BlaB from *C. meningosepticum*, respectively. Thus, in addition to the previously characterized clavulanic acid-inhibited extended-spectrum  $\beta$ -lactamase CGA-1 of Ambler class A, *C. gleum* produces a very likely chromosome-borne class B  $\beta$ -lactamase.

Metallo- $\beta$ -lactamases of Ambler class B are zinc-dependent enzymes that possess the property of hydrolyzing most  $\beta$ -lactam antibiotics, including carbapenems (7). Some of these enzymes are emerging worldwide in gram-negative pathogens as a result of plasmid- and integron-location of their genes that are of the *bla*<sub>IMP</sub> and *bla*<sub>VIM</sub> series (13, 14, 20, 25). However, several metallo- $\beta$ -lactamases have been reported as a source of intrinsic resistance to carbapenems in bacterial species less frequently isolated in clinical microbiology, such as L-1 from *Stenotrophomonas maltophilia* (27), Bc-II from *Bacillus cereus* 569/H (11), CcrA from *Bacteroides fragilis* (21), CphA from *Aeromonas hydrophila* (15), FEZ-1 from *Legionella gormanii*, (6), and THIN-B from *Janthinobacterium lividum* (22).

*Flavobacteria* species are mostly a source of nosocomial infections (26). Metallo- $\beta$ -lactamases have been identified so far in two *Flavobacterium* species, *Chryseobacterium meningosepticum* and *Chryseobacterium indologenes* (1, 2, 4, 23). *C. meningosepticum* usually expresses two unrelated metallo-enzymes of the BlaB and GOB-like series that share less than 12% amino acid identity (1). *C. indologenes* expresses IND-like enzymes that share less than 45 and 15% amino acid identity with BlaB and GOB-like enzymes, respectively (2, 4).

A search for a potential natural reservoir of acquired carbapenem-hydrolyzing  $\beta$ -lactamases is interesting, particularly in understanding the mechanism(s) of integration of these genes in an integron. Thus, we proceeded to study the  $\beta$ -lactamase content of another *Flavobacterium* species, *Chryseobacterium gleum* that is a phylogenetically related species to *C. indologenes* (10, 12, 28).

From *C. gleum* strain CIP 103039, we have reported a cla-

vanic-acid inhibited Ambler class A expanded-spectrum  $\beta$ -lactamase (ES $\beta$ L), CGA-1 (3). However, since *C. gleum* CIP 103039 had also reduced susceptibility to carbapenems and CGA-1 hydrolyzes weakly these  $\beta$ -lactam group, the aim of our study was to analyze the mechanism sustaining carbapenem resistance in *C. gleum*. We have cloned and identified genetically and biochemically a novel metallo- $\beta$ -lactamase that is closely related to metallo-enzymes of *C. indologenes*.

## MATERIALS AND METHODS

**Bacterial strains.** *C. gleum* reference strain CIP 103039 was from the Institut Pasteur Collection (3). *Escherichia coli* DH10B and nalidixic acid-resistant *E. coli* JM109 were used for cloning and conjugation assays, respectively. All strains were stored at  $-70^{\circ}\text{C}$  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 were obtained in the form of standard laboratory powders and were used immediately after their solubilization. The agents and their sources have been described previously (19). Antibiotic disks were used for routine antibiograms (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France).

MICs of selected  $\beta$ -lactams were determined by an agar dilution technique on Mueller-Hinton plates (Sanofi Diagnostics Pasteur) with an inoculum of  $10^4$  CFU per spot, as described previously (17).

**Cloning and analysis of recombinant plasmids.** Whole-cell DNA of *C. gleum* CIP103039 was extracted as described previously (19). All enzymes used in cloning experiments were from Amersham Pharmacia Biotech (Orsay, France). Fragments from whole-cell DNA partially digested with *Sau*3AI were ligated into *Bam*HI-restricted phagemid pBK-CMV (Stratagene, Amsterdam, The Netherlands), as previously reported (24). Ligations were performed at a 1:5 vector/insert ratio, in 30  $\mu\text{l}$  of a ligation mixture containing 5 U of T4 DNA ligase at  $4^{\circ}\text{C}$  incubated overnight. Recombinant plasmids were transformed by electroporation (Gene Pulser II; Bio-Rad, Ivry-sur-Seine, France) into *E. coli* DH10B electrocompetent cells (Gibco BRL, Life Technologies, Cergy Pontoise, France). Antibiotic-resistant colonies were selected onto amoxicillin (30  $\mu\text{g/ml}$ )- and kanamycin (30  $\mu\text{g/ml}$ )-containing TS agar plates, and their antibiotic resistance patterns were analyzed according to the results of antibiogram performed by disk diffusion.

Recombinant plasmid DNAs were obtained from 100-ml TS broth cultures grown overnight in the presence of amoxicillin (30  $\mu\text{g/ml}$ ) at  $37^{\circ}\text{C}$ . Plasmid DNAs were recovered by using Qiagen (Courtabouef, France) columns. Plasmid mapping was performed after double-restriction analysis (24). Fragment sizes

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were estimated by comparison with the 1-kb DNA ladder (Amersham Pharmacia Biotech). One recombinant *E. coli* strain that had reduced susceptibility to imipenem and harbored recombinant plasmid pCGB-1 with the smallest 2-kb insert was retained for further analysis.

**Plasmid content, conjugation assay, and Southern hybridization.** Extraction of plasmid DNA from *C. gleum* CIP 106039 and direct transfer by conjugation of any  $\beta$ -lactam resistance marker into in vitro-obtained nalidixic acid-resistant *E. coli* JM109 were attempted as previously described (5). Southern hybridization was performed as previously described (24) with whole-cell DNA of *C. gleum* CIP 103039 by using the enhanced chemiluminescence nonradioactive labeling and detection kit (Amersham Pharmacia Biotech) with a 628-bp PCR-obtained probe with primers internal to *bla*<sub>CGB-1</sub> (primer 1: 5'-GCAAACGCCCGGATA CAACAG-3'; primer 2: 5'-TTCCATTCATCATGTCCGGG-3')

**$\beta$ -Lactamase purification.** A culture of *E. coli* DH10B harboring recombinant plasmid pCGB-1 was grown overnight at 37°C in 4 liters of TS broth containing amoxicillin (30  $\mu$ g/ml). Bacterial suspensions were pelleted, resuspended in 60 ml of 30 mM Tris-HCl buffer (pH 8.2), disrupted by sonification (three times at 30 W for 30 s with a Vibra Cell 75022 Phospholyser [Biolock, Illkirch, France]), and centrifuged for 1 h at 48,000  $\times$  g at 4°C. The supernatant ultracentrifuged at 100,000  $\times$  g for 1 h at 4°C and the supernatant was dialyzed overnight against 30 mM Tris-HCl buffer (pH 8.2).

This extract was loaded onto a preequilibrated Q-Sepharose column (Amersham Pharmacia Biotech). The enzyme recovered in the flowthrough and determined qualitatively by nitrocefin hydrolysis (Oxoid, Dardilly, France) was dialyzed overnight at 4°C against 50 mM sodium phosphate buffer (pH 7.0). The  $\beta$ -lactamase extract was then loaded onto a preequilibrated S-Sepharose column (Amersham Pharmacia Biotech) preequilibrated with the same buffer. The enzyme was eluted by a 100-ml linear NaCl gradient (0 to 500 mM) in sodium phosphate buffer (pH 7.0). The  $\beta$ -lactamase was eluted at a concentration of 260 mM NaCl. The fractions containing the highest  $\beta$ -lactamase activities were pooled, dialyzed overnight against 50 mM sodium phosphate buffer (pH 7.0) containing 50  $\mu$ M ZnCl<sub>2</sub> and stored at -80°C. Purity of the enzyme was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (24).

**IEF analysis and N-terminal sequencing.** Purified enzyme preparation from culture of *E. coli* DH10B harboring pCGB-1 and  $\beta$ -lactamase crude extracts from 100 ml-culture of *C. gleum* CIP 103039 were subjected to analytical isoelectric focusing (IEF) on a pH 3.5 to 9.5 ampholine polyacrylamide gel (Ampholin PAG plate; Amersham Pharmacia Biotech) as described previously (4).

In order to determine the cleavage site of the CGB-1 mature protein, the purified enzyme was subjected to an Edman analysis (9) at the laboratory for protein microsequencing at the Pasteur Institute, Paris, France. Purified enzyme and marker proteins were subjected to SDS-PAGE (25 mA, 4 h, room temperature). Proteins were then electrotransferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore) by using the Mini Protean II transfer cell (8 by 7.3 cm; Bio-Rad) in 50 mM Tris-borate buffer (pH 8.7) at room temperature (3.5 V/cm, overnight). The membrane was then rinsed in distilled water and stained with a solution made of 0.1% Coomassie brilliant blue R-250 in methanol and water (50:40 [vol/vol]). The protein band was then excised with a razor blade and allowed to air dry. The amino-terminal sequence of the mature  $\beta$ -lactamase was determined with an automated Edman sequencer on a 473A model gas phase sequencer (Applied Biosystems).

**Kinetic measurements of  $\beta$ -lactamase CGB-1 and induction study.** *C. gleum* CIP103039 crude extract was used to detect imipenem hydrolysis in preliminary experiments. Purified metallo- $\beta$ -lactamase CGB-1 was used for kinetic measurements performed at 30°C in 100 mM sodium phosphate (pH 7.0) containing 50  $\mu$ M ZnCl<sub>2</sub>. Determinations of specific activities of purified enzyme and of  $\beta$ -lactamase extract from *C. gleum* CIP 103039 were performed with 100  $\mu$ M imipenem as a substrate as described previously (1). The total protein content was measured with bovine albumin as the standard (Bio-Rad DC protein assay kit).  $K_m$  and  $k_{cat}$  values were determined with a spectrophotometer ULTROSPEC 2000 (Amersham Pharmacia Biotech) by analyzing the  $\beta$ -lactam hydrolysis under initial rate conditions by using the Eadie-Hofstee linearization of the Michaelis-Menten equation as previously described previously (1).

Various concentrations of EDTA and clavulanic acid were preincubated with the enzyme for 3 min at 30°C before testing the rate of imipenem hydrolysis. Fifty percent inhibitory concentrations (IC<sub>50</sub>) were determined for EDTA and clavulanic acid, and results were expressed in micromolar units. Induction experiments were performed with 1 and 4  $\mu$ g of ceftaxime per ml and 0.1  $\mu$ g of imipenem per ml as inducer with cultures of *C. gleum* CIP 103039 and *E. coli* DH10B(pCGB-1), as previously described (1).

**DNA sequencing and protein analysis.** The cloned DNA fragment of recombinant plasmids pCGB-1 was sequenced on both strands, with an Applied Bio-

systems sequencer (ABI 377). The nucleotide and the deduced protein sequences were analyzed with software available over the Internet as reported previously (3). The relative molecular mass of the  $\beta$ -lactamase expressed by a culture of *E. coli* DH10B(pCGB-1) was estimated by SDS-PAGE analysis, as previously described (5).

**Nucleotide sequence accession number.** The nucleotide sequence and deduced  $\beta$ -lactamase amino acid sequence reported in this work have been assigned to the GenBank and EMBL databases under the accession no. AF339734.

## RESULTS AND DISCUSSION

**Cloning experiments.** Preliminary experiments of imipenem hydrolysis with a  $\beta$ -lactamase extract of *C. gleum* CIP 103039 suggested the presence of a carbapenem-hydrolyzing  $\beta$ -lactamase in addition to a previously characterized class A  $\beta$ -lactamase (3). Forty recombinant *E. coli* DH10B clones harboring plasmids with inserts that varied in size (2.0 to 10 kb) were obtained after shotgun cloning of *Sau3AI*-restricted DNA of *C. gleum* CIP 103039. Among them, 28 clones had decreased susceptibility to ceftazidime and a  $\beta$ -lactam resistance phenotype that was susceptible to clavulanic acid inhibition that was consistent with the presence of the previously characterized class A enzyme CGA-1 (3). Twelve other recombinant *E. coli* clones had a slight decrease of susceptibility to carbapenems. Among them, *E. coli* DH10B(pCGB-1) harboring a recombinant plasmid which possessed the smallest 2.0-kb insert was retained.

**DNA and protein sequence analysis of CGB-1.** DNA sequence analysis of the 2,004-bp insert of pCGB-1 revealed an open reading frame (ORF) (named *bla*<sub>CGB-1</sub>) of 729 bp (from nucleotide 461 to 1,189) encoding a 243-amino-acid preprotein (Fig. 1). A putative cleavage site was found by computer analysis after the Ala-Asn-Ala motif (18). N-terminal sequence analysis of the protein confirmed that the first amino acids residues of the mature protein were QDTQ (Fig. 1). The resulting mature protein contained 223 amino acid residues. The overall G+C content of this ORF was 36%, which lies within the expected range of G+C ratio of the *Chryseobacterium* genes (36 to 38%) (10).

Another truncated ORF was detected on this same insert (nucleotide 1,240 to 2,004) of recombinant plasmid pCGB-1 located downstream and in opposite orientation of *bla*<sub>CGB-1</sub> (Fig. 1). It encoded 254 amino acids of the carboxyl end of a putative protein that possessed 31.5% amino acid identity with the capreomycin acetyltransferase from *Streptomyces capreolus* (accession number no. JC4557). A similar capreomycin resistance gene had been identified downstream and in opposite orientation of the class B  $\beta$ -lactamase gene *bla*<sub>IND-1</sub> from *C. indologenes* 001 (unpublished data) and in *Mycobacterium tuberculosis* genome (accession no. NC 000962). Thus, the *bla*<sub>CGB-1</sub> gene may be part of a cluster of antibiotic resistance genes. A putative DNA sequence, located from nucleotides 1,187 to 1,229 between the stop codon of *bla*<sub>CGB-1</sub> and the stop codon of the putative capreomycin acetyltransferase gene, might play a role as a transcription terminator (Fig. 1).

**Genetic location of *bla*<sub>CGB-1</sub>.** No plasmid was detected in *C. gleum* CIP 103039, and direct conjugation experiments failed to transfer any  $\beta$ -lactam resistance marker from *C. gleum* CIP 103039 to nalidixic acid-resistant *E. coli* JM109. Using an internal probe for *bla*<sub>CGB-1</sub>, Southern hybridization was posi-

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1
65 CCGATGATGCTTTCCTGACCTACCTAAATGACAGAAAACCTCTCCTCAACAGTAAGGTAATAAATATTAAAATTGAAAGTTTGTATTATCGATGACGA
164 TAGAAATTGATGGTGAACCTGAAATTTTAAAGCAAAAAGCTACGGAGAAGATACTCGGTAAGAAATAAATCATTAATAAAGCTGAAAAAACCTAACAGGT
263 TTTTAAAACCTGTTAGGTTTACTATTTTATACCAATCTACACTTACCTGGAAAAATACATTTATCCCTTAAACTGTTAAAAAACGGTTAAACTCAATCTC
362 TTTAAAGTCGAAAGATTTATTTTAAATAGGTTTGCAAAACAATCTGCAGACTTTGTCTTTTCTGACAAATACAACATTAATTATTACTCAAAAAATT

461 ATG AAA AAA AGC ATT CCG TTT TTT ATT ATT TCG ATG TTG CTA AGC CCA TTG GCA AAC GCC CAG GAT ACA CAG GTA
M K K S I P F F I I S M L L S P L A N A Q D T Q V
blaCGB-1 →
536 AGA GAT TTT GTA ATT GAG CCT CAA ATT CAA CCC AAC TTT TAT ATT TAC AAA ACA TTC GGA GTA TTC GGA GGG AAA
R D F V I E P Q I Q P N F Y I Y K T F G V F G G K
611 GAA TAT TCT ACC AAT GCC GTG TAT CTG GTA ACT AAA AAA GGA GTT GTC CTG TTT GAT GTT CCA TGG CAG AAA ACC
E Y S T N A V Y L V T K K G V V L F D V P W Q K T
686 CAA TAC CAA AGT CTG ATG GAT ACA ATT CAA AAA CGT CAT CAC CTT CCG GTT ATA GCT GTA TTT GCT ACC CAT TCT
Q Y Q S L M D T I Q K R H H L P V I A V F A T H S
761 CAT GAA GAC AGA GCG GGA GAT TTA AGC TTT TAT AAT AAG AAA GGA ATC AAA ACC TAC GCT ACA GCA AAA ACC AAT
H E D R A G D L S F Y N K K G I K T Y A T A K T N
836 GAA ATT TTA AAG AAA GAA GGA AAA GCA ACT TCT ACA GAA ATT ATA AAA ACC GGT AAA CCT TAC CGT ATT GGT GGA
E I L K K E G K A T S T E I I K T G K P Y R I G G
911 GAA GAA TTT GTA GTA GAC TTT CTT GGT GAA GGT CAT ACG GCG GAT AAT GTA GTG GTT TGG TTT CCC AAA TAT AAG
E E F V V D F L G E G H T A D N V V V W F P K Y K
986 ATA CTG GAC GGA GGC TGT CTT GTA AAA AGT AAA GCA GCA GCC GAT CTT GGC TAC ACC GGC GAA GCC AAC GTT GCA
I L D G G C L V K S K A A A D L G Y T G E A N V A
1061 CAA TGG CCA AAA ACA ATG GAA AAG TTA AAA TCC AAA TAC GCT CAG GCA ACC CTG ATT ATT CCC GGA CAT GAT GAA
Q W P K T M E K L K S K Y A Q A T L I I P G H D E
1136 TGG AAA GGC GGC GGC CAT GTA GAG CAC ACA CTT GAT CTT TTA AAT AAA AAA TAA ATATTTCGGGCCCGCAAAATGCCGGG
W K G G G H V E H T L D L L N K K *
1217 GCCCGAAATATTFAAACAGCATGTTA ATG ACT GAT ACT TTT TAA AAG CTC TGA TAT TCT TTC AAT TTC TTC CAT TGT ATT AAA
* H S I S K L L E S I R E I E E M T N F
1300 ATA ATG AGG AGA CAA ACG GAT TGA ATG TTC CAC ATT CTT AGA AGT AAA ATC AAT TAA TGC ATC CCC TCT CCG GTT
Y H P S L R I S H E V N K S T F D I L A D G R R N
1375 TTC CTT AAA AAA CAC ATT GTT ATC ACT CAG TAT TTT GTG AAT TTT TTC AAC CTT TCC GTC CGG CTG GCA GAA GGC
E K F F V N N D S L I K H I K E V K G D P Q C F A
1450 AAT AAT ACT GCT TAA TCT GTT CCC TTT ATC CAG AAC GGT GAA ACC ACT ATT CTG AAG ATG AGC TCT TAA AGT CTC
I I S S L R N G K D L V T F G S N Q L H A R L T E
1525 TGA CAA CGT ACG ATT GTA ACT TTC AAT CTT ATC CAT ACC AAT CGT ATT CGC ATA ATG CAA AGC TTC TGC AAA TCC
S L T R N Y S E I K D M G I T N A Y H L A E A F G
1600 CAG CAA CGC TGA ATA TGG TCT TTC AAA AAG TTC AAA TTT TCT GGC GGT ATC ATT AAG CTG AAA ATC ATC AAA GGA
L L A S Y P R E F L E F K R A T D N L Q F D D F S
1675 AGA CCA TCT GGC TCC GAT ACT ATC TAA AAA TAA AGG ATA CAT ATT TCT ATC CAA AGC TTT ATC CGA AAC ATA TAA
S W R A G I S D L F L P Y M N R D L A K D S V Y L
1750 AAA ACC TGT TCC TCT TGG TCC ACG CAT AAA TTT CCT TCC GGT GGC CGT CAG AAA ATC ACA ATG GAT TTT TTC AAC
F G T G R P G R M F K R G T A T L F D C H I K E V
1825 ATC TAC CAC GAG CTG TCC TAC CGA CTG ACA GGC ATC TAC AAG ATA AAG AAT ATC ATA TTG CTT ACA TAA CTT ACC
D V V L Q G V S Q C A D V L Y L I D Y Q K C L K G
1900 CAC TCC TTC TAT ATT CTG AAT CAA TCC GGA ATT GGT AGG AAT ATG AGT GAC TGC GAT TAA TTT AGG ATG ATG CTT
V G E I N Q I L G S N T P I H T V A I L K P H H K
1975 TCT GAT TAA GTT TTC AAA ATC CTC CAG ATC 2004
R I L N E F D E L D ← partial sequence of a capreomycin resistance determinant

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FIG. 1. Nucleotide sequence of the cloned 2,004-bp fragment of recombinant plasmid pCGB-1 containing the *bla<sub>CGB-1</sub>* coding region and a truncated ORF encoding a putative capreomycin resistance determinant. The deduced amino acid sequence is designated in the single-letter code below the nucleotide sequence. The putative terminator sequence is indicated by two inverted arrows, and stop codons are indicated by stars.

tive at the chromosomal position of migration of whole-cell DNA of *C. gleum* CIP 103039 (data not shown)  
**Susceptibility testing.** MICs of β-lactams for *C. gleum* CIP 103039 showed that it was resistant to amino- and carboxy-

penicillins, narrow-spectrum cephalosporins, cefotaxime, aztreonam, had intermediate susceptibility to carbapenems and remained susceptible to piperacillin (Table 1). *E. coli* DH10B (pCGB-1) was resistant to amoxicillin, ticarcillin, some restrict-

TABLE 1. MICs of  $\beta$ -lactams for *C. gleum* CIP 103039, *E. coli* DH10B(pCGB-1) expressing CGB-1, *E. coli* DH10B(pSO-1) expressing IND-1 from *C. indologenes* OO1, and reference strain *E. coli* DH10B

$\beta$ -Lactam(s) <sup>a</sup>	MIC ( $\mu$ g/ml) for:			
	<i>C. gleum</i> CIP 103039	<i>E. coli</i> DH10B (pCGB-1)	<i>E. coli</i> DH10B (pSO-1) <sup>b</sup>	<i>E. coli</i> DH10B
Amoxicillin	256	512	>512	2
Amoxicillin-CLA	32	256		2
Ticarcillin	256	>512	>512	2
Ticarcillin-CLA	256	>512		2
Piperacillin	2	8	64	1
Piperacillin-TZB	2	8		1
Cephalothin	256	32	128	2
Cefuroxime	>512	256		4
Cefoperazone	4	0.5		0.12
Ceftazidime	4	0.5	8	0.06
Cefotaxime	32	1	16	0.06
Cefepime	1	0.06	0.06	0.06
Cefpirome	2	0.06		0.06
Ceftriaxone	16	0.12		0.06
Cefoxitin	8	4	16	4
Moxalactam	16	0.12	0.12	0.12
Aztreonam	>512	0.06	0.12	0.06
Imipenem	2	0.25	2	0.06
Meropenem	8	0.25	0.5	0.06

<sup>a</sup> CLA, clavulanic acid at a fixed concentration of 2  $\mu$ g/ml; TZB, tazobactam at a fixed concentration of 4  $\mu$ g/ml.

<sup>b</sup> Data are from reference 4.

ed-spectrum cephalosporins, and had a reduced susceptibility to piperacillin, ceftazidime and carbapenems compared to that of *E. coli* DH10B. MICs of  $\beta$ -lactams for *E. coli* DH10B (pCGB-1) were not lowered by addition of clavulanic acid and tazobactam (Table 1). Resistance to aztreonam in *C. gleum* CIP103039 as found in other flavobacterial species (1–5) may be partially explained by expression of the previously characterized clavulanic acid-inhibited CGA-1 enzyme (3). Other resistance mechanisms such as low outer membrane permeability, efflux, and penicillin binding protein affinity may explain also resistance to this compound.

Once cloned onto a plasmid vector and expressed in *E. coli*, both enzymes, CGB-1 and IND-1, provided a similar pattern of decreased susceptibility to  $\beta$ -lactams, although in the latter case the degree of resistance was more important (Table 1). The overall susceptibility of *C. gleum* to  $\beta$ -lactams was similar to that reported for *C. meningosepticum* (1, 5). As for *C. meningosepticum* that possesses a class A  $\beta$ -lactamase (CME) and two class B  $\beta$ -lactamases (BlaB and GOB) (1, 5, 23), *C. gleum* possesses, in addition to the class A  $\beta$ -lactamase CGA-1, another very likely chromosomal-encoded class B enzyme CGB-1.

**Biochemical properties of CGB-1.** IEF analysis revealed that cultures of *E. coli* DH10B(pCGB-1) produced a  $\beta$ -lactamase activity with a pI value of 8.6. However, only one  $\beta$ -lactamase activity was detected from crude extracts of a culture of *C. gleum* CIP103039, at a pI of 8.9 that corresponded to the previously identified class A  $\beta$ -lactamase CGA-1 (3). Thus, expression of CGB-1 from *C. gleum* CIP 103039 might be weak. The specific activity of the purified CGB-1 enzyme was 8.3  $\mu$ mol $\cdot$ min<sup>-1</sup> $\cdot$ mg of protein<sup>-1</sup>, determined with 100  $\mu$ M imipenem as a substrate with a 174-fold purification coefficient.

Its purity was estimated to be 95% by SDS-PAGE (data not shown).

The mature protein (named CGB-1 for *C. gleum* class B  $\beta$ -lactamase) expressed in *E. coli* DH10B had a relative molecular mass determined experimentally to be ca. 26 kDa (data not shown).

Kinetic parameters of CGB-1 revealed a strong activity against amino- and carboxy-penicillins and restricted-spectrum cephalosporins (Table 2). Surprisingly, cefotaxime was a good substrate for CGB-1 due to a low  $K_m$ , whereas ceftazidime that possesses a  $K_m$  superior to 1 mM was a very poor substrate. This difference in hydrolysis activity mirrored the MICs for both  $\beta$ -lactams for *C. gleum* CIP 103039 (but not for *E. coli* DH10B(pCGB-1)) (Table 1). CGB-1  $\beta$ -lactamase had poor affinity for imipenem ( $K_m$ , >1,000  $\mu$ M), resulting in a weak catalytic efficacy of imipenem (Table 2). Therefore, its  $k_{cat}/K_m$  value for imipenem was at least 10-fold lower than that of IND-2, whereas these values for meropenem and for benzylpenicillin were only twofold lower (Table 2). These results were consistent with the low level of resistance to imipenem of *C. gleum* CIP 103039 (Table 1). Hydrolysis of aztreonam was not detectable, as found for all metallo-enzymes (20). Catalytic efficacy of CGB-1 was lower than that of GOB-1 from *C. meningosepticum* for all  $\beta$ -lactams except for benzylpenicillin which were similar (1). CGB-1 had  $k_{cat}/K_m$  values which were 4-fold lower for benzylpenicillin, 10-fold lower for cephaloridine and for cefoxitin, 60-fold lower for imipenem, and 3-fold higher for cefotaxime, compared to those of BlaB from *C. meningosepticum* (1). The hydrolytic activity of CGB-1 was inhibited by EDTA ( $IC_{50}$ , 37  $\mu$ M) but not by class A  $\beta$ -lactamase inhibitors such as clavulanic acid ( $IC_{50}$  > 1 mM). These kinetic parameters lead to include CGB-1 in the biochemical group 3a of the Bush  $\beta$ -lactamase classification for metallo-enzymes (7). This group includes all characterized metallo- $\beta$ -lactamases with a very broad substrate hydrolysis profile except FEZ-1 and Cph-A-like enzymes from *L. gormanii* and *Aeromonas* sp., respectively.

TABLE 2. Kinetic parameters of purified CGB-1 from cultures of *E. coli* DH10B(pCGB-1)<sup>a</sup>

$\beta$ -Lactam	$k_{cat}$ (s <sup>-1</sup> )	$K_m$ ( $\mu$ M)	$k_{cat}/K_m$ (s <sup>-1</sup> $\cdot$ mM <sup>-1</sup> )
Benzylpenicillin	45	20	2,200
Amoxicillin	145	220	660
Ticarcillin	60	107	570
Piperacillin	ND <sup>b</sup>	>1,000	ND
Cephalothin	20	40	430
Cephaloridine	10	180	50
Cefuroxime	10	9	890
Cefoperazone	10	115	90
Ceftriaxone	3	20	150
Ceftazidime	2	>1,000	<2
Cefotaxime	10	19	610
Cefepime	0.2	>1,000	<0.2
Cefpirome	2	>1,000	2
Cefoxitin	5	160	25
Meropenem	90	>1,000	>90
Imipenem	53	>1,000	<50
Aztreonam	<0.1	ND	ND

<sup>a</sup> Values are geometric means of three independent measures (standard deviations were within 15%).

<sup>b</sup> ND, not determinable.

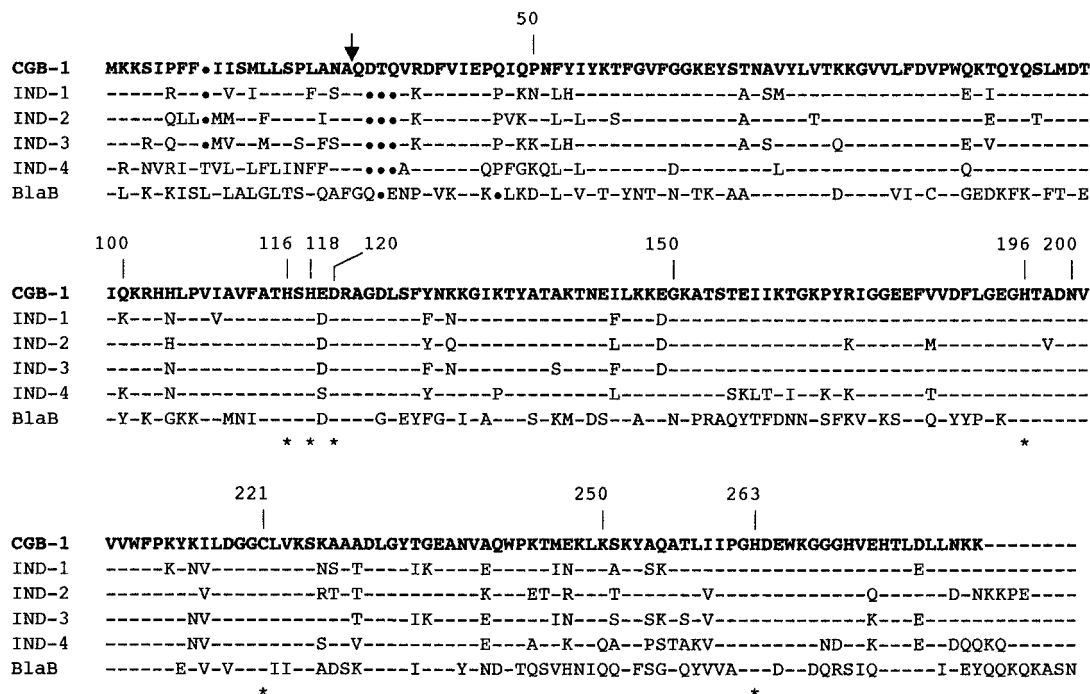


FIG. 2. Comparison of the amino acid sequence of β-lactamase CGB-1 with those of IND-1, IND-2, IND-3, and IND-4 from *C. indologenes* (4) and BlaB from *C. meningosepticum* (23). The BBL numbering scheme is indicated above the sequences (8). Broken lines and dots indicated identical and deleted amino acid residues, respectively. The vertical arrow indicates the putative cleavage site for the leader peptide of CGB-1. Amino acids that may be involved in binding of Zn<sup>2+</sup> or/and water are indicated by a star.

**Induction experiments.** Induction studies with cefoxitin and imipenem as β-lactam inducers failed to detect induction of β-lactamase expression with cultures of *C. gleum* CIP 103039 and of *E. coli*(pCGB-1) with imipenem as substrate. These results are consistent with the absence of any sequence for regulatory gene, such as LysR-type regulator gene upstream of *bla*<sub>CGB-1</sub> (16).

**Amino acid sequence analysis.** The comparison of amino acid sequence of CGB-1 with those of other class B β-lactamases revealed 83% identity with IND-1, 82% identity with IND-2 and IND-3, and 73% identity with IND-4 from *C. indologenes*, and only 42 and 12% identity with BlaB and GOB-1 from *C. meningosepticum*, respectively (1–4, 23). Thus, the metallo-enzyme of *C. gleum* was most closely related to those of *C. indologenes* thus paralleling the taxonomic position of both *Chryseobacterium* species (28). The six conserved amino acid residues implicated in the Zn<sup>2+</sup> or water molecule binding (His116, His118, Asp120, His196, Cys221, and His 220) (8) of metallo-β-lactamases were found in the CGB-1 sequence (Fig. 2). However, an insertion of three amino acids (Asp, Thr, and Gln) was detected at the second, third and fourth position of the N-terminal sequence of the mature protein CGB-1 (positions 22, 23, and 24) compared to IND-like (Fig. 2). β-Lactamase CGB-1 may be classified in the molecular subclass B1 of the structural classification that groups IND-like, Bc-II, CfiA, BlaB, IMP, and VIM β-lactamases (1, 2, 20). A phylogenetic analysis of CGB-1 from *C. gleum*, BlaB variants from *C. meningosepticum* (and not GOB variants from *C. meningosepticum* also), and IND variants from *C. indologenes*, showed that they belong to a same phylogenetic lineage (data not shown).

Comparison of amino acid sequence of CGB-1 with those of IND-1 and IND-2 did not provide evidence of any particular amino acid residue that may explain a lower affinity of CGB-1 for carbapenems.

In conclusion, this report identified a novel bacterial species for which β-lactam resistance is at least mediated by an Ambler class A expanded-spectrum β-lactamase and an Ambler class B metallo-enzyme, as found in *S. maltophilia* and *C. meningosepticum*.

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