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Characterization of an Integron Carrying bla_{IMP-1} and a New Aminoglycoside Resistance Gene, *aac(6′)-31*, and Its Dissemination among Genetically Unrelated Clinical Isolates in a Brazilian Hospital▽

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Seven bla_{IMP-1}-harboring *Acinetobacter* sp. isolates and one *Pseudomonas putida* clinical isolate were recovered from hospitalized patients. All isolates possessed a class 1 integron, named In86, carrying the same cassette array [bla_{IMP1}, *aac(6′)-31*, and *aadA1*], which was plasmid located in five of the isolates. This report describes the ability of nonfermentative nosocomial pathogens to acquire and disseminate antimicrobial resistance determinants.

Metallo-β-lactamases (MβLs) and aminoglycoside-modifying enzymes (AgMEs) represent a new challenge to antimicrobial therapy of nosocomial infections, since they confer phenotypic resistance to nearly all clinically available β-lactams and aminoglycosides, respectively (1). Several MβL and aminoglycoside resistance genes in nosocomial isolates recovered from Latin American countries have been described previously (7, 8, 9); however, there is limited information regarding the dissemination of these genes in this region. In the present study, we describe a new bla_{IMP-1}-carrying integron that contains a new aminoglycoside resistance gene and its dissemination among genetically unrelated clinical isolates recovered from a Brazilian hospital.

As part of the SENTRY Antimicrobial Surveillance Program (12), gram-negative bacilli recovered from Latin American hospitals between March 2001 and April 2003 were tested for antimicrobial susceptibility by reference methods according to standard guidelines (3, 4). Strains showing combined resistance to ceftazidime (MIC, ≥16 μg/ml), imipenem (MIC, ≥16 μg/ml), and meropenem (MIC, ≥16 μg/ml) were routinely screened for MβL genes by standards PCRs (2, 9, 11). Seven *Acinetobacter* sp. isolates and one *Pseudomonas putida* clinical isolate recovered from a 600-bed tertiary university hospital located in São Paulo, Brazil, were found to harbor bla_{IMP-1} and were further evaluated in the present study (Table 1).

In general, the evaluated isolates showed a decreased susceptibility or resistance phenotype to mostly all the antimicrobial agents tested, showing susceptibility only to polymyxin B, and some isolates also showed susceptibility to quinolones (Table 1). A genotypic comparison of the bla_{IMP-1}-harboring *Acinetobacter* sp. isolates was performed using the RiboPrinter microbial characterization system and pulsed field-gel electrophoresis (PFGE), as previous described (12). *Acinetobacter* sp. isolates 694 and 696 showed identical PFGE patterns, while isolates 9043 and 695 differed from each other in less than five bands (similar PFGE patterns), and they were considered to belong to the same ancestor. The remaining *Acinetobacter* spp. showed distinct PFGE patterns and were considered to be unrelated (16). In summary, five different clones were observed among the seven *Acinetobacter* sp. isolates (Table 1).

The bla_{IMP-1}-containing integrons were amplified by PCR and sequenced using primers targeting the 5′ conserved sequences (CS) and 3′ CS of class 1 integrons (2, 9). All isolates possessed the same class 1 integron cassette arrangement, designated In86. This integron harbored bla_{IMP-1} at the first position downstream of the 5′ CS, followed by an open reading frame of 519 bp identified as a new AgME gene cassette. This gene, designated *aac(6′)-31*, was followed by another AgME gene cassette, namely, *aadA1* (Fig. 1). *aac(6′)-31* potentially encoded a protein of 173 amino acids (19.1 kDa), which exhibited the highest identity (82.1%) to AAC(6′)-Ib′ (GenBank accession number CAE48336) (Fig. 2), encoded by the bla_{IMP-16}-carrying *Pseudomonas aeruginosa* integron isolated from Brasília, Brazil (9), 630 miles from São Paulo, suggesting that these two genes could be derived from a common ancestor.

To evaluate the flanking DNA sequences upstream of In86, these regions were sequenced using a random primer PCR approach as previously described (15). The 5′ CS of In86 in the *P. putida* isolate contained the integrase gene, which was bound by a Tn*402*-like 25-bp IR_i sequence. The DNA sequence upstream of the 25-bp IR_i did not show any homology with previously deposited sequences in the GenBank database (Fig. 1 and 3). However, all the *Acinetobacter* sp. isolates showed the same DNA insertion just downstream of the In86 integrase gene, which was inserted between the i2 and i3 19-bp repeats that are usually present in the internal region of the IR_i

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TABLE 1. Clinical and antimicrobial profile and molecular information from the clinical isolates harboring bla_{IMP-1}-carrying In86 evaluated in the present study

Parameter	Value							
	A. baumannii 694	*A. baumannii* 696	*A. baumannii* 9043	*A. baumannii* 695	*A. baumannii* 501	*Acinetobacter* sp. strain 5227	*Acinetobacter* sp. strain 5248	*P. putida* 12346
Sample collection date (mo/day/yr)	05/28/2002	06/04/2002	06/22/2001	06/02/2002	05/06/2001	08/14/2002	09/28/2002	07/23/2001
Source	SSTI[b]	SSTI[b]	BSI[c]	SSTI[b]	BSI[c]	LRTI[d]	LRTI[d]	BSI[c]
MIC (μg/ml)[a]								
β-Lactam								
Aztreonam	>16	>16	>16	>16	>16	>16	>16	>16
Ampicillin	>16	>16	>16	>16	>16	>16	>16	>16
Amoxicillin-clavulanate	>16	>16	>16	>16	16	>16	>16	>16
Aztreonam	>16	>16	>16	>16	>16	>16	>16	>16
Ceftazidime	>16	>16	>16	>16	>16	>16	>16	>16
Piperacillin	>128	>128	>128	128	32	>128	>128	64
Piperacillin-tazobactam	32	64	>64	64	64	>64	64	64
Ticarcillin	>128	>128	>128	>128	>128	>128	>128	>128
Ticarcillin-clavulanate	>128	>128	>128	>128	>128	>128	>128	>128
Cefepime	>16	>16	>16	>16	>16	>16	>16	>16
Imipenem	>8	>8	>8	>8	>8	>8	>8	>8
Meropenem	>8	>8	>8	>8	>8	>8	>8	>8
Aminoglycoside								
Amikacin	>32	>32	>32	32	32	>32	32	>32
Gentamicin	>8	>8	>8	>8	>8	>8	>8	>8
Netilmycin	32	32	>32	8	>32	>32	16	>32
Tobramycin	16	16	16	>16	>16	16	8	>16
Quinolone								
Levofloxacin	0.25	0.25	>4	>4	0.25	2	4	>4
Gatifloxacin	0.25	0.06	>4	4	0.12	1	4	>4
Ciprofloxacin	1	0.5	>2	>4	0.12	2	>4	>2
Polymyxin B	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
bla_{IMP-1} location	Plasmidial	Plasmidial	Plasmidial	Plasmidial	Plasmidial	Chromosomal	Plasmidial	Chromosomal
Ribotyping	258.105.2	258.105.2	105.815.4	105.815.4	252.43.4	258.148.6	258.148.2	NA[e]
PFGE	PSA48A	PSA48A	PSA48B	PSA48B1	PSA48C	PSA48D	PSA48E	NA[e]

[a] Interpretive criteria for the antimicrobial tested were those published by the Clinical and Laboratory Standard Institute (CLSI) (formerly National Committee for Clinical Laboratory Standards) (4).
[b] SSTI, skin and soft tissue infection.
[c] BSI, bloodstream infection.
[d] LRTI, lower respiratory tract infection.
[e] NA, not applicable.

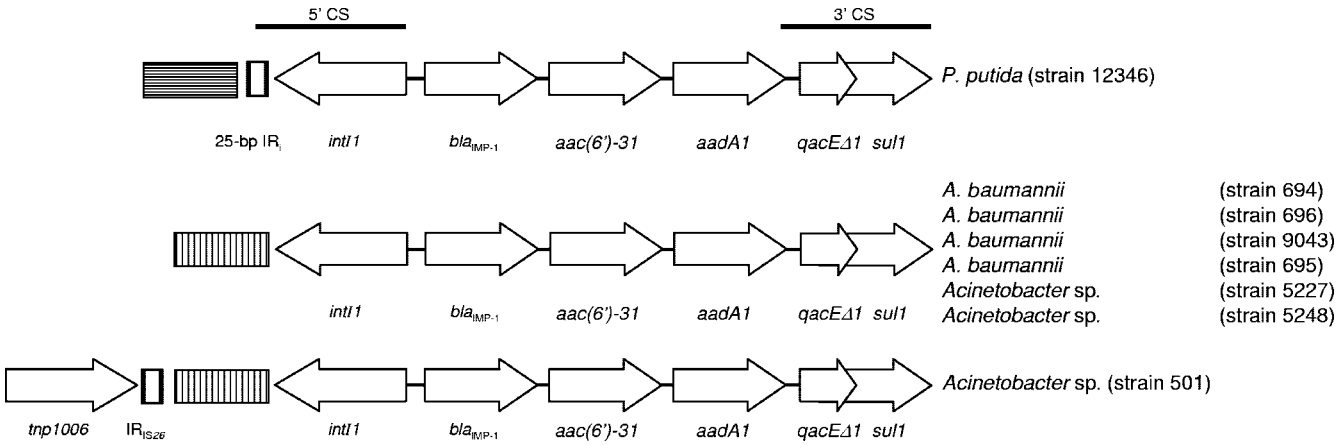

FIG. 1. Schematic representation of the bla_{IMP-1}-containing class 1 integron found in *P. putida* and *Acinetobacter* sp. Inserted genes are indicated by boxes, and the arrows indicate their transcriptional orientations. Boxes with horizontal and vertical bars represent the inserted DNA sequences found upstream of the In86 25-bp IR_i in the *P. putida* strain and in the In86 5′ CS in the *Acinetobacter* sp. strains, respectively.

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AAC(6')-I31   MTTTISFVTLRLMTEHDLPMLHDWLNRPHIVEWWGGEETRPTLAEVLEQYLPSALAKESVTPYIAMLDEEPIGYA  75
AAC(6')-Ib'   ..------------....A..YE....S.........A.....D.Q......V..Q..........NG......  63
AAC(6')-Ib''  ..NSNDS..........A..YE....S.........A.....D.Q......V..Q..........NG......  75

AAC(6')-I31   QSYIALGSGDGWWEDETDPGVRGIDQSLANPSQLGKGLGTKLVCALVEMLFKDAEVTKIQTDPSPNNLRAIRCYE  150
AAC(6')-Ib'   ...V..........E..............A............R....L..N.P..........S.........  138
AAC(6')-Ib''  ...V..........E..............A............R....L..N.P..........S.........  150

AAC(6')-I31   KAGFVAQRTINTPDGPAVYMVQTRQAFEQARSAV.      185
AAC(6')-Ib'   ....ER.G.VT.................RT..-DA     172
AAC(6')-Ib''  ....ER.G.VT.................RT..-.A     184
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FIG. 2. Comparison of the deduced amino acid sequences of the most similar proteins compared to AAC(6′)-31. Differences in the amino acid sequences are noted by the insertion of a single letter representing the amino acid change within that particular sequence. GenBank accession numbers for each sequence are as follows: AAA25685 for AAC(6′)-Ib′ (6) and CAE48336 for AAC(6′)-Ib″ (9).

(13) (Fig. 1 and 3). This sequence also did not possess homology with previously reported DNA sequences. Moreover, *Acinetobacter* sp. isolate 501 showed a second DNA insertion just upstream of the previous one, which was revealed to be a terminal inverted repeat, IS*1006.1*, located 138 bp upstream of the IR_i. IS*1006.1* was closely related to the terminal repeat of IS*26*, IS*1006*, IS*1007*, IS*1009*, and IS*1010* followed by the exact last 39 nucleotides of the *tnp1006* gene (Fig. 3). This structure showed the highest identity to a similar region in the *Acinetobacter lwoffii* plasmid pKLH202 (GenBank accession number AJ486857) (5). These findings suggest that the integron found in the *P. putida* strain was likely to be the progenitor bla_{IMP-1}-carrying integron circulating in this nosocomial environment.

Repeated electroporation and conjugation experiments of putative plasmid DNA extracts from the bla_{IMP-1}-containing strains, performed as previously described (9), failed. Despite several preparations, the presence of plasmid DNA was not observed in *P. putida* and in *Acinetobacter* sp. isolate 5227, suggesting a chromosomal location. The remaining isolates showed several plasmids (data not shown), which were recognized by a bla_{IMP-1}-specific probe in a Southern blot experiment (14), suggesting that In86 was plasmid located in those isolates (data not shown). The bla_{IMP-1} probe recognized plasmids showing similar sizes, apart from *Acinetobacter* sp. isolate 501, which seemed to possess a slightly smaller bla_{IMP-1}-carrying DNA plasmid. These hybridization profiles were in agreement with the sequencing results, which showed an identical structure downstream of the integrase gene in those *Acinetobacter* sp. isolates except for *Acinetobacter* sp. isolate 501, suggesting a distinct genetic locus in the latter strain.

To determine the functionality of *aac(6′)-31*, the gene was amplified by PCR and ligated into a pPCRScriptCam SK(+) vector to construct the recombinant plasmid pIMPAR-31 as previously described (9). This plasmid was subsequently trans-

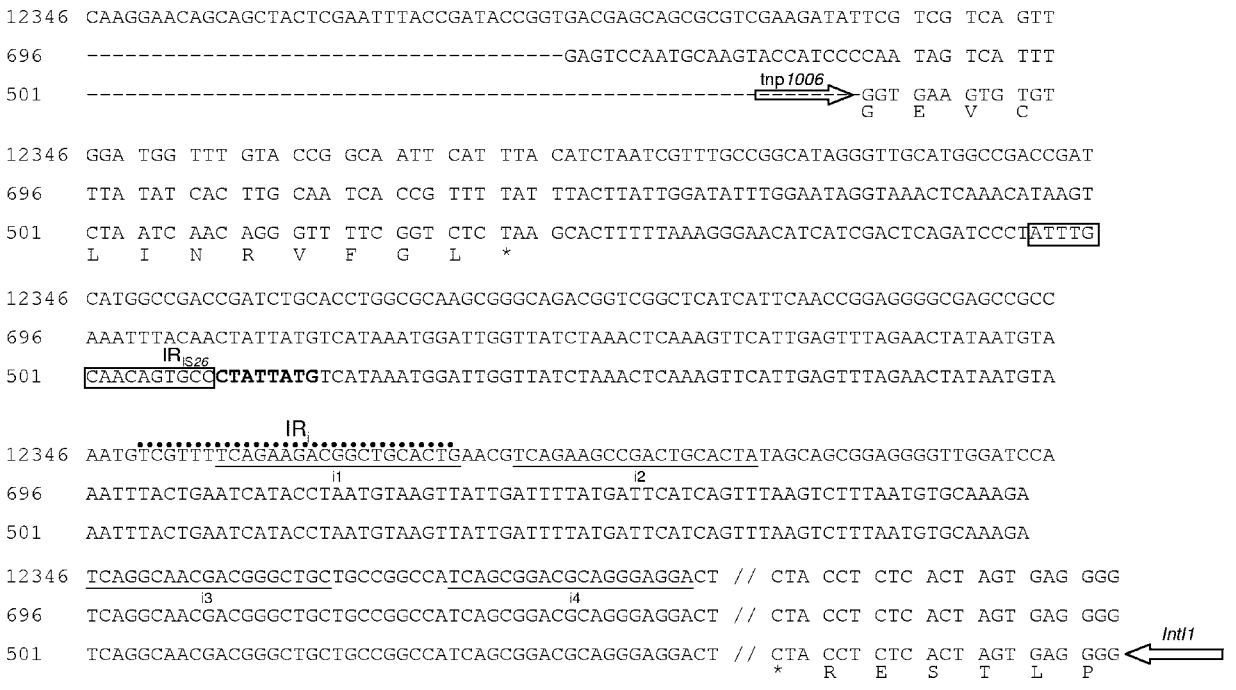

FIG. 3. Nucleotides and amino acid sequences of the In86 5′ CS found in the studied strains. Asterisks and horizontal arrows indicate the stop codon and the transcription direction of the partial open reading frames, respectively. The corresponding predicted protein translation is reported below the DNA sequence. The IS*26* 15-bp inverted repeat is boxed, and the IR_i and the i1 to i4 inverted repeats typical of the 5′ CS of Tn*402*-like elements are also indicated. The hypothetical 8-bp direct repeat of IS*26* is in boldface type.

TABLE 2. Aminoglycoside susceptibility profiles of *E. coli* DH5α-harboring recombinant plasmid pIMPAR-31 and the recipient strain *E. coli* DH5α

E. coli plasmid or strain	MIC (μg/ml)							
	Gentamicin	Amikacin	Kanamycin	Neomycin	Netilmicin	Sisomicin	Isepamicin	Tobramycin
pIMPAR-31	4	8	16	8	4	8	4	4
DH5α	0.25	0.5	0.5	≤0.25	0.5	≤0.25	0.12	0.25

ferred into *Escherichia coli* DH5α cells, and the recombinant strain was tested for susceptibility against several aminoglycosides (Table 2). *aac(6′)-31* expressed in *E. coli* DH5α cells conferred decreased susceptibility to all aminoglycosides evaluated, including gentamicin, tobramycin, kanamycin, amikacin, neomycin, netilmicin, sisomicin, and isepamicin. MICs were 8- to ≥32-fold higher than those for *E. coli* DH5α (Table 2). This phenotype has not been described previously but was observed only when different AgME genes were expressed in combinations. This suggests that the expression of *aac(6′)-31* may be sufficient to confer resistance to all clinically available aminoglycosides, and this gene may gradually replace other *aac(6′)* family genes commonly found among nonfermentative pathogens. A similar situation was previously observed among bacterial strains recovered from Turkey (10).

The presence of five genetically unrelated *Acinetobacter* sp. strains and one *P. putida* strain, sharing a common bla_{IMP-1}-carrying integron during the study period, shows the ability of nonfermentative nosocomial strains to acquire and subsequently spread antimicrobial resistance determinants. This may be the reason for the continuing recovery of bla_{IMP-1}-harboring *P. aeruginosa* and *Acinetobacter* sp. strains in this hospital.

Nucleotide sequence accession numbers. The nucleotide sequences of the bla_{IMP-1}-containing integron described in this paper have been submitted to the EMBL/GenBank/DNA Data Bank of Japan sequence databases and assigned the accession numbers AM283489 (*P. putida*), AM283490 (*Acinetobacter baumannii* strain 501), and AJ640197 (*Acinetobacter* sp. strains 695, 696, 5227, 9043, and 5248).

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