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Genetic Diversity of Carbapenem-Hydrolyzing Metallo-β-Lactamases from *Chryseobacterium* (*Flavobacterium*) *indologenes*

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The class B carbapenem-hydrolyzing β-lactamase IND-1 has been characterized for *Chryseobacterium indologenes* strain 001. With internal primers for the *bla* gene for IND-1 (bla_{IND-1}) and an internal bla_{IND-1} probe, PCR amplifications failed, while hybridization results were positive when DNA from another *C. indologenes* isolate, strain CIP101026, was used as a template. Thus, a bla_{IND}-related gene was cloned from this *C. indologenes* reference strain. Sequencing of the insert of a recombinant plasmid conferring resistance to carbapenems revealed an open reading frame with a G + C content of 39.9% and coding for a 243-amino-acid preprotein named IND-2. IND-2 shared 80% amino acid identity with IND-1 and had a similar broad-spectrum resistance profile, including resistance to carbapenems. It was classified in functional subgroup 3a of class B carbapenem-hydrolyzing β-lactamases. IND-1 and IND-2, despite their genetic diversity, possessed similar kinetic parameters, except that ceftazidime was hydrolyzed less by IND-2. To obtain the entire bla_{IND}-related gene sequences of eight other *C. indologenes* isolates, PCR was performed using internal and external primers, followed by inverse PCR techniques. The likely chromosome-mediated metallo-β-lactamases of the 10 *C. indologenes* isolates were divided into several groups and subgroups. IND-1, IND-2, IND-2a, IND-3, and IND-4 shared 77 to 99% amino acid identity.

The genus *Chryseobacterium*, defined in 1994 by Vandamme et al., comprises six species, including *Chryseobacterium meningosepticum* (previously *Flavobacterium meningosepticum*) and *Chryseobacterium indologenes* (previously *Flavobacterium indologenes*), which are the most common clinical species of this genus (29). *C. meningosepticum* isolates are associated with meningitis in newborns or in immunocompromised patients (4). *C. indologenes* is responsible mostly for nosocomial infections linked to the use of intravascular devices (10, 28). In 1997, Hsueh et al. reported 36 cases of infections caused by *C. indologenes* over a 3-year period at the National Taiwan University Hospital (11). These infections were intra-abdominal infections, biliary tract infections, or wound sepsis (11). *C. indologenes* is also implicated in pneumonia (9). *C. indologenes* is resistant to nearly all penicillins, except piperacillin, to restricted-spectrum cephalosporins, to aztreonam, and to carbapenems (1). Among β-lactams, only extended-spectrum cephalosporins show in vitro activity against *C. indologenes* (8). Minocycline and ciprofloxacin seem to be the most effective antibiotics and have been successfully used to treat *C. indologenes* infections (10).

Recently, two Ambler class B β-lactamases have been characterized for *C. meningosepticum*, which is also resistant to all β-lactams, including carbapenems (2, 26). The unrelated BlaB and GOB-1 exhibit a broad-spectrum profile and are chromosome encoded. These carbapenem-hydrolyzing β-lactamases (CHβLs) have molecular and biochemical heterogeneity among *C. meningosepticum* isolates. We have characterized a β-lactamase, IND-1, from *C. indologenes* strain 001 (1). Since plasmid-mediated CHβLs have been increasingly reported for gram-negative species worldwide (5, 12, 18, 23, 24, 25), it was of interest to identify naturally occurring CHβLs of gram-negative species and to search for their degree of identity with the plasmid-mediated CHβLs. Thus, we performed an extended genetic and biochemical study of β-lactamase-mediated resistance to carbapenems in *C. indologenes*.

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MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this work are listed in Table 1. *C. indologenes* 002, 007, 008, and 009 were isolated from bronchoalveolar brush border, blood culture, rectal swab, and biliary liquid drainage, respectively, at the Hôpital de Bicêtre (Le Kremlin-Bicêtre, France) from 1997 to 1999. *C. indologenes* 003 and 004 were isolated from blood cultures at the Hôpital Robert Debré (Paris, France). *C. indologenes* 005 was from the Assistance Publique-Hôpitaux de Marseille (Marseille, France). *C. indologenes* 006 was isolated at the Hôpital de la Pitié Salpêtrière (Paris, France), and the *C. indologenes* reference strain CIP101026 was from the Pasteur Institute (Paris, France) strain collection. Each *C. indologenes* isolate was identified according to standard biochemical techniques (19, 29, 31), and their identification was checked at the Center for Bacterial Identification at the Pasteur Institute. These *C. indologenes* isolates were epidemiologically unrelated, as assessed by pulsed-field gel electrophoresis of *Xba*I-restricted DNA (data not shown).

Escherichia coli DH10B and rifampin-resistant *E. coli* JM109 were used for cloning and conjugation, respectively (Table 1). All strains were stored at −70°C in Trypticase soy (TS) broth supplemented with 15% glycerol until testing.

Antimicrobial agents and MIC determinations. The antimicrobial agents used in this study have been described elsewhere (21). MICs were determined by an agar dilution technique with Mueller-Hinton agar (Sanofi-Diagnostics Pasteur, Marnes-la-Coquette, France) and an inoculum of 10^4 CFU per spot. The plates were incubated at 35°C for 18 h before MIC determinations (16).

Cloning experiments and analysis of recombinant plasmids. Genomic DNA was extracted as described previously (3). Fragments from *Sau*3AI partially digested genomic DNA from *C. indologenes* CIP101026 were cloned into the pBK-CMV phagemid (Ozyme, Saint Quentin-en-Yvelines, France) and expressed in *E. coli* DH10B as described previously (3). Antibiotic-resistant colonies were selected on amoxicillin (30 μg/ml)- and kanamycin (30 μg/ml)-containing TS agar plates.

Recombinant plasmid DNA was obtained from 100-ml TS broth cultures grown overnight in the presence of amoxicillin (30 μg/ml) at 37°C. Plasmid DNAs were recovered by using Qiagen (Courtaboeuf, France) columns before restriction digest analyses.

Conjugation assays and plasmid content. Direct transfer of resistance markers into in vitro-obtained rifampin-resistant *E. coli* JM109 was attempted by liquid and solid conjugation assays (21). Transconjugants were selected on TS agar

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
E. coli DH10B	F′ *mcrA* Δ(*mrr-hsdRMS-mrcBC*) Φ80Δ*lac*ZDM15 Δ*lacX74 deoR recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK1 rpsL nupG*	Gibco BRL
E. coli JM109	*endA1 hsdR17 gyrA96* Δ(*lac proA*) *recA1 relA supE44 thi* F′ (*lacI*^{q} *lacZ*ΔM15 *proAB*$^+$ *traD36*)	32
In vitro-obtained rifampin-resistant *E. coli* JM109	Rifampin resistant	This study
C. indologenes CIP101026	Carbapenem resistant	Pasteur Institute
C. indologenes 001	Carbapenem resistant	1
C. indologenes 002	Carbapenem resistant	This study
C. indologenes 003	Carbapenem resistant	This study
C. indologenes 004	Carbapenem resistant	This study
C. indologenes 005	Carbapenem resistant	This study
C. indologenes 006	Carbapenem resistant	This study
C. indologenes 007	Carbapenem resistant	This study
C. indologenes 008	Carbapenem resistant	This study
C. indologenes 009	Carbapenem susceptible	This study
Plasmids		
pPCR-Script Amp SK(+)	Ampicillin resistance	Stratagene
pPCR-Script Cam SK(+)	Chloramphenicol resistance	Stratagene
pBK-CMV phagemid	Neomycin and kanamycin resistance	Stratagene
pSO-1	$bla_{\text{IND-1}}$ in the *Bam*HI site of pBK-CMV	1
pSO-2	$bla_{\text{IND-2}}$ in the *Bam*HI site of pBK-CMV	This study
pSO-3	$bla_{\text{IND-3}}$ in the *Sac*I/*Hin*dIII site of pBK-CMV	This study
pSO-4	$bla_{\text{IND-4}}$ in the *Sac*I/*Hin*dIII site of pBK-CMV	This study

plates containing rifampin (200 μg/ml) and amoxicillin (30 μg/ml). Plasmid DNA extraction of *C. indologenes* isolates was attempted by using methods reported previously (2).

Hybridization experiments and PCR strategy. The electrophoresis gel containing *Ssp*I-restricted genomic DNAs of *C. indologenes* isolates was transferred on a nylon membrane (Hybond N$^+$; Amersham Pharmacia Biotech, Les Ullis, France) by the Southern method (22), and the transferred DNAs were UV cross-linked (Stratalinker; Stratagene) (21). The probe made from a PCR-generated 695-bp internal fragment of the *bla* gene for IND-1 ($bla_{\text{IND-1}}$) was labeled with an ECL nonradioactive labeling and detection kit (Amersham Pharmacia Biotech).

In order to amplify IND-like β-lactamase genes from other *C. indologenes* isolates, we designed primers based on the external regions of $bla_{\text{IND-2}}$: primer 1, 5′-GGTTTGCATATCTATCTGCC-3′; and primer 2, 5′-ATCCAAAGAGAGG CTGGAGT-3′. PCR products were obtained for only four *C. indologenes* isolates. Thus, we designed other primers based on the conserved regions of $bla_{\text{IND-1}}$ and $bla_{\text{IND-2}}$ (primer 3, 5′-GCCCAGGTTAAAGATTTTGTAAT-3′; and primer 4, 5′-CATGGCCACCGCCTTTCCATTC-3′) to characterize the other IND-like β-lactamase genes (Fig. 1).

After an initial denaturation step (5 min at 95°C), 42 cycles of amplification were performed as follows: denaturation at 95°C for 1 min, annealing at 53°C for 1 min, and DNA extension at 72°C for 1 min. A terminal DNA extension step was also performed at 72°C for 10 min. After sequencing of the PCR products, 580-bp-long sequences were characterized. In order to obtain the entire sequences of the β-lactamase genes, the inverse PCR (IPCR) technique was used (20). This technique enabled us to amplify, by PCR, flanking regions of a DNA fragment. Primers in inverted orientation with regard to the gene were used to amplify the restricted DNA fragment, which had been previously circularized by ligation (Fig. 1). Briefly, 4 μg of genomic DNA was restricted by *Ava*II (known to be absent from the $bla_{\text{IND-1}}$ and $bla_{\text{IND-2}}$ coding sequences). Since an *Ava*II restriction site was present in another bla_{IND}-like gene (later identified as $bla_{\text{IND-4}}$), *Pst*I was used in IPCR for *C. indologenes* 009. After heat inactivation of the restriction enzyme, T4 DNA ligase was added to the reaction mixture and incubation was performed at 4°C overnight. IPCR was performed using the degenerate primers revA (5′-CCAYGGGACRTCAAATAAGAC-3′) and revB (5′-CWGCMACYGAYCTKGGATATA-3′) (where Y was C or T, R was A or G, W was A or T, M was A or C, and K was G or T), designed by comparison of $bla_{\text{IND-1}}$ and $bla_{\text{IND-2}}$ sequences and exhibiting orientations opposite those of primers 3 and 4, respectively (Fig. 1). Since IPCR with revA and revB failed, primers revC (5′-CTTTGCCGTCAAAAACTCCG-3′) and revD (5′-GCTAAT GTAGAACAATGGCC-3′) were used in IPCR for *C. indologenes* 009. A 40-cycle amplification protocol was used, each cycle consisting of a denaturation step at 95°C for 1 min, a primer annealing step at 53°C, and an elongation step at 72°C for 3 min; 3 U of *Taq* polymerase was added per tube. The 1.4- or 1.7-kb IPCR products were purified, cloned into pPCR-Script Amp SK(+) as described by the manufacturer (Stratagene), and sequenced using T3 and T7 universal primers. DNA sequences adjacent to the *Ava*II (or *Pst*I) restriction site in the cloned IPCR product were added to the PCR products obtained with primers 3 and 4 in order to deduce the entire bla_{IND}-like gene sequences from five *C. indologenes* isolates.

In order to establish a valid comparison of MICs of β-lactams for *E. coli* DH10B harboring either $bla_{\text{IND-1}}$, $bla_{\text{IND-2}}$, $bla_{\text{IND-3}}$, or $bla_{\text{IND-4}}$, PCR products of $bla_{\text{IND-3}}$ from *C. indologenes* 005, obtained using external primers 5 (5′-CCC AGCAAGTCCTAACTTTAATTAC-3′) and 6 (5′-CTAGTTACCTAGAGATA GCACG-3′), and of $bla_{\text{IND-4}}$ from *C. indologenes* 009, obtained using external primers 7 (5′-TTATGAGGAAAAATGTTAGG-3′) and 8 (5′-GAACAGTTAA TAGAAAAGCGGG-3′), were cloned first into pPCR-Script Cam SK(+) (Stratagene) and then into pBK-CMV and transformed back into *E. coli* DH10B by electroporation.

DNA sequencing and protein analysis. Once the bla_{IND}-like gene sequences were reconstituted using the above-described PCR strategy, a series of external primers (primers 1 and 2, 5 and 6, and 7 and 8) were used to obtain a PCR fragment for each *C. indologenes* strain as a template; the fragments were sequenced directly using an Applied Biosystems sequencer (ABI 373). Sequencing was also performed for the inserts of recombinant plasmids pSO-2, pSO3, and pSO4. The nucleotide and deduced protein sequences were analyzed with software available over the Internet from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) and from Pedro's BioMolecular Research Tools website (http://www.fmi.ch/biology/research_tools.htlm).

Multiple nucleotide or protein sequence alignments were carried out using the program ClustalW, available over the Internet from the University of Cambridge website (http://www2.ebi.ac.uk/clustalw). Among the Ambler class B β-lactamases, nine were compared to IND-like β-lactamases: GOB-1 and BlaB from *C. meningosepticum* (2, 26), VIM-1 and VIM-2 from *Pseudomonas aeruginosa* (12, 23), CphA from *Aeromonas hydrophila* (14), IMP-1 from *Serratia marcescens* (18), CcrA from *Bacteroides fragilis* (25), B-II from *Bacillus cereus* (13), and L-1 from *Stenotrophomonas maltophilia* (30).

β-Lactamase purification. A culture of *E. coli* DH10B(pSO-2) was grown overnight at 37°C in 6 liters of TS broth containing kanamycin (30 μg/ml) and amoxicillin (30 μg/ml). Bacterial suspensions were pelleted, resuspended in 60 ml of 100 mM phosphate buffer (pH 6.0), disrupted by sonification (three times at 50 W for 30 s each time using a Vibra Cell 75022 Phospholyser; Bioblock, Illkirch, France), and centrifuged for 1 h at 48,000 × *g* and 4°C. Nucleic acids were precipitated by the addition of 0.2 M spermin (7% [vol/vol]; Sigma, Saint-Quentin Fallavier, France) overnight at 4°C. This suspension was ultracentrifuged at 100,000 × *g* for 1 h at 4°C. Similar unpurified β-lactamase extracts were obtained from 10-ml cultures of *C. indologenes* isolates and subsequently resuspended in 0.5 ml of sodium phosphate buffer.

The β-lactamase extract from *E. coli* DH10B(pSO-2) was filtered through a 0.45-μm-pore-size filter (Millipore) prior to being loaded onto a preequilibrated S-Sepharose column (Amersham Pharmacia Biotech). The enzyme recovered in the flowthrough was dialyzed overnight at 4°C against 20 mM Tris-HCl buffer (pH 8.0). The enzyme fraction was then loaded onto a preequilibrated Q-Sepharose column (Amersham Pharmacia Biotech). The enzyme was eluted with a linear NaCl gradient (0 to 1 M) in Tris-HCl buffer (pH 8.0). The β-lactamase was eluted at a concentration of 350 mM NaCl. The fraction containing the β-lac-

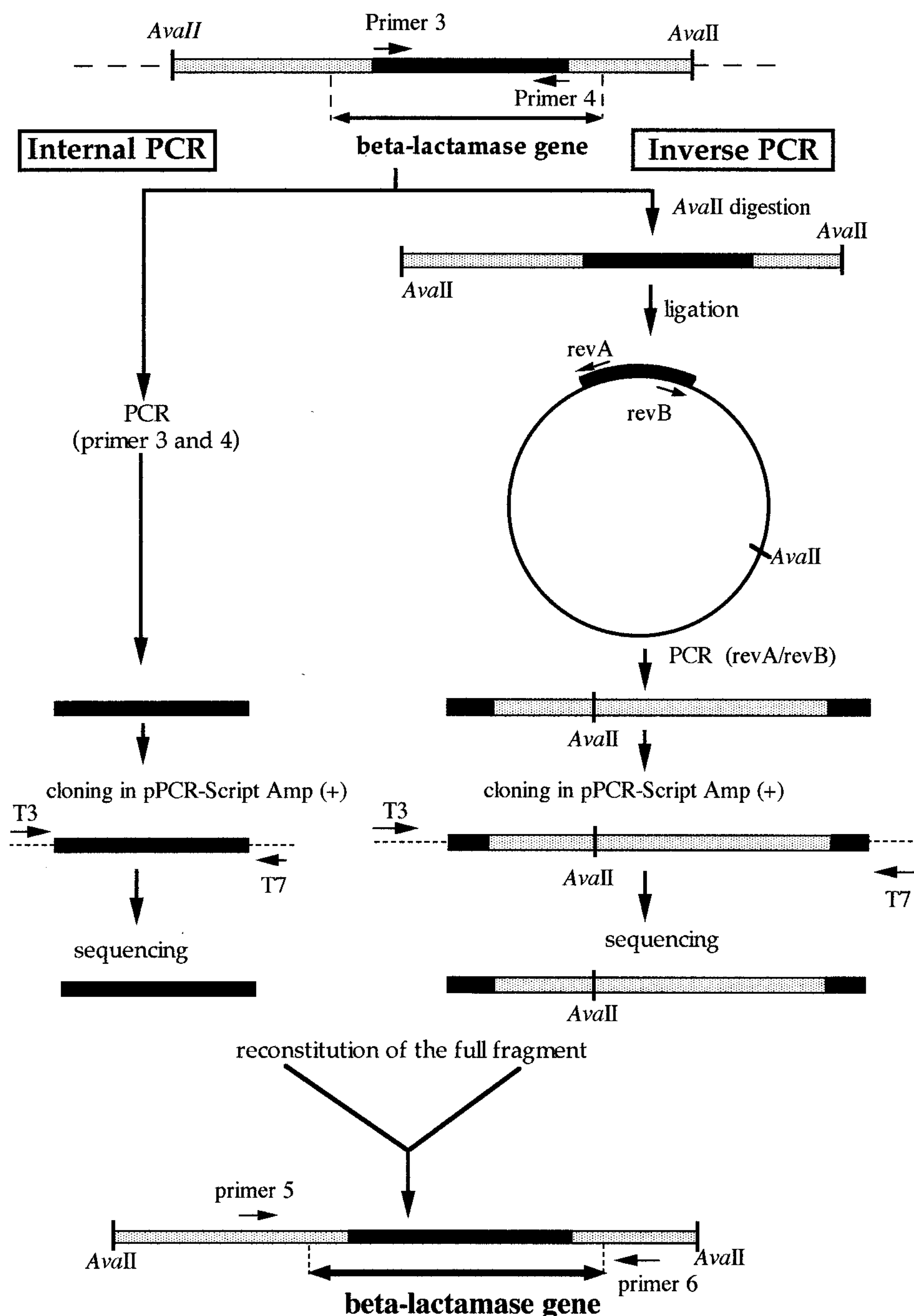

FIG. 1. Strategies of direct PCR and IPCR. Black bars represent the internal PCR product sequence obtained with primers 3 and 4. Grey bars represent known sequences of IPCR products. revA and revB (or revC and revD for *C. indologenes* 009) represent primers used for amplification of flanking regions. T3 and T7 primers were used for sequencing the IPCR products. Primers 5 and 6 were used for cloning the entire $bla_{\mathrm{IND\text{-}3}}$ gene into pPCR-Script Cam SK(+) and into pBK-CMV. Primers 7 and 8 (same positions as primers 5 and 6, respectively) were used for cloning the $bla_{\mathrm{IND\text{-}4}}$ gene. Double-headed arrows represent the entire bla_{IND}-related gene fragment.

tamase activity was dialyzed overnight against 100 mM phosphate buffer (pH 7.0) containing 50 μM $ZnCl_2$. The specific activities of the β-lactamase extract and of the β-lactamase purified from *E. coli* DH10B(pSO-2) were compared using 100 μM imipenem as the substrate as described previously (2).

N-terminal sequencing and IEF analysis. In order to determine the site for cleavage of the mature protein for IND-2 β-lactamase, the purified enzyme was submitted to Edman analysis as described previously (2) but with the following modifications in the protein transfer technique. The buffer consisted of 10 mM 3-cyclohexylamino-propane sulfonic acid (CAPS) (pH 11)–10% (vol/vol) methanol, and the transfer was carried out for 30 min at 50 V.

A purified enzyme preparation from a culture of *E. coli* DH10B(pSO-2) and extracts from cultures of 10 *C. indologenes* isolates were subjected to analytical isoelectric focusing (IEF) on a pH 3.5 to 9.5 Ampholine polyacrylamide gel (Ampholine PAG plate; Amersham Pharmacia Biotech) for 90 min at 1,500 V, 50 mA, and 30 W. The focused β-lactamases were detected by overlaying the gel with a 0.2% (wt/vol) starch agar gel containing 1% (wt/vol) benzylpenicillin in 100 mM phosphate buffer (pH 7.0) as described previously (2, 14). The pI values were determined and compared to those of known β-lactamases.

Kinetic measurements and identification of relative molecular mass. The relative molecular mass of the β-lactamase IND-2 from *E. coli* DH10B(pSO-2) was estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis. Purified enzyme and marker proteins were boiled for 5 min in a 1%

SDS–3% β-mercaptoethanol solution and then subjected to electrophoresis on a 12% polyacrylamide gel (25 mA, 4 h) (3). Renaturation of β-lactamase activity after denaturing electrophoresis and revelation by overlaying the gel with 1 mM nitrocefin were performed as described previously (14).

Purified β-lactamase was used for kinetic measurements performed at 30°C with 100 mM phosphate buffer (pH 7.0) supplemented with 50 μM $ZnCl_2$ (1). The rates of hydrolysis were determined with an ULTROSPEC 2000 UV spectrophotometer (Amersham Pharmacia Biotech) and analyzed by computer with SWIFT II software (Amersham Pharmacia Biotech). K_m and k_{cat} values were determined by analyzing β-lactam hydrolysis under initial rate conditions using the Eadie-Hoffstee linearization of the Michaelis-Menten equation as described previously (7).

Various concentrations of EDTA or clavulanic acid were preincubated with the enzyme for 10 min at 30°C before the rate of imipenem hydrolysis was tested. Then, the 50% inhibitory concentration was determined for each inhibitor.

Induction experiments and specific activity. The inducibility of CHβL expression in *C. indologenes* clinical isolates was examined as described previously (22) with cefoxitin (5 μg/ml) or imipenem (1 μg/ml) as the inducer. Hydrolysis measurements were recorded with imipenem as the substrate. The total protein content was measured with bovine serum albumin as the standard (Bio-Rad DC protein assay kit).

Specific activities with benzylpenicillin, imipenem, or meropenem as substrates were determined for culture extracts from *E. coli* DH10B harboring either pSO-2 or pSO-4 in order to compare the efficacies of carbapenem hydrolysis by the β-lactamases IND-2 and IND-4.

Nucleotide sequence accession numbers. The IND-like nucleotide and deduced amino acid sequences reported in this work will appear in the GenBank/EMBL database under accessions no. AF219127 to AF219135.

RESULTS AND DISCUSSION

Identification of the β-lactamase IND-2 and comparison with IND-1. Several species belonging to the *Chryseobacterium* genus express a CHβL. Among them, *C. meningosepticum* expresses two nonrelated CHβLs, BlaB and GOB-1, with genetic and biochemical heterogeneity (2). Recently, a CHβL, IND-1, from *C. indologenes* strain 001 was characterized (1). This enzyme shares 43% amino acid identity with the most related metallo-β-lactamase, BlaB, from *C. meningosepticum*, its gene being very likely located on the chromosome. In order to investigate the distribution of *bla*$_{\text{IND-1}}$ among several *C. indologenes* isolates, we performed PCR amplification and hybridization experiments. Negative PCR results indicated the likely absence of the *bla*$_{\text{IND-1}}$ gene among the tested *C. indologenes* isolates, except for *C. indologenes* 001. However, positive hybridization results obtained with a *bla*$_{\text{IND-1}}$ internal probe revealed the presence of *bla*$_{\text{IND}}$-related genes in all *C. indologenes* isolates studied (data not shown).

We cloned the *bla*$_{\text{IND}}$-like gene from *C. indologenes* CIP101026, which showed a hybridization pattern different from that of *C. indologenes* 001 (data not shown). Only one recombinant *E. coli* DH10B clone was obtained after electroporation of ligation products of *Sau*3AI partially restricted genomic DNA of *C. indologenes* CIP101026 into phagemid pBK-CMV, followed by selection on amoxicillin-containing plates. The recombinant plasmid (pSO-2) possessed a 1.5-kb insert and expressed a β-lactam resistance phenotype similar to that of *E. coli* DH10B(pSO-1), expressing β-lactamase IND-1.

Sequencing of the insert revealed an open reading frame of 729 bp, from bp 477 to 1,205, encoding a putative 243-amino-acid preprotein named IND-2 (Fig. 2). A putative cleavage site was found between alanine and glutamine at positions 20 and 21, respectively (17). This site was confirmed, since Edman analysis (six cycles) determined the N-terminal sequence of the purified IND-2 β-lactamase from cultured *E. coli* DH10B (pSO-2) cells to be QVKD (Fig. 2). No sequence typical of a class 1 integron and no open reading frame likely coding for a regulatory protein were found within the 1.5-kb insert. Amino acid sequence analysis revealed a G+C content of 39.9%, similar to that found for other *C. indologenes* genes, as recorded in the EMBL sequence database, indicating the likely

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         1        10        20        30        40        50        60        70        80        90        100       110       120
         |        |         |         |         |         |         |         |         |         |         |         |         |
IND-1    MKKSIRFFIVSILLSPFASAQVKDFVIEPPIKNNLHIYKTFGVFGGKEYSANSMYLVTKKGVVLFDVPWEKIQYQSLMDTIKKRHNLPVVAVFATHSHDDRAGDLSFFNNKGIKTYATAKTN
IND-2    -----QLLMM-MF---LIN-----------V-P--YL--S------------AV--T---------V--Q-E---T-----Q---H---I-----------------Y-Q------------
IND-2a   -----QLLMM-MF---LIN-----------V-P--YL--S------------AV--T---------V--Q-E---T-----Q---H---I-----------------Y-Q------------
IND-3    ---R-Q--M--MM--SLF--------------K--------------------V----Q------------V---------Q-------I----------------------------S---
IND-4   MR-NVRI-TVL-LF-IN-FN---R-----Q-FGKQ-YL-------D-----T-AL---------------Q-T-----------------I--------S--------Y-K---P--------

                130       140       150       160       170       180       190       200       210       220       230       240
                |         |         |         |         |         |         |         |         |         |         |         |
IND-1    EFLKKDGKATSTEIIKTGKPYRIGGEEFVVDFLGEGHTADNVVVWFPKYNVLDGGCLVKSNSATDLGYIKEANVEQWPKTINKLKAKYSKATLIIPGHDEWKGGGHVEHTLELLNKK
IND-2    -L-------------------K------M---------V----------K----------RT------TG----K---E-MR---T--AQ---V-------------Q---D--D-NKKPE
IND-2a   -L-------------------K------M---------V----------K----------RT------TG----K---E-MR---T--AQ---V----E--------Q---D--D-NKKPE
IND-3    ------------------------------------------------------------KA-----------------------S-----S-V-------------K---------
IND-4    -L---E-----SKLT-I--K-KI-----T-------------------------------SAAV----TG--------A-MK--Q---PSTAKV---------ND--K------DQQKQ
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FIG. 2. Amino acid sequence comparison of the IND-like β-lactamases of the 10 *C. indologenes* isolates. Broken lines indicate identical amino acids. IND-1 was from *C. indologenes* 001; IND-2 was from *C. indologenes* CIP101026, 002, and 003; IND-2a was from *C. indologenes* 004; IND-3 was from *C. indologenes* 005, 006, 007, and 008; and IND-4 was from *C. indologenes* 009. Shading correspond to conserved residues in CHβLs (25). Numbering is according to that for the IND-1 β-lactamase. The arrow indicates the peptide leader cleavage site determined for IND-2 by N-terminal sequence analysis of *E. coli* DH10B(pSO-2) cultures.

TABLE 2. MICs of β-lactams for several *C. indologenes* isolates, *E. coli* DH10B harboring recombinant plasmids, and *E. coli* DH10B (reference strain)[a]

β-Lactam	MIC (μg/ml) for:								
	C. indologenes 001	*C. indologenes* CIP101026	*C. indologenes* 005	*C. indologenes* 009	*E. coli* DH10B (pSO-1)	*E. coli* DH10B (pSO-2)	*E. coli* DH10B (pSO-3)	*E. coli* DH10B (pSO-4)	*E. coli* DH10B
Amoxicillin	256	256	256	256	>512	>512	>512	>512	2
Ticarcillin	256	512	512	512	>512	>512	>512	>512	2
Piperacillin	16	16	8	8	64	64	64	64	1
Cephalothin	256	256	128	128	128	128	128	128	4
Cefepime	1	2	0.5	1	0.06	0.06	0.06	0.06	0.03
Cefoxitin	8	16	8	16	16	16	16	16	1
Ceftazidime	32	8	8	8	8	4	4	8	0.12
Cefotaxime	64	32	32	32	16	16	8	16	0.12
Aztreonam	>128	>128	>128	>128	0.25	0.25	0.25	0.25	0.25
Imipenem	32	64	32	4	2	2	1	2	0.06
Meropenem	32	32	32	16	0.5	0.5	0.5	0.5	0.06

[a] *C. indologenes* 001 and *E. coli* DH10B(pSO-1) produced IND-1, *C. indologenes* CIP101026 and *E. coli* DH10B(pSO-2) produced IND-2, *C. indologenes* 005 and *E. coli* DH10B(pSO-3) produced IND-3, and *C. indologenes* 009 and *E. coli* DH10B(pSO-4) produced IND-4.

chromosome location of $bla_{\text{IND-2}}$. A comparison of the IND-2 amino acid sequence with that of IND-1 revealed only 80% amino acid identity (83% without the leader peptide sequence), showing the heterogeneity of CHβLs in *C. indologenes*, like that found in *C. meningosepticum* (2).

Despite relatively low amino acid identity between β-lactamases IND-2 and IND-1, the amino acids that may be involved in the catalytic site of these metalloenzymes were identical (Fig. 2).

The MICs of β-lactams for *C. indologenes* CIP101026 (IND-2) showed that it was resistant or had reduced susceptibility to most β-lactams tested, including carbapenems, but not to piperacillin and cefepime (Table 2). *E. coli* DH10B(pSO-2) showed a broad-spectrum profile of resistance, with resistance to aminopenicillins and to early cephalosporins. *E. coli* DH10B (pSO-2) also showed reduced susceptibility to ureidopenicillins, cephalosporins, and carbapenems but remained fully susceptible to cefepime, aztreonam, and moxalactam. The addition of clavulanic acid to amoxicillin or tazobactam to piperacillin did not modify the MICs (data not shown). Despite the sequence heterogeneity of the CHβLs IND-1 and IND-2, the MICs of β-lactams for *C. indologenes* 001 and CIP101026 on the one hand and for *E. coli* DH10B(pSO-1) and *E. coli* DH10B(pSO-2) on the other hand remained similar (Table 2). However, *E. coli* DH10B(pSO-1) was slightly less susceptible to ceftazidime than *E. coli* DH10B(pSO-2). The level of resistance to carbapenems remained low in *E. coli*, as previously described for other CHβLs, confirming the major role of the permeability coefficient for each β-lactam in gram-negative bacteria (15).

Specific activity before and after purification showed a purification factor of 420-fold for IND-2 from *E. coli* DH10B (pSO-2) cultures. The specific activity of the purified enzyme was 129 μmol/min/mg of protein with imipenem as a substrate, and the relative molecular mass of the purified enzyme was estimated by SDS-polyacrylamide gel electrophoresis to be 31 kDa. β-Lactamase activity with a pI of 8.8 was detected in *E. coli* DH10B(pSO-2), and a similar pI was found in *E. coli* DH10B(pSO-1). A second and weaker β-lactamase activity with a pI of 7.2 was also detected and likely reflects protein degradation during extraction, as described previously (1). β-Lactamase IND-2 showed a broad-spectrum profile of hydrolysis (Table 3). The kinetic parameters of purified β-lactamase IND-2 revealed strong activities against benzylpenicillin and piperacillin. Ampicillin, cephalothin, cefotaxime, and imipenem were also effectively hydrolyzed by IND-2, but ceftazidime and cefepime were poor substrates for this CHβL. Fifty percent inhibitory concentrations obtained with imipenem as a substrate showed that IND-2 activity was inhibited by EDTA (0.5 μM) but not by clavulanic acid (>100 μM), like other class B β-lactamases. The broad hydrolysis spectrum of this CHβL enabled us to classify IND-2 in functional subgroup 3a (5, 6).

TABLE 3. Kinetic parameters of β-lactam antibiotics for the purified CHβL IND-2 compared to those available for IND-1 (1)[a]

Substrate	IND-2					IND-1		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	$V_{max\ rel}$	V_{max}/K_m[b]	K_m (μM)	$V_{max\ rel}$	V_{max}/K_m[b]
Ampicillin	95	70	0.7	22	15	363	33	2.4
Benzylpenicillin	70	320	4.6	100	100	26.4	100	100
Cefepime	2,200	1	<0.001	0.1	<0.01	—	—	—
Cefotaxime	10	9	0.9	2.8	19	60	19	8.7
Cefoxitin	40	2	0.05	0.8	1.4	30	1.6	1.3
Ceftazidime	440	2	0.005	0.7	0.1	765	15	0.5
Cephalothin	16	19	1.2	6	24	79	47	16
Imipenem	170	100	0.6	30	12	198	90	12
Meropenem	920	170	0.2	53	4	845	105	3.3
Piperacillin	210	430	2	130	43	365	255	18.4

[a] Standard deviations were within 10%. —, not done.
[b] Relative to the value for benzylpenicillin, which was set at 100.

In order to establish a valid comparison between the kinetic parameters of IND-1 and IND-2, the values for the maximum rate of metabolism (V_{max}), relative to that for benzylpenicillin ($V_{max\ rel}$), for IND-2 were added in the table (Table 3). Hydrolysis efficacy did not vary significantly for both CHβLs, especially for carbapenems. However, the V_{max}/K_m value of IND-1 for ceftazidime (relative to that for benzylpenicillin) was fivefold higher than that of IND-2, whereas this value of IND-2 for ampicillin was sixfold higher than that of IND-1. Thus, amino acid sequence changes in these CHβLs may be linked to variability in their biochemical properties.

Identification of other IND-like β-lactamases. PCR products were obtained from *C. indologenes* isolates CIP101026, 002, 003, and 004 and sequenced by using external primers 1 and 2. Therefore, in order to characterize other bla_{IND}-related genes, partial nucleotide sequences of bla_{IND}-related genes from five other *C. indologenes* isolates were obtained using PCR amplification of a 580-bp bla_{IND}-like internal fragment and PCR primers 3 and 4 (Fig. 1). IPCR was then performed in order to obtain the entire β-lactamase gene sequence and to characterize flanking regions from the five other *C. indologenes* isolates, 005, 006, 007, 008, and 009. Sequencing of internal PCR and IPCR products enabled us to obtain the entire nucleotide sequences of all the bla_{IND}-related genes. A comparison of the deduced amino acid sequences of these genes enabled us to determine four groups of IND-like β-lactamases (Fig. 2). *C. indologenes* 001 produced IND-1; *C. indologenes* CIP101026, 002, and 003 produced IND-2; *C. indologenes* 004 produced an IND-2 variant, named IND-2a; *C. indologenes* 005, 006, 007, and 008 produced an IND-like β-lactamase, named IND-3, sharing 91% amino acid identity with IND-1 (94% if the leader peptide sequence was excluded); and *C. indologenes* 009, which was susceptible to carbapenems, produced an IND-related β-lactamase, named IND-4, displaying 72% amino acid identity with IND-1 (77% if the leader peptide sequence was excluded). None of the IND-like sequences shared more than 43% amino acid identity with the most closely related CHβL, BlaB, as described for IND-1 β-lactamase. β-Lactamases IND-1, IND-2, IND-3, and IND-4 could be classified on a molecular basis in subclass B1 (24). Within a given IND-1-like gene group (IND-1, IND-2, IND-3, and IND-4), the DNA sequences surrounding the β-lactamase gene were identical, whereas they were different from one group to another. Thus, IND-like genes of a given group lie within a specific genetic context, indicating possible subspecies within *C. indologenes*.

From a molecular point of view, these four main groups of CHβLs showed extensive heterogeneity of amino acid sequences. However, all of the six conserved amino acid residues for metallo-β-lactamases were conserved in the IND-related β-lactamases. Only one amino acid substitution (Asp99Ser) was located within the active site of β-lactamase IND-4 (Fig. 2). A serine residue at this position is also found in CHβL IMP-1, originally identified in *S. marscescens* (18).

Cloning of the PCR products of the entire bla_{IND-3} and bla_{IND-4} sequences into pBK-CMV and electroporation of the obtained plasmids, pSO-3 and pSO-4, into *E. coli* DH10B were performed in order to compare the MICs of β-lactams conferred by the four groups of CHβLs (IND-1, IND-2, IND-3, and IND-4).

A β-lactamase activity with a pI of 8.4 to 9 was detected in the *C. indologenes* isolates by IEF. A similar pI of ca. 8.8 was detected for cultures of *E. coli* DH10B harboring recombinant plasmids pSO-1 (IND-1), pSO-2 (IND-2), pSO-3 (IND-3), and pSO-4 (IND-4). No evidence of β-lactamase inducibility was detected for *C. indologenes* isolates by a spectrophotometric assay with imipenem or cefoxitin as an inducer and imipenem as a substrate for CHβL.

The MICs of β-lactams for the 10 *C. indologenes* isolates were similar (within a two-dilution range), except for *C. indologenes* 009, which was fully susceptible to imipenem (Table 2). The four β-lactamase groups expressed in *E. coli* DH10B, including *E. coli* DH10B(pSO-4), conferred similar MICs (within a two-dilution range) (Table 2). In order to explain this surprising result, we determined the specific activity of a β-lactamase extract from *E. coli* DH10B harboring either pSO-2 or pSO-4 with benzylpenicillin, imipenem, or meropenem as a substrate. The ratio of specific activity with imipenem as a substrate divided by the specific activity with benzylpenicillin as a substrate was sevenfold lower for *E. coli* DH10B(pSO-4), expressing IND-4, than for *E. coli* DH10B(pSO-2), expressing IND-2. Similarly, the ratio of the specific activity of meropenem divided by that of benzylpenicillin was 2.5-fold lower for *E. coli* DH10B(pSO-4) than for *E. coli* DH10B(pSO-2). Further investigations are necessary to evaluate the role of the amino acid changes observed for IND-4 in the decreased hydrolysis of carbapenems.

A recent study has reported the major role of permeability in the activity of β-lactams against gram-negative bacteria that produce group 3 β-lactamases (15). Therefore, susceptibility to imipenem in parental strain *C. indologenes* 009 (IND-4) could be due to a low level of carbapenem hydrolysis by IND-4, together with an increased permeability coefficient for carbapenems.

Conclusion. IND-1-like sequences in *C. indologenes*, like L-1 from *S. maltophilia* (27), BlaB and GOB-1 from *C. meningosepticum* (2), B-II from *B. cereus*, or CcrA from *B. fragilis* (25), confirmed the variability of chromosome-located class B β-lactamases within a bacterial species. As opposed to *C. meningosepticum*, *C. indologenes* may possess only one CHβL. No pI values other than those of the CHβLs were detected in *C. indologenes* isolates (data not shown). Thus, the naturally occurring resistance to monobactams, such as aztreonam, observed in *C. indologenes* may not be linked to β-lactamase.

The origin of the metallo-β-lactamases recently identified as being integron located in gram-negative species (IMP-1, VIM-1, and VIM-2) remains unknown. None of them is related to the CHβLs described in this report. Cloning and expression of IND-like sequences in *E. coli* DH10B conferred only slightly decreased susceptibility to carbapenems. Thus, if expressed in gram-negative clinical isolates, the CHβLs of *C. indologenes* would be difficult to detect on the basis of the sole carbapenem resistance pattern.

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