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Integron Carrying a Novel Metallo-β-Lactamase Gene, bla_{IMP-16}, and a Fused Form of Aminoglycoside-Resistant Gene *aac(6′)-30/aac(6′)-Ib′*: Report from the SENTRY Antimicrobial Surveillance Program

Rodrigo E. Mendes,[1,2]* Mark A. Toleman,[2] Julival Ribeiro,[3] Helio S. Sader,[1,4] Ronald N. Jones,[4,5] and Timothy R. Walsh[2]

Disciplina de Doenças Infecciosas e Parasitárias, Universidade Federal de São Paulo, São Paulo,[1] and Hospital de Base do Distrito Federal, Brasília,[3] Brazil; Department of Pathology & Microbiology, University of Bristol, Bristol, United Kingdom[2]; The JONES Group/JMI Laboratories, North Liberty, Iowa[4]; and Tufts University School of Medicine, Boston, Massachusetts[5]

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Since January 2002 *Pseudomonas* sp. strains resistant to carbapenems and ceftazidime have been routinely screened as part of the SENTRY Antimicrobial Surveillance Program for metallo-β-lactamase production, and their resistance determinants have been analyzed. *Pseudomonas aeruginosa* index strain 101–4704, which harbors a novel bla_{IMP} variant, bla_{IMP-16}, was isolated in April 2002 from a 60-year-old man in Brasília, Brazil. bla_{IMP-16} was found on the chromosome of the *P. aeruginosa* index strain, and the deduced amino acid sequence (IMP-16) showed the greatest identities to IMP-11 (90.3%) and IMP-8 (89.5%). Sequence analysis revealed that bla_{IMP-16} was associated with a class 1 integron, which also encoded aminoglycoside-modifying enzymes. Downstream of bla_{IMP-16} resided an open reading frame, which consisted of a new aminoglycoside-modifying gene, namely, *aac(6′)-30*, which was fused with *aac(6′)-Ib′*. The amino acid sequence of the *aac(6′)-30* putative protein showed the most identity (52.7%) to the sequence of AAC(6′)-29b described previously. The fourth gene cassette constituted *aadA1*. The steady-state kinetics of IMP-16 demonstrated that the enzyme preferred cephalosporins and carbapenems to penicillins. The main functional difference observed among the kinetic values for IMP-16 compared to those for other IMPs was a lack of cefoxitin hydrolysis and a lower k_{cat}/K_m value for imipenem (0.36 $\mu M^{-1} \cdot s^{-1}$). This report further emphasizes the spread of metallo-β-lactamase genes and their close association with various aminoglycoside resistance genes.

The carbapenems represent important therapeutic options for serious infections caused by *Pseudomonas aeruginosa*, especially multidrug-resistant strains. The activities of these compounds are mainly related to their rapid permeation across bacterial membranes and their stabilities against most β-lactamases (3). However, as with other antimicrobial agents, carbapenem-resistant *P. aeruginosa* strains continue to be isolated with an increasing frequency from nosocomial infection sources. Trends have been observed that demonstrate a decrease in carbapenem susceptibility among *P. aeruginosa* strains isolated from hospitalized patients in Latin America (from 83.0 to 64.4% between the years 1997 and 2001 [$P < 0.001$; odds ratio = 2.70; 95% confidence interval = 1.88 to 3.89]) (2).

The mechanisms responsible for carbapenem resistance include decreased outer membrane permeability (porin mutations), up-regulation of multidrug efflux pumps, substantial production of a chromosomal *ampC* β-lactamase or a class D β-lactamase (often accompanied by porin changes), and production of a class B β-lactamase (1, 22). The class B enzymes, or metallo-β-lactamases (MβLs), are zinc-dependent enzymes that catalyze the hydrolysis of a broad range of β-lactams, including carbapenems, using zinc ions as metal cofactors (44). To date, three types of mobile MβL genes have been reported. In 1994, the IMP-type MβL gene was first reported in Japan (29), and since then, IMP MβL-producing strains have been reported from many different countries worldwide (4, 6, 7, 13, 46, 51). The VIM-type enzymes were first reported from Italy in 1999, and strains producing these enzymes have now been reported from European countries as well as Asia and the Americas (21, 25, 35, 47, 50). Some studies have indicated the presence of MβL genes among multidrug-resistant strains in Brazil (10, 31), and this evidence was later confirmed by the report of a *P. aeruginosa* strain producing a new MβL subclass, namely, SPM-1 (26, 48).

Most mobile MβL genes are part of a gene cassette consisting of a single gene and a downstream recombination site, known as a 59-base element (59-be) (36). The genes are usually associated with class 1 and 3 integrons (5), apart from SPM-1, for which the genetic environment has recently been described (34). Among MβL genes, the class 1 integron is the most commonly encountered and consists of a 5′ conserved sequence (5′-CS), which constitutes an *intI1* gene coding for an integrase; a recombination site, *attI1*; a promoter; and usually, a 3′-CS that possesses the *qacEΔ1* and *sul1* genes (9). Integrons are able to capture gene cassettes by a site-specific recombination event between two recombination sites, one in the integron and one in the cassette. Both recombination sites confer mobility due to their recognition by the integrase that catalyzes the integration of the gene cassette between *attI1* in

* Corresponding author. Mailing address: Laboratório Especial de Microbiologia Clínica, Division of Infectious Diseases, Universidade Federal de São Paulo, Rua Leandro Dupret, 188 São Paulo, SP 04025-010, Brazil. Phone: (55-11) 5081-2819. Fax: (55-11) 5571-5180. E-mail: rodrigo.mendes@lemc.com.br.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source or reference
Strains		
P. aeruginosa 101-4704	Carbapenem-hydrolyzing clinical isolate	This study
Rifampin-resistant *P. aeruginosa* pAO1	*algU*$^+$ *algD*$^+$ *algW*$^+$ *mucD*$^+$ Alg (wild type) Rif	14
Rifampin-resistant *E. coli* K-12	Rif Nal^r	This study
XL10-Gold Kan *E. coli* cell	Tet^r (*mcrA*) 183 (*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lac1*^{q}*Z* · *M15* Tn*10* (Tet^r) Tn*5* (Kan^r) Amy]	Stratagene
E. coli DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	48
Plasmids		
pPCRScriptCam SK(+)	Chloramphenicol resistant	Stratagene
pREM-1	819-bp PCR product from *aac(6′)-30* cloned into pPCRScriptCam SK(+)	This study
pREM-2	1429-bp PCR product from *aac(6′)-30*/*aac(6′)-Ib′* cloned into pPCRScriptCam SK(+)	This study
pREM-3	748-bp PCR product from *aac(6′)-Ib′* cloned into pPCRScriptCam SK(+)	This study
pREM-4	1,817-bp PCR product from $bla_{\text{IMP-16}}$ and *aac(6′)-30* cloned into pPCRScriptCam SK(+)	This study

the integron and the 59-be in the gene cassette. Although recombination between different sites has been documented, it occurs at a very low frequency (30, 36).

In the present study, we describe the $bla_{\text{IMP-16}}$ variant, the kinetic parameters for IMP-16, and the genetic context of the $bla_{\text{IMP-16}}$-carrying integron, which also included a fused form of aminoglycoside-modifying resistance gene *aac(6′)-30*/*aac(6′)-Ib′*, found in a *P. aeruginosa* strain (strain 101-4704) isolated from the Latin American component of the SENTRY Antimicrobial Surveillance Program.

MATERIALS AND METHODS

Clinical context of carbapenem-hydrolyzing *P. aeruginosa* index strain (strain 101–4704). The SENTRY Program was established in 1997 to monitor the most important pathogens and antimicrobial resistance patterns of the organisms causing nosocomial and community-acquired infections via a broad network of sentinel hospitals distributed by geographic location and bed capacity. As part of that study, a *P. aeruginosa* index strain (strain 101-4704) was isolated from a 60-year-old man with bronchogenic carcinoma who was first admitted to the Hospital de Base do Distrito Federal, Brasília, Brazil, in May 2001. A pneumonectomy of the right lung was performed in July 2001, and in August 2001 the patient developed severe pneumonia and required mechanical ventilation. An empyema was also diagnosed, and drainage was performed. The patient's symptoms improved, but he had three other episodes of pneumonia and remained in the intensive care unit for 151 days.

Several pathogens were recovered from respiratory specimens, including *P. aeruginosa*, an *Acinetobacter* sp., and *Stenotrophomonas maltophilia*, but all blood cultures were negative. When the patient was in the intensive care unit he received meropenem at 1 g every 8 h (for 54 days), amikacin at 500 mg every 12 h (q12h; for a total of 42 days), trimethoprim-sulfamethoxazole at 800 mg every 6 h (for 16 days), ciprofloxacin at 400 mg q12h (for 14 days), and vancomycin at 1 g q12h (for 21 days). The patient was subsequently discharged. In April 2002, he was readmitted with cough and dyspnea. Pulmonary secretions collected during a bronchoscopy yielded the *P. aeruginosa* index strain, strain 101-4704. No antimicrobial was administered to the patient, and he was discharged in May 2002. Strain 101-4704 was resistant to several β-lactams, including meropenem and imipenem (MICs $>$ 16 μg/ml), but was susceptible to aztreonam, piperacillin, and piperacillin-tazobactam. The microorganism was also resistant to most aminoglycosides, including gentamicin, tobramycin, and netilmicin, but remained susceptible to amikacin and the fluoroquinolones.

Susceptibility testing. The susceptibilities of all isolates collected in the SENTRY Program were tested by the reference broth microdilution method described by the National Committee for Clinical Laboratory Standards (NCCLS) (27). The aminoglycoside resistance profiles of *Escherichia coli* DH5α harboring recombinant plasmids were assayed by MIC determinations by either the reference agar dilution method or Etest (AB Biodisk, Solna, Sweden) methodology, according to the guidelines of NCCLS and the manufacturer, respectively. Antimicrobial agents were obtained from the respective manufacturers; and quality control was performed by concurrent testing of *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212.

Phenotypic detection of β-lactamase enzymes. Isolates were initially screened for the production of MβLs by the modified disk approximation test. Briefly, a 100-mm-diameter Mueller-Hinton agar plate was inoculated with a 0.5 McFarland standard suspension from a fresh overnight culture. Imipenem, meropenem, and ceftazidime disks were strategically aligned around disks that contained either EDTA (750 μg) or thiolactic acid (0.3 μl) as MβL inhibitors. The test result was read after 20 h of incubation at 35°C. Any appearance of either an elongated or a phantom zone between the carbapenems and/or ceftazidime and either one of the disks containing an MβL inhibitor was considered a positive test result. IMP-2-producing *Acinetobacter baumannii* 54/97 was used as a positive control. MβL Etest strips were used to confirm the disk approximation test results. In addition, ceftazidime–ceftazidime-clavulanic acid and cefepime–cefepime-clavulanic acid ESBL Etest strips were used to evaluate the organisms for the production of extended-spectrum β-lactamases (ESBLs).

Bacterial strains, plasmids, conjugation, and transformation. The bacterial strains and plasmids used in this study are described in Table 1. Plasmid DNA from *P. aeruginosa* 101-4704 was extracted with a plasmid DNA Midi kit (Qiagen, Chatsworth, Calif.). The transfer of β-lactam resistance markers from strain 101–4704 into *E. coli* DH5α and a rifampin-resistant (Rif^r) mutant of *P. aeruginosa* pA01 was performed with a Gene Pulser apparatus (Bio-Rad, Watford, United Kingdom) that was set at 2.5 kV, 25 μF, and 400 Ω. The transfer of resistance to Rif^r mutant *E. coli* K-12 and *P. aeruginosa* pAO1 was also performed by conjugation experiments, as described previously (40). Strains DH5α, pAO1, and K-12 harboring the possible plasmids of strain 101-4704 were selected by plating the strains onto nutrient agar plates containing ceftazidime (10 μg/ml) or ceftazidime and rifampin (500 μg/ml).

Analytical IEF. The β-lactamase extract from *P. aeruginosa* strain 101-4704 was obtained by cell lysis with a BugBuster apparatus (Novagen, Nottingham, United Kingdom), and isoelectric focusing (IEF) was performed with a NOVEX apparatus (Invitrogen, Paisley, United Kingdom). The focused β-lactamases were detected by overlaying the gel with nitrocefin solution (150 μM; Microbiology Systems, Cockeysville, Md.). The β-lactamase pIs were estimated by linear regression, obtained by comparison to the pIs of reference proteins by using a standard IEF marker containing proteins with a pI range of 4.5 to 9.5 (Bio-Rad).

PCR and DNA sequencing. The SENTRY Program strains with positive MβL phenotypic test results were screened for MβL genes by standard PCRs (40) with Extensor Hi-Fidelity PCR Master Mix (ABgene, Surrey, United Kingdom) and primers targeting conserved regions of bla_{VIM}, bla_{IMP}, and bla_{SPM}. Additional primers designed to target the 5′-CS and 3′-CS regions of the class 1 integron were used to amplify the $bla_{\text{IMP-16}}$-containing integron resident in *P. aeruginosa* 101-4704. These primers yielded PCR products, and both strands were sequenced on a Perkin-Elmer system 377 DNA sequencer (Advanced Biotechnology Centre, London, United Kingdom). The DNA sequences were found to overlap, and these were assembled to produce a contiguous sequence of 4,333 bp.

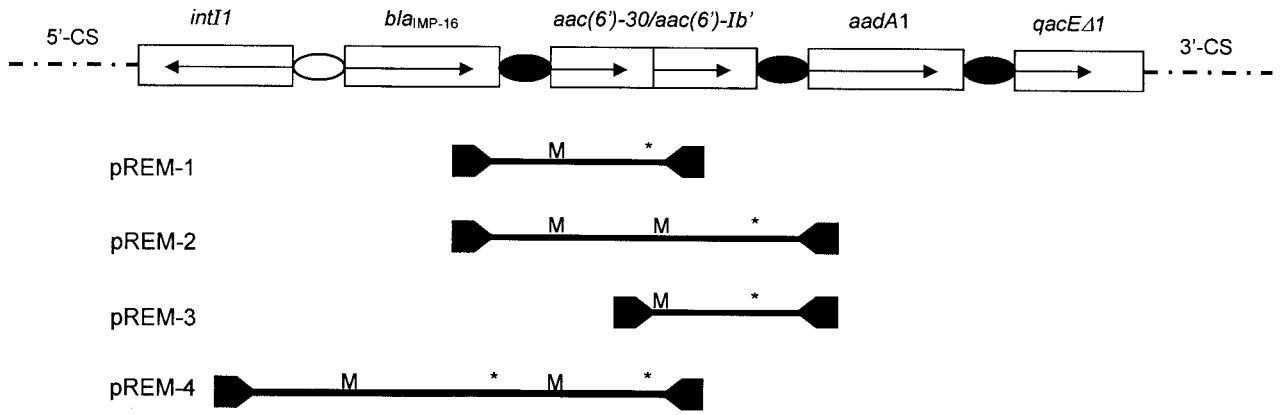


FIG. 1. Schematic representation of the class 1 integron-containing bla_{IMP-16} gene cassette from clinical isolate *P. aeruginosa* 101–4704. Boxes, inserted genes; arrows, transcriptional orientations; black circles, 59-be's; white circle, *attI1* recombination site; lines, DNA of the inserts contained within recombinant plasmids pREM-1, pREM-2, pREM-3, and pREM-4; arrowheads, primer positions and their orientations; M, start codon; asterisk, the location of the stop codon for the particular gene.

Recombinant DNA methodology. The bla_{IMP-16} and aminoglycoside resistance genes were amplified by PCR. Primers were designed to amplify specific individual genes or sets of genes that could subsequently be cloned into pPCRScript-Cam SK(+) (Stratagene Cloning Systems, La Jolla, Calif.). bla_{IMP-16} and the downstream region were amplified with primer set Int1-1F–aacA4FR. The fused-form gene *aac(6′)-30/aac(6′)-Ib′* was amplified with primer set IMP-16FF–aadA1FR. Additionally, *aac(6′)-30* and *aac(6′)-Ib′* were separately amplified with primer sets IMP-16FF–aacA4FR and aacA30FF-aadA1FR, respectively (Fig. 1). The ribosome-binding site and the stop codon were included in order to allow gene expression. This technique yielded several subclones of the original integron that were subsequently screened by PCR with primer set M13F-M13R, and the presence of the insertion and its orientation were confirmed by sequencing. Because XL10-Gold Kan ultracompetent *E. coli* cells are intrinsically resistant to streptomycin due to a chromosomal mutation, the recombinant plasmids were transferred into *E. coli* DH5α, and their respective antimicrobial resistance profiles were evaluated.

β-Lactamase purification. A single colony of *E. coli* harboring recombinant plasmid pREM-4 was grown overnight in 10 ml of nutrient broth containing ceftazidime (10 μg/ml) and chloramphenicol (30 μg/ml) at 37°C. The cells were harvested by centrifugation (4,000 × *g* for 10 min at 4°C) and then added to 4 liters of terrific broth (12% tryptone, 20% yeast extract, 0.4% glycerol, 0.17 M monopotassium phosphate, 0.72 M dipotassium phosphate) for aerobic growth in an orbital shaker for 24 h at 37°C. The cells were again harvested, as described above, and were resuspended in 100 ml of 10 mM HEPES buffer containing 50 μM $ZnCl_2$ (pH 7.5). The periplasm preparation was obtained by the addition of 400 μl of lysozyme solution (20 mg/ml; Sigma-Aldrich, Poole, United Kingdom) and 400 μl of calcium chloride (147 mg/ml). Cells debris was removed by centrifugation (9,000 × *g* for 20 min at 4°C), the supernatant was loaded onto a Q-Sepharose (quaternary ammonium) HR 16/50 column (Amersham Pharmacia Biotech, Uppsala, Sweden), and then the proteins were eluted with a linear NaCl gradient (0 to 1 M) at a flow rate of 2 ml/min. Fractions containing β-lactamase activity were pooled, concentrated, and then injected onto a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech) that had previously been equilibrated with HEPES buffer containing 50 μM $ZnCl_2$ and 0.2 M NaCl. Proteins were eluted at a flow rate of 0.7 ml/min. During the purification procedure the presence of β-lactamase activity was monitored with nitrocefin solution.

Protein electrophoretic technique. The enzyme that was obtained was submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a Mini-Protein II apparatus (Bio-Rad) in order to confirm its purity.

Kinetic measurements. The reactions for kinetic measurements were performed at 20°C with 20 μl of enzyme in 1 ml of HEPES buffer containing 50 μM $ZnCl_2$ (pH 7.0). Hydrolysis was measured by observing the changes in absorbance due to the opening of the β-lactam ring at a range of concentrations in a Lambda 35 spectrophotometer (Perkin-Elmer, Cambridge, United Kingdom). The steady-state kinetic parameters K_m (in micromolar units) and k_{cat} (per second) were deduced from the initial rates of hydrolysis by using the Hanes-Woolf plot (41). The extinction coefficients and wavelengths for each antimicrobial agent evaluated were those described previously (26).

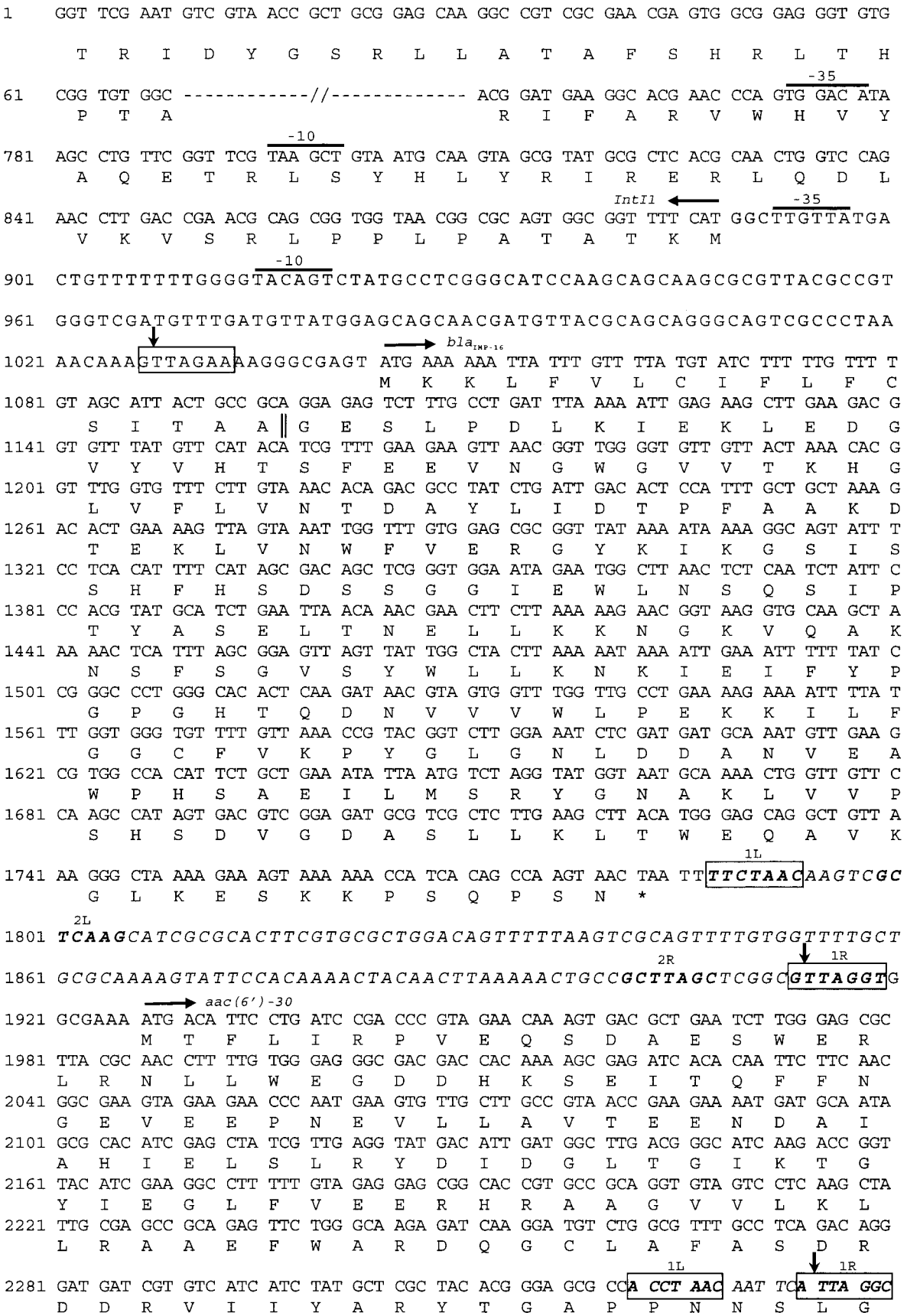
Computer sequence analysis. The nucleotide sequences were compared by using software available over the Internet (http://www.ebi.ac.uk/fasta33/). The nucleotide sequences and their deduced protein products, alignments, and phylogenetic relationships were determined with the Lasergene software package (DNASTAR, Madison, Wis.). The putative cleavage site of the signal sequence of IMP-16 was identified by computer analysis with software available at the Center for Biological Sequence Analysis website (http://www.cbs.dtu.dk).

Nucleotide sequence accession number. The nucleotide sequence of the bla_{IMP-16}-containing integron described in this paper has been submitted to the EMBL/GenBank/DDBJ sequence databases and assigned accession number AJ584652.

RESULTS

Genetic environment of bla_{IMP-16}. The bla_{IMP-16} gene was located in a class 1 integron that contained a 5′-CS composed of integrase gene *intI1*, the *attI1* recombination site, and a promoter region (Fig. 2). The P1 promoter (−35 [TGGACA]; −10 [TAAGCT]) was identified and was found to contain two hexamers separated by 17 bp, followed by a second promoter, P2 (−35 [TTGTTA]; −10 [TACAGT]), 82 bp downstream of P1. The fused disinfectant determinant and sulfonamide gene *qacEΔ1/sul1* was found at the 3′-CS end. The integron consisted of four antimicrobial resistance gene cassettes (Fig. 1). Located at the first position downstream of the 5′-CS was the MβL bla_{IMP-16}, which was flanked by typical features of a gene cassette, namely, a core site (GTTACGC), an inverse core site (TTCTAAC), and a 59-be (Fig. 2). This 59-be was 133 bp in length and showed the greatest identity to the 59-be from IMP-11, which has been found in *P. aeruginosa* (EMBL/GenBank/DDBJ accession no. AB074437) and *A. baumannii* (EMBL/GenBank/DDBJ accession no. AB074436) strains isolated in Japan. The bla_{IMP-16} 59-be sequence differed by 11 of 132 bp (90.6% identity) from the bla_{IMP-11} 59-be sequence.

***bla*$_{IMP-16}$ sequence analysis and its deduced protein sequence.** bla_{IMP-16} encoded a putative protein of 246 amino acids and presented a G+C content of 38.5%. The N terminus of the protein showed features typical of bacterial signal peptides that target proteins to the periplasmic space, and the most likely cleavage site was identified between the alanine and glycine residues (Fig. 2). This produced a mature protein


-35
-10
IntI1
-35
-10
bla IMP-16
1L
2L
2R
1R
aac(6')-30
1L
1R

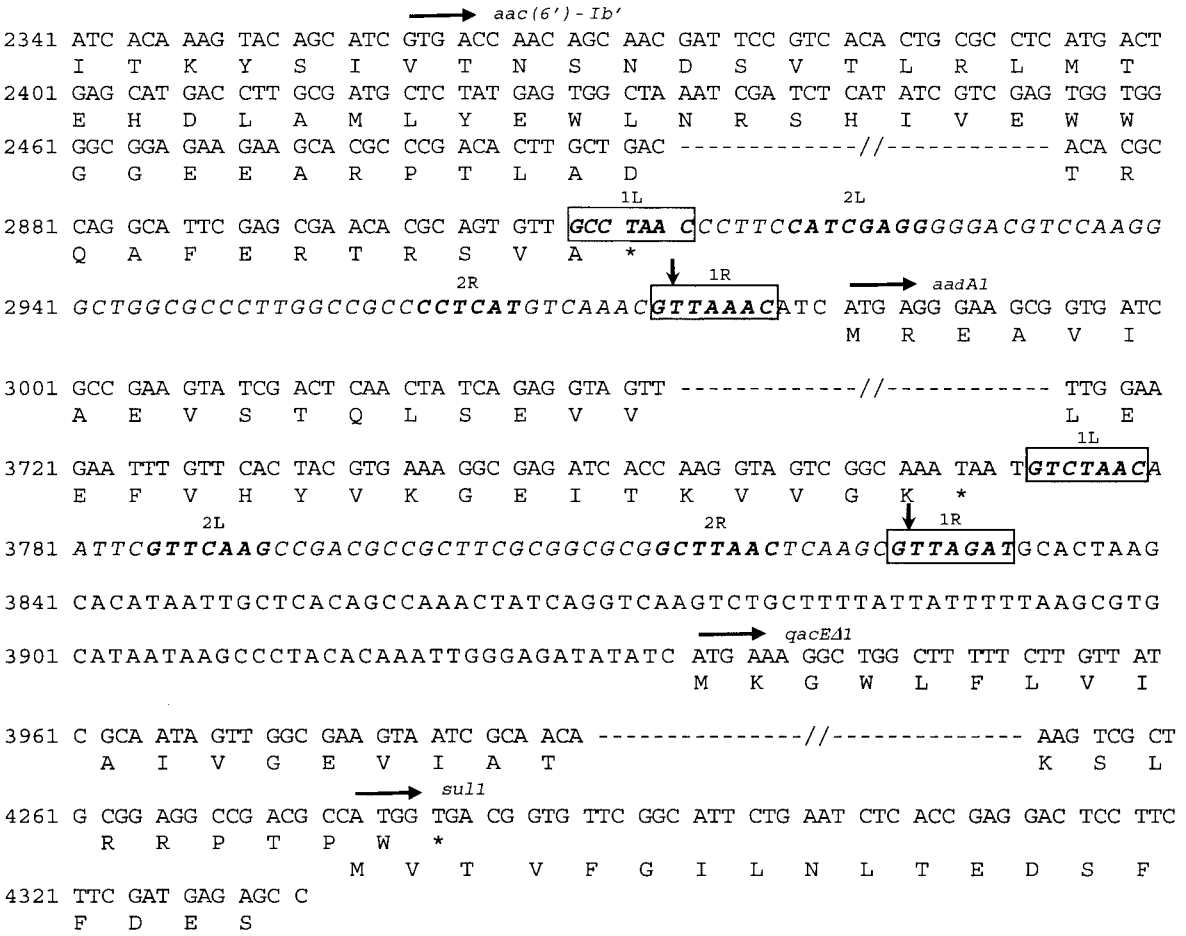


FIG. 2. DNA and amino acid sequences of the bla_{IMP-16}-carrying integron and its gene. The start and stop codons of the ORFs are indicated by horizontal arrows and asterisks, respectively. The corresponding predicted protein translation is reported below the DNA sequence. The double slashes represent the putative cleavage site of the signal sequence of IMP-16. The conserved 7-bp core sites located at the cassette boundaries and the 7-bp inverse core sites located at the left end of each 59-be are boxed. The recombination crossover sites are indicated by vertical arrows. The 59-bes are in italic; and their conserved sequences are highlighted in boldface and are labeled 1L, 2L, 2R, and 1R. The entire sequence has been assigned EMBL/GenBank/DDBJ accession number AJ584652.

of 25,266 Da with a theoretical pI of 6.5. The results of the analytical IEF experiment gave a pI that was in accordance with the theoretical pI of bla_{IMP-16} (data not shown). IMP-16 showed six unique amino acid differences compared to the sequences of the other IMP variant, namely, E50D, V74F, T88A, T122S, V183L, and K252R, and displayed the greatest identities to IMP-11 (90.3%) and IMP-8 (89.5%), with 21 and 24 amino acid differences at the level of the mature protein, respectively (Fig. 3). The MβL active site implicated in the binding of zinc ions, as well as residues known to not tolerate substitutions, were conserved, as previously described for all the other IMP-type enzymes (Fig. 3).

Antimicrobial resistance pattern of *E. coli* DH5α harboring recombinant plasmid pREM-4. bla_{IMP-16} was amplified by PCR and ligated into a vector, pPCRScriptCam SK(+), in order to create recombinant plasmid pREM-4, which was expressed in *E. coli* DH5α (Fig. 1). *E. coli* DH5α harboring recombinant plasmid pREM-4 showed an antimicrobial resistance profile consistent with that observed for the index strain, strain 101-4704. It showed some degree of resistance to benzylpenicillin, ampicillin, amoxicillin-clavulanate, ceftazidime, and cefotaxime and decreased susceptibility to cefepime, piperacillin, piperacillin-tazobactam, imipenem, and meropenem. The index strain as well as *E. coli* DH5α(pREM-4) remained highly susceptible to the monobactam aztreonam (Table 2).

MβL resistance marker transfer experiments. Conjugation experiments between rifampin-susceptible (Rif^s) strain *P. aeruginosa* 101-4704 and Rif^r strain *E. coli* K-12, as well as *P. aeruginosa* 101-4704 and Rif^r strain *P. aeruginosa* pA01, did not yield transconjugants. Despite repeated attempts, analysis of plasmid DNA from *P. aeruginosa* 101-4704 did not identify any plasmid, and transformation experiments were unsuccessful. As a β-lactam resistance marker was not transferred to the recipient strains, even in the conjugation experiments, bla_{IMP-16} is likely encoded by the chromosome and is not carried on a plasmid.

Genetic context of fused gene *aac(6')-30/aac(6')-Ib'*. An open reading frame (ORF) of 984 bp was detected immediately downstream of bla_{IMP-16}. The ORF was preceded by a ribosome-binding site and potentially encoded a protein of

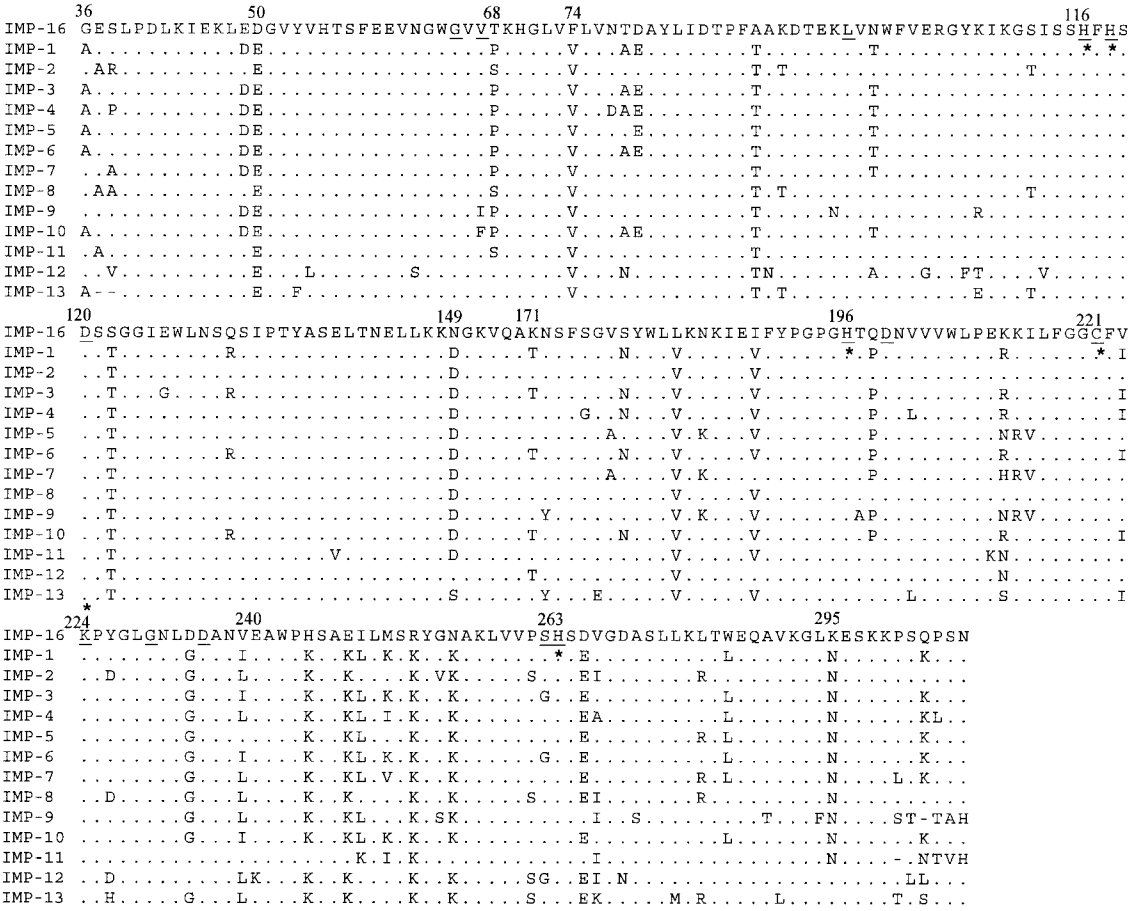


FIG. 3. Amino acid alignment of the IMP-16 protein sequence with those of IMP-type enzymes. Differences in the amino acid sequences are noted by insertion of a single letter representing the amino acid change within that particular sequence. Asterisks under the IMP-16 sequence represent amino acids involved in the coordination of zinc ions, and residues known to not tolerate substitutions are underlined. References for each sequence are as follows: IMP-1 (20), IMP-2 (37), IMP-3 (15), IMP-4 (4), IMP-5 (7), IMP-6 (52), IMP-7 (13), IMP-8 (51), IMP-9 (EMBL/GenBank accession no. AY033653), IMP-10 (16), IMP-11 (EMBL/GenBank accession no. AB074437), IMP-12 (8), IMP-13 (46), and IMP-16 (this study). Numbering is according to the scheme for class B β-lactamases (12).

36.7 kDa. This ORF consisted of a novel gene cassette, namely, *aac(6′)-30* fused with the *aac(6′)-Ib′* gene. *aac(6′)-30* was also flanked by typical features, but it presented a shortened 59-be of 19 bp, including the core and inverse core sites (Fig. 2). *aac(6′)-Ib′* had a core site with a 1-bp mismatch, an A residue (in boldface) instead of the usual G residue (**A**TTAGGC) and an inverse core site (GCCTAAC), and the translation could start at the GTG codon located 19 bp downstream from its core site or at either one of the ATG codons located farther downstream (Fig. 2) (11, 28).

***aac(6′)-30*/*aac(6′)-Ib′* sequence analysis and its deduced protein sequence.** The deduced amino acid sequence of the *aac(6′)-30* product possessed the highest similarity (52.7%) to previously described protein AAC(6′)-29b (EMBL/GenBank/DDBJ accession no. AAK26254) (Fig. 4) (32). Except for the substitution aspartate-171→valine, AAC(6′)-Ib′ contained a sequence homologous to the previously described product of *aac(6′)-Ib′* (EMBL/GenBank/DDBJ accession no. AAA25685), which encodes the aminoglycoside 6′-*N*-aminoglycoside acetyltransferase. This gene was characterized by a leucine-90→serine substitution and specified a type II enzyme that conferred resistance to gentamicin but not to amikacin (19).

IMP-16 purification and kinetics. The IMP-16 enzyme was overproduced and purified from *E. coli*(pREM-4) by fast-performance liquid chromatography, followed by a gel permeation chromatography step. The yield of the IMP-16 purified protein preparation was 0.3 μM, which appeared to contain a single band just under the band at 28.8 kDa, and it was estimated to be >95% pure (data not shown). Steady-state kinetics demonstrated that IMP-16 was able to hydrolyze several β-lactams, including penicillins, narrow- to expanded-spectrum cephalosporins, and carbapenems. Kinetic values showed that the cephalosporins and the carbapenems were the best substrates (k_{cat}/K_m ratios, ≥0.15 $\mu M^{-1} \cdot s^{-1}$), while penicillins were uniformly poorer substrates (k_{cat}/K_m ratios, ≤0.13 $\mu M^{-1} \cdot s^{-1}$) (Table 3). No hydrolysis of cefoxitin or aztreonam was observed.

Expression of aminoglycoside-modifying genes in *E. coli* DH5α. The *P. aeruginosa* index strain was resistant to kanamycin, tobramycin, gentamicin, and netilmicin but was suscep-

TABLE 2. Antimicrobial susceptibility profiles of bla_{IMP-16}-carrying clinical isolate *P. aeruginosa* 101-4704; *E. coli* DH5α harboring recombinant plasmid pREM-1, pREM-2, pREM-3, or pREM-4; and the *E. coli* DH5α recipient strain

Antimicrobial class and agent	MIC (μg/ml)					
	P. aeruginosa 101-4704	*E. coli* DH5α (pREM-1) *aac(6′)-30*	*E. coli* DH5α (pREM-2) *aac(6′)-30/aac(6′)-Ib′*	*E. coli* DH5α (pREM-3) *aac(6′)-Ib′*	*E. coli* DH5α (pREM-4) bla_{IMP-16}	*E. coli* DH5α
β-Lactams						
Benzylpenicillin	>256	—[a]	—	—	64	0.5
Ampicillin	>256	—	—	—	128	2
Amoxacillin-clavulanate	>256/>128	—	—	—	128/64	2
Aztreonam	4	—	—	—	1	0.5
Ceftazidime	>256	—	—	—	32	0.06
Cefotaxime	>256	—	—	—	64	0.12
Piperacillin	32	—	—	—	4	1
Piperacillin-tazobactam	4/4	—	—	—	4/4	0.5
Ticarcillin	128	—	—	—	128	2
Ticarcillin-cavulanate	128	—	—	—	128	2
Cefepime	64	—	—	—	4	0.06
Imipenem	256	—	—	—	0.5	0.12
Meropenem	128	—	—	—	0.25	0.06
Aminoglycosides						
Gentamicin	16	1	4	1	—	0.25
Amikacin	4	2	8	2	—	0.5
Kanamycin	128	8	32	16	—	0.5
Neomycin	8	4	8	2	—	≤0.25
Netilmicin	128	1	4	2	—	0.5
Sisomicin	64	1	4	4	—	≤0.25
Isepamicin	2	1	1	1	—	0.12
Tobramycin	32	4	8	8	—	0.25

[a] —, not applicable.

tible to amikacin and isepamicin (Table 2). In order to evaluate the functional status of AAC(6′)-30 and AAC(6′)-Ib′, their respective genes, as well as the fused form, were amplified by PCR and ligated into the pPCRScriptCam SK(+) vector, creating recombinant plasmids pREM-1, pREM-2, and pREM-3 (Fig. 1). *E. coli* harboring pREM-1 [AAC(6′)-30] showed decreased susceptibilities to amikacin, kanamycin, tobramycin, and neomycin but remained susceptible to gentamicin, sisomicin, isepamicin, and netilmicin. *E. coli* harboring pREM-2 [*aac(6′)-30/aac(6′)-Ib′*] showed decreased susceptibilities to all aminoglycosides tested, apart from isepamicin. Strikingly, *E. coli* harboring pREM-2 [AAC(6′)-Ib′] did not confer the expected AAC(6′)-II phenotype, since it remained susceptible to gentamicin. Increases in the MICs of kanamycin, tobramycin, and sisomicin were observed (Table 2).

DISCUSSION

Multidrug-resistant *Pseudomonas* sp. isolates continue to be a growing concern, particularly for institutions where carbapenems are readily used. Many hospitals in Brazil routinely use carbapenems and expanded-spectrum cephalosporins to treat infections caused by gram-negative organisms, which provides selective pressure for the development of resistance. *P. aeruginosa* 101-4704 remained susceptible to amikacin, aztreonam, piperacillin, and fluoroquinolones, yet it displayed high-level resistance to most other aminoglycosides and β-lactams (including carbapenems), a resistance phenotype that is becoming common in Brazil.

The kinetics of IMP-16 showed that the enzyme hydrolyzes β-lactams less efficiently than IMP-1 does (Table 3). However, similar to other IMP variants, IMP-16 demonstrated an overall preference for cephalosporins and carbapenems rather than penicillins. The poor hydrolytic activity of IMP-16 toward piperacillin (k_{cat}/K_m ratio, 0.09 $\mu M^{-1} \cdot s^{-1}$) was consistent with the relatively low increase in the piperacillin MIC for *E. coli* harboring recombinant plasmid pREM-4. The k_{cat}/K_m ratios of IMP-16 for penicillins showed very similar results (0.09 to 0.13 $\mu M^{-1} \cdot s^{-1}$, which may suggest that the structure of the C-6 side chain, which varies among penicillins, did not affect the hydrolytic activity of IMP-16.

The kinetic data also revealed a lower k_{cat}/K_m value for imipenem (0.36 $\mu M^{-1} \cdot s^{-1}$), essentially due to a higher K_m value. Other IMP variants, such as IMP-3 and IMP-12, also showed similar k_{cat}/K_m ratios (0.08 and 0.26 $\mu M^{-1} \cdot s^{-1}$, respectively), apparently due to the S262G substitution (8, 15). This amino acid mutation was not present in IMP-16, and no other amino acid changes were present in IMP-3, IMP-12, and IMP-16 which were absent from the other IMP variants with higher levels of imipenem hydrolysis. However, IMP-16 showed six unique amino acid substitutions (Fig. 3), which included V74F, located seven residues after the IMP loop site, and T122S, just adjacent to the zinc ligand Asp120. These changes may have contributed to the IMP-16 hydrolytic activity.

The 5′ region of the contiguous gene *aac(6′)-30/aac(6′)-Ib′*, located downstream from bla_{IMP-16}, was associated with a shortened 59-be. To date, there have been only three cases in which a complete cassette was also found in an alternative form with a shorter 59-be (30). *aac(6′)-30* is the second case in which the complete version of the gene has not yet been found (33). It appears likely that the creation of the short *aac(6′)-30*

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AAC(6')-30  MTFLIRPVEQSDAESWERLRNLLWEG---DDHKSEITQFFNGEVEEPNEVLLAVTEENDA      57
AAC(6')-29a .SI....KEQ..AD.LA......LA------...A...E.Y.S.GL.GLV...I.RDATGA.  55
AAC(6')-29b .SI....KEQ..AD.LA......LA------...A...E.Y.S.GF...A...I.RDATGA.  55
AAC(6')-Ic  .-IV--ICDHDNLDA.LA..TA..PSGSPE..RA.MREILAS--PHH-TAFM.RGLDGAF    54
AAC(6')-If  .D-------EASLSM.VG..SQ..PDHSYE..ILDSQHILSC--PDKYVSF..INNQSQ.    51
AAC(6')-Ig  .NIK--.ASEASLKD.LE...K..SDSE-AS.LQ.MH.LLAE--KYAL-Q...YSD-HQ.    53
AAC(6')-Ih  .NIM--.ISE.QLSD.LA..C...PDHE-.V.LQ.MR.LITQ--AHRL-Q...Y.DTQQ.    54
AAC(6')-Ij  .NIM---.SE.LMAD.LG..K...PDHD-EA.LQ.MQRLLQQ--TQSL-Q...YSDTQQ.    54
AAC(6')-Ik  .NIK--.ASEASLKD.LK..IK..NDLE-ES.LQ.MH.LLAE--KHAL-Q..VYSD-DH.    53
AAC(6')-Ir  .KIM---.SEPFLAD.LQ..I...PDHE-.A.LL.MR.LLEQ--PHTL-Q..SYNDQQQ.    54
AAC(6')-Is  .NIM--.ISE.QLSD.LA..S...PDHE-.A.LL.MRHVLKQ--TDTL-Q..VYS.TQL.    54
AAC(6')-It  .HIM--.ITE.QLSD.LV..C...PDHE-.ADLQ.MR.LITQ--AHCL-Q...Y.NTQK.    54
AAC(6')-Iu  .NI.--.ISE.QLSD.LA..S...PDHE-EA.LQ.MR.LLKQ--TDTL-Q...YS.TQH.    54
AAC(6')-Iv  .KIM---.ISE.QLSD.LV..C...PDHE-EQ.LQ.MR.LITQ--AHCL-Q...Y.DTQQ.    54
AAC(6')-Iw  .KIM--.ISEALLAD.LQ..I...PDHE-.A.LL.MR.LLTR--TDSL-Q...YS.TQQP    54
AAC(6')-Ix  .NIM---.ISE.QLSD.LA..S...PDHD-.A.LL.MH.LLKQ--TDTL-Q...Y.DSQQ.    54
AAC(6')-Iy  .DIR--QMNKTHL.H.RG..KQ..P.HPD.A.LADGEEILQ---ADHLASFI.MAD-GV.    54

AAC(6')-30  IAHIELSLRYD-IDGLTGIKTGYIEGLFVEERHRAAGVVLKLLRAAEFWARDQGCLAFAS    116
AAC(6')-29a VG.V...I.H.-LEE.Q..........Y.APS..STDL.RRF..ES.K..LE...S....    114
AAC(6')-29b VG.V...I.H.-LEE.Q..........Y.APS..STDL.RRF..ES.K..LE...S....    114
AAC(6')-Ic  V.FA.VA....YVN.CESSPVAFL..IYTA..A.RQ.WAAR.IAQVQE..KQ...SEL..    114
AAC(6')-If  ..FADAAV.H.YVN.CESSPVV.L..I..IPEQ.GH..AKL.VA.VQD.GVAK..TEM..    111
AAC(6')-Ig  ..ML.A.I.FEYVN.TETSPV.FL..IY.LPA..RS..ATM.I.Q..V..KQFS.TE...    113
AAC(6')-Ih  ..ML.A.I..EYVN.TQTSPVAFL..I..LPEY.RS.IATG.VQQV.I..KQFA.TE...    114
AAC(6')-Ij  ..ML.A.I..EYVN.TQTSPVAFL..IY.LPDY.RS.IATH.VQQV.A..KPF..IE...    114
AAC(6')-Ik  VGML.A.I..EYVN.TETSPVAFL..IY.LPEY.RL..ATL.V.QV.A..KQFS.TE...    113
AAC(6')-Ir  V.ML.A.I..EYVN.QQSSPVAFL..IY.LPEY.RL..AST.VQQV.H..KQFA.TE...    114
AAC(6')-Is  ..ML.A.I.HEYVN.TQTSPVAFL..IY.LPEY.RS.IATQ.VQCV.E..KQFA.TE...    114
AAC(6')-It  .GML.A.I..EYVN.TQTSPVAFL..IY.LPEY.RS.IATG.VQHV.I..KQFA.TE...    114
AAC(6')-Iu  ..ML.A.I.HEYVN.TQTSPVAFL..IY.LPEY.RS.IATQ.VQCV.E..KQFA.TE...    114
AAC(6')-Iv  ..ML.A.I..EYVN.TQTSPVAFL..IY.LPEY.RS.IATG.VQHV.I..KQFS.TE...    114
AAC(6')-Iw  ..ML.A.I.HEYVN.TQTSPVAFL..IY.LPE..RS.IATQ.VQQV.Q..KQYA.TE...    114
AAC(6')-Ix  V.ML.A.I.HEYVN.TQTSPVAFL..IYILPEY.RS.IATQ.VQYV.E..KQFA.TE...    114
AAC(6')-Iy  .GFADA.I.H.YVN.CDSSPVVFL..I..LPSF.QR..AKQ.IA.VQR.GTNK..REM..    114

AAC(6')-30  DRD-DRVIIYARYTGAPPNNSLGITKYSIVTNSNDFRHTAPS          157
AAC(6')-29a ..S-....THRKFA.SAV                                   131
AAC(6')-29b ..S-....THRKFA.SAV                                   131
AAC(6')-Ic  .T.IANLDSQ------RLHAA..FAETER.VF---Y.K.L-G          146
AAC(6')-If  .AAL.NH.S.------QMHQA..FEETER.VF----..KRIAG         144
AAC(6')-Ig  .AAL.N..SH------AMHR...FQETEK.VY----.SKKI-D         145
AAC(6')-Ih  .AAL.NQ.SH------AMHQA..FHETER.VY----.KKNI-G         146
AAC(6')-Ij  .AAL.NR.SH------AMHQA..FHETER.VY----.KKHI-G         146
AAC(6')-Ik  .AAL.N..SH------AMHRA..FQETER.VY----.SKKI-D         145
AAC(6')-Ir  .AAL.NT.SH------AMHRA..FQETEC.VY----.KKNI-.         146
AAC(6')-Is  .AALENT.SH------AMHRA..FHETER.VY----.KKNI-G         146
AAC(6')-It  .ATL.NQ.SH------AMHRA..FHETER.VY----.KKNI-G         146
AAC(6')-Iu  .AAL.NT.SH------AMHRA..FHETER.VY----.KKNI-.         146
AAC(6')-Iv  .AAL.NQ.TH------AMHQA..FQETER.VY----.KKNI-G         146
AAC(6')-Iw  .AAI.NT.SH------AMHQA..FHETER.VY----.KKNI-.         146
AAC(6')-Ix  .AAI.NT.SH------AMHRA..FHETER.VY----.KKNI-G         146
AAC(6')-Iy  .TSPENT.SQ------KVHQA..FEETER.IF---Y.KRC            145
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FIG. 4. Comparison of the deduced amino acid sequences of AAC(6′)-30 with those members of the AAC(6′) family. Differences in the amino acid sequences are noted by insertion of a single letter representing the amino acid change within that particular sequence. Motifs A, B, C, and D are conserved among all members of the AAC(6′) family, while motifs E, F, and G are conserved among most of the members of the AAC(6′) subfamily. References for each sequence are as follows: AAC(6′)-Ic (43); AAC(6′)-If (45); AAC(6′)-Ig (18); AAC(6′)-Ih and AAC(6′)-Ij (17); AAC(6′)-Ik (38); AAC(6′)-Ir, AAC(6′)-Is, AAC(6′)-It, AAC(6′)-Iu, AAC(6′)-Iv, AAC(6′)-Ix, and AAC(6′)-Iw (39); AAC(6′)-Iy (23); AAC(6′)-29a and AAC(6′)-29b (32); and AAC(6′)-30 (this study).

59-be occurred through misreading of the 2L 59-be conserved sequence for the natural core sequence during an excision event, as described previously (49). Given the short 59-be, it is possible that the *aac(6′)-30* and *aac(6′)-Ib′* genes were fused and may move as a single unit on excision.

The *E. coli* strain harboring recombinant plasmid pREM-1 [AAC(6′)-30] did not demonstrate an obvious resistance profile; however, a slight increase in aminoglycoside MICs was observed for the strain, suggesting that its complete form may be functional and expresses an AAC(6′)-I phenotype. This phenotype can be inferred because (i) this class of proteins is more active against tobramycin and amikacin than gentamicin (42), and (ii) AAC(6′)-30 revealed a large number of the same conserved residues present in all related members of the AAC(6′) family (motifs A, B, E, and F) (Fig. 4). However, like AAC(6′)-29a and AAC(6′)-29b, AAC(6′)-30 did not contain the G conserved motif, commonly present in most of the AAC(6′) subfamily members (24), probably due to a truncation event in the C-terminal region of these proteins (Fig. 4) (32).

Comparison of the MICs for *E. coli* harboring the three recombinant plasmids showed that pREM-2 [AAC(6′)-30/AAC(6′)-Ib′] conferred broad aminoglycoside-modifying enzyme activity, similar to that of the index strain (strain 101-4704), and a resistance profile similar to that of strain 101-4704. The MICs for *E. coli* harboring plasmid pREM-2 were between two- and fourfold higher than those observed for *E. coli* harboring plasmid pREM-1 [AAC(6′)-30] and *E. coli* har-

TABLE 3. Kinetic parameters of purified IMP-1, IMP-2, IMP-12, and IMP-16

Antimicrobial agent	IMP-1 (20)[e]			IMP-2 (37)[e]			IMP-12 (8)[e]			IMP-16		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
Penicilllin	320	520	0.62	—[b]	—	—	—	—	—	800	7,805	0.10
Ampicillin	950	200	4.8	23	110	0.21	18	1,500	0.012	137	1,065	0.13
Carbenicillin	ND[a]	ND	0.02	252	700	0.36	3.7	175	0.021	433	3,331	0.13
Cloxacillin	—	—	—	—	—	—	—	—	—	33	380	0.09
Piperacillin	ND	ND	0.72	—	—	—	ND	ND	0.023	250	2,804	0.09
Nitrocefin	63	27	2.3	275	95	2.9	570	72	7.9	1,166	115	10.2
Cephalothin	48	21	2.4	—	—	—	118	16	7.4	77	42	1.8
Cefuroxime	8	37	0.22	—	—	—	61	7	8.7	52	49	1.06
Cefoxitin	16	8	2	7	7	1.0	—	—	—	NH[d]	ND	ND
Ceftazidime	8	44	0.18	21	111	0.19	6.7	15	0.45	13	87	0.15
Cefotaxime	1.3	4	0.35	—	—	—	56	22	2.5	35	36	0.97
Cefepime	7	11	0.66	4	7	0.57	15	26	0.58	20	88	0.23
Imipenem	46	39	1.2	22	24	0.92	240	920	0.26	133	365	0.36
Meropenem	50	10	0.12	1	0.3	3.3	9.5	7.2	1.3	23	72	0.32
Aztreonam	<0.01	>1,000	$<1 \times 10^{-5}$	NH	ND	ND	NH	ND	ND	NH	ND	ND

[a] ND, data could not be determined.
[b] —, data not available.
[c] K_m was obtained as the K_i value.
[d] NH, no hydrolysis detected.
[e] The corresponding references for the enzymes whose kinetics were measured previously are provided.

boring plasmid pREM-3 [AAC(6′)-Ib′]. The phenotype expressed by the strain harboring recombinant plasmid pREM-2 may be characterized as an AAC(6′)-II type with an additional decreased susceptibility to amikacin.

Although it is still not clear, the phenotype expressed by the strain harboring recombinant plasmid pREM-2 may be due to the expression of the fused enzyme [AAC(6′)-30/AAC(6′)-Ib′] alone or even expression of the fused enzyme accompanied by the additional expression of AAC(6′)-Ib′, since *aac(6′)-Ib′* contains its own promoter and ribosome-binding site, which are essential for transcription and translation. Thus, the aminoglycoside resistance profile may be maximized due to the expression of both proteins, leading to a synergistic effect. However, the gentamicin and amikacin MICs for the strain harboring recombinant plasmid pREM-2 were fourfold higher than those for the strain harboring pREM-1 and pREM-3, suggesting the activity of a unique protein rather than the additive effects of two enzymes.

The association of mobile MβL genes with aminoglycoside resistance genes has become very common. bla_{IMP}, bla_{VIM}, and the recently discovered gene bla_{GIM-1} (M. Castanheira, R. E. Mendes, F. Schmitz, M. A. Toleman, R. N. Jones, and T. R. Walsh, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-669, p. 76, 2003) all represent MβL genes associated with aminoglycoside-modifying enzymes. The exception among the MβL genes is bla_{SPM-1}, which does not appear to be associated with an integron (34, 48). In the majority of cases the aminoglycoside resistance genes appear to be functional and confer significant resistance. Their mobilization with β-lactamase genes that confer broad-spectrum β-lactam resistance and the fact that both classes of enzymes cannot be neutralized by clinically available enzyme inhibitors result in a situation of great concern regarding the treatment of infections caused by multidrug-resistant gram-negative bacilli.

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