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Characterization of Two Novel 6'-N-aminoglycoside Acetyltransferase Genes, *aac(6')-30* and *aac(6')-31*, Found in bla_{IMP-16} and bla_{IMP-1} *Pseudomonas aeruginosa* Strains Carrying Integron

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SENTRY ANTIMICROBIAL SURVEILLANCE

ABSTRACT

Objectives. Metallo-ß-lactamase (MßL) genes were found embedded in class 1 integrons that also encoded aminoglycoside-modifying enzymes (AgMEs). The aim of this study was to further characterize the two novel AgME genes.

Methods. Primers targeting the 5'CS and 3'CS regions of class 1 integron were used to amplify the bla_{IMP-16} and bla_{IMP-1} containing integron. These primers yielded PCR products, which were sequenced on both strands using DuPont Automated systems. After integron sequence analysis, the AgME genes were amplified by PCR and cloned into the expression vector pPCRScriptCam SK. The recombinant plasmids were transferred into *Escherichia coli* DH5α and their respective aminoglycoside resistance profile evaluated against gentamicin, amikacin, kanamycin, neomycin, netilmicin, sisomicin, isepamicin and tobramycin. Nucleotide sequences and their deduced protein products, alignments and phylogenetic relationships were determined using the Lasergene software package.

Results. Sequence analysis revealed the presence of two novel aminoglycoside genes just downstream of the bla_{IMP-16} and bla_{IMP-1}, namely *aacA(6')-30* and *aacA(6')-31*, respectively. The *aacA(6')-30* was fused with the following gene, *aacA(6')-Ib'*, which formed an open reading frame of 984-bp and potentially encodes a protein of 36.7 kDa. The AAC(6')-30 possessed most identity (52.7%) to the previously described AAC(6')-29b. The *aacA(6')-31* was 555-bp long, encoded a putative protein of 21.2 kDa and was most similar (77.2%) to the *aacA(6')-Ib'* found in the bla_{IMP-16} carrying integron strain. *E. coli* strains harboring the fused form *aac(6')-30/aac(6')-Ib'* and *aacA(6')-31* showed MICs three- to five-fold higher than the recipient *E. coli* DH5α strain, including to gentamicin and amikacin. The MICs remained unaltered to isepamicin.

Conclusions. The fused form AAC(6')-30/AAC(6')-Ib' is likely to be a bifunctional protein rather than the expression of both AAC(6')-30 and AAC(6')-Ib'. The AAC(6')-30/AAC(6')-Ib' and AAC(6')-31 conferred a resistance profile called AAC(6')-IV phenotype. The association of mobile MßL genes with AgME genes presents an immense concern since the enzymes modified by these genes cannot be neutralized by commercially available ß-lactamase inhibitors.

INTRODUCTION

Metallo-ß-lactamases (MßL) represent a new challenge to antimicrobial chemotherapy due to their broad substrate specificity, which includes the carbapenems and nearly all other ß-lactams commercially available. The association of mobile MßL genes with aminoglycoside resistance genes has become very common.

Three classes of aminoglycoside-modifying enzymes (AgME) have been described: nucleotidyltransferases, phosphotransferases and acetyltransferases. The latest is the largest class, which acetylate the 1', 2', 3' and 6' positions of aminoglycosides. Members of the 6'-N-acetyltransferase family [AAC(6')] modify kanamycin, tobramycin, netilmicin, sisomicin, and according to additional antimicrobial modification, the enzyme might also be classified into different types: (i) Type I members [AAC(6')-I] modify amikacin and, in a lesser degree, isepamicin, but not gentamicin; (ii) type II members acetylate gentamicin, but not amikacin or isepamicin; (iii) type III members were recently described and they confer high-level resistance to amikacin and isepamicin; and (iv) type IV confer resistance to gentamicin and amikacin.

The aim of this study was to further characterize two novel aminoglycoside resistance genes found in unrelated MßL-producing *Pseudomonas aeruginosa* clinical isolates collected through the SENTRY Antimicrobial Surveillance Program.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are described in Table 1. Two clinically unrelated *P. aeruginosa* strains, 101-4704 and 48-696, were isolated from hospitalized patients in Brasilia and Sao Paulo, respectively. MßL genes, bla_{IMP-16} (101-4704) and bla_{IMP-1} (48-696), were detected in these strains and further gene sequence analysis revealed that these genes were harbored by a class 1 integron, which also contained aminoglycoside resistance genes.

Susceptibility testing. Aminoglycoside resistance profiles of *Echerichia coli* DH5α harboring recombinant plasmids were evaluated by reference agar dilution or Etest® methodology according to the NCCLS and the manufacturer's guidelines, respectively. Antimicrobial agents were obtained from the respective manufacturers or purchased from Sigma (St. Louis, MO, USA). 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.

PCR and DNA sequencing. MßL genes were detected using primers for conserved regions of bla_{IMP}. Additional primers designed against the 5'CS and 3'CS regions of class 1 integron were used to amplify the bla_{IMP-16} and bla_{IMP-1} containing integron. PCR products were sequenced on both strands using Perking Elmer systems 377 DNA Sequencer.

Recombinant DNA methodology. Aminoglycoside resistance genes found in both MßL-carrying integrons were amplified by PCR. Primers were designed to amplify specific individual or sets of genes that could subsequently be cloned into pPCRScriptCam SK+. The fused form *aac(6')-30/aac(6')-Ib'* was amplified using the primers set IMP-16FF – aadA1FR. Additionally, the *aac(6')-30* and *aac(6')-Ib'* were individually amplified using the primers set IMP-16FF – aacA4FR and aacA30FF – aadA1FR, respectively. The *aac(6')-31* was amplified using the primers set IMP-1FF – aadA1FR (Figure 1). The ribosome binding-site and the stop codon were included in order to allow the gene expression. This technique yielded several sub-clones of the original integron that were subsequently screened by PCR using primer set M13F – M13R and their insert and orientation confirmed by sequencing. Since 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 profile evaluated.

Computer sequence analysis. The nucleotide sequences were deduced using software available over the internet (http://www.ebi.ac.uk/fasta33/). Nucleotide sequences and their deduced protein products, alignments and phylogenetic relationships were determined using the Lasergene software package (DNASTAR, Madison, WI, USA).

RESULTS

Genetic context of the *aac(6')-30* and *aac(6')-31*.

- Immediately downstream of the bla_{IMP-16}, there was an open reading frame (ORF) of 984-bp that potentially encoded a protein of 36.7 kDa. This ORF consisted of a novel gene cassette, namely *aac(6')-30*, fused with the *aac(6')-Ib'* gene. The *aac(6')-30* was flanked by typical features, but it presented a shortened 59-be of 19-bp, including the core and inverse core sites (Figure 1).

- Downstream of the bla_{IMP-1} resided an ORF of 555-bp and further sequence analysis identified it as a new AgME gene, designated *aac(6')-31*. This ORF was flanked by a core site (GTTAGGC), an inverse core site (GTCTAAC) and a 59-be. The translation could start at the ATG codon located 19-bp downstream from its core site or at either one of the ATG codons located further downstream.

Table 1. Bacterial strains and plasmids used in this study.

Strain	Genotype/phenotype
P. aeruginosa 101-4704	bla_{IMP-16}/Carbapenem-hydrolyzing clinical isolate
P. aeruginosa 48-696	bla_{IMP-1}/Carbapenem-hydrolyzing clinical isolate
XL10-Gold® Kan *E. Coli* cell	TetR *(mcrA)183 (mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB lacIqZ.M15* Tn10 (TetR) Tn5 (KanR) Amy].
E. coli DH5α	*SupE44ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*
Plasmid	
pPCRScriptCam SK+	Chloramphenicol
pREM-1	819-bp PCR product from *aac(6')-30* cloned into pPCRScriptCam SK+
pREM-2	1429-bp PCR product from *aac(6')-30/aac(6')-Ib'* cloned into pPCRScriptCam SK+
pREM-3	748-bp PCR product from *aac(6')-Ib'* cloned into pPCRScriptCam SK+
pREM-31	819-bp PCR product from *aac(6')-31* cloned into pPCRScriptCam SK+

Table 2. Aminoglycoside susceptibility profile of the *P. aeruginosa* 101-4704 and *E. coli* DH5α harboring recombinant plasmids pREM-1, pREM-2, pREM-3, pREM-31 and the recipient strain *E. coli* DH5α.

	MIC (mg/L)						
Aminoglycosides	*P. aeruginosa* 101-4704	pREM-1 *aac(6')-30*	pREM-2 *aac(6')-30/aac(6')-Ib'*	pREM-3 *aac(6')-Ib'*	*P. aeruginosa* 48-696	pREM-31 *aac(6')-31*	*E. coli* DH5α
Gentamicin	16	1	4	1	8	4	0.25
Amikacin	4	2	8	2	>32	8	0.5
Kanamycin	128	8	32	16	>256	16	0.5
Neomycin	8	4	8	2	128	8	≤0.25
Netilmicin	128	1	4	2	8	4	0.5
Sisomicin	64	1	4	4	16	8	≤0.25
Isepamicin	2	1	1	1	>256	4	0.12
Tobramycin	32	4	8	8	4	4	0.25

Figure 1. Schematic representation of the class 1 integron containing bla_{IMP-16} and bla_{IMP-1} gene cassette from *P. aeruginosa* 101-4704 and 48-696 clinical isolates, respectively. The DNA of the inserts contained within the recombinant plasmids pPREM-1, pPREM-2, pPREM-3 and pPREM-31 are represented by lines. Inserted genes are indicated by boxes and the arrows indicate their transcriptional orientation. The 59-be's are represented by black circles and the *attI1* recombination site by white circle. The arrowheads represent the primer positions and their orientation. M represents the start codon and asterisk indicates the location of the stop codon for the particular gene.

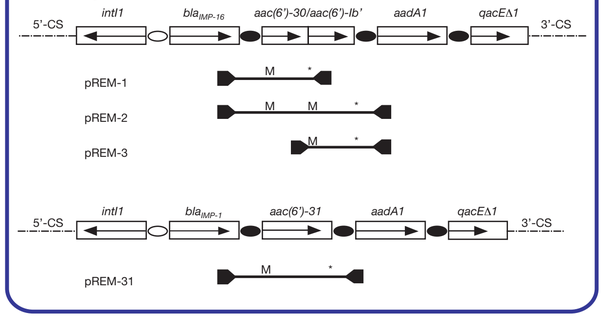


Figure 2. Phylogenetic tree of the AAC(6') family of enzymes and percent amino acid similarity of the AAC(6')-31 when compared with enzymes with the highest identity, AAC(6')-II, AAC(6')-Ib' and AAC(6')-Ib, as well as the percent amino acid similarity of the AAC(6')-30 when compared with enzymes with the highest identity, AAC(6')-29a and AAC(6')-29b. Alignment of protein sequences was performed by the Lasergene software package using ClustalV methods.

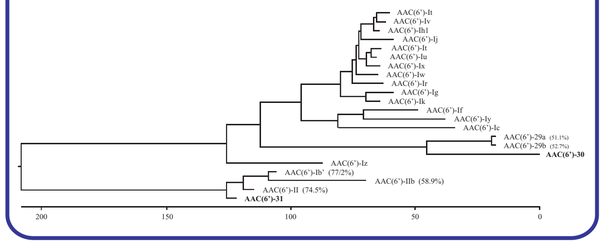
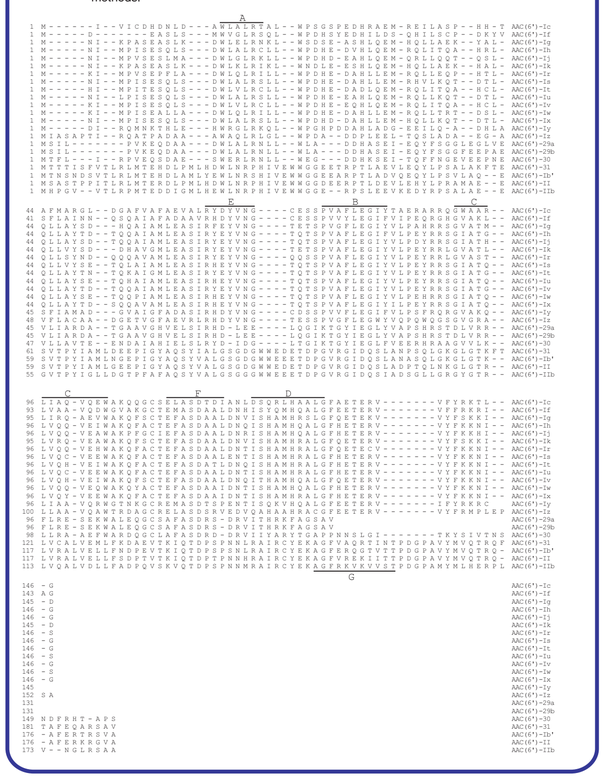


Figure 3. Comparison of the deduced amino acid sequences of the AAC(6')-30 and AAC(6')-31 with those members of the AAC(6') family. Motifs A, B, C and D are conserved among all AAC(6') family, while motifs E, F and G are conserved among most of the members of AAC(6') subfamily. Alignment of protein sequences was performed by the Lasergene software package using ClustalV methods.



***aac(6')-30* and *aac(6')-31* sequence analysis and their deduced protein sequences.**

- The deduced amino acid sequence of the *aac(6')-30* product showed highest identity (52.7%) to the previously described AAC(6')-29b (AAK26254) (Figure 2).

- The *aac(6')-31* potentially encoded a protein of 190 amino acids (20.5 kDa). Nucleotide sequence analysis revealed that the gene product showed highest identity (77.2%) to the AAC(6')-Ib' (AAA25685), which was produced by the bla_{IMP-16}-carrying *P. aeruginosa*. Both proteins encoded the aminoglycoside 6'-N-aminoglycoside acetyltransferase (Figure 2).

- Both enzymes, AAC(6')-30 and AAC(6')-31, revealed a large number of the same conserved residues present in all related members of the AAC(6') family (Figure 3).

Expression of the AgMEs in *E. coli* DH5α.

- *E. coli* harboring the pREM-1 recombinant plasmid [*aac(6')-30*] showed a decreased susceptibility to kanamycin, tobramycin and neomycin, and remained susceptible to gentamicin, sisomicin, isepamicin and netilmicin (Table 2).

- *E. coli* harboring the pREM-2 recombinant plasmid [*aac(6')-30/aac(6')-Ib'*] showed a decreased susceptibility to all aminoglycosides tested, except isepamicin (Table 2).

- Strikingly, the pREM-3 recombinant plasmid [*aac(6')-Ib'*] did not confer the expected AAC(6')-II phenotype, since the *E. coli* strain harboring this plasmid remained susceptible to gentamicin. Increased MIC was observed for kanamycin, tobramycin and sisomicin (Table 2).

- The pREM-31 recombinant plasmid [*aac(6')-31*] conferred resistance to all aminoglycosides evaluated, including gentamicin, amikacin and isepamicin (Table 2).

CONCLUSIONS

- Like AAC(6')-29a and AAC(6')-29b, the AAC(6')-30 did not contain the conserved motif G, commonly present in most of the AAC(6') subfamily members, probably due to a truncation event in the C-terminal region of these proteins (Figure 3).

- The fused form AAC(6')-30/AAC(6')-Ib' (pREM-2) conferred a broad aminoglycoside-modifying enzyme activity and a resistance profile similar to that of the index strain (101-4704).

- pREM-2 recombinant strains showed MIC values two- to four-fold higher than those observed when AAC(6')-30 (pREM-1) and AAC(6')-Ib' (pREM-3) were expressed individually. This phenotype may be characterized as an AAC(6')-II type with an additional decrease in the susceptibility to amikacin.

- The high degree of amino acid similarity between AAC(6')-31 and AAC(6')-Ib', strongly suggests that these two enzymes were derived from a common ancestral gene.

- The strain harboring the pREM-31 recombinant plasmid [*aac(6')-31*] showed decreased susceptibility to all aminoglycosides tested, including gentamicin, amikacin and isepamicin, a phenotype which has not been described before.

- The association of mobile MßL genes with aminoglycoside resistance genes has become very common. The dissemination of integrons carrying these genes is of great concern and may jeopardize the treatment of infections caused by multi-drug resistant gram-negative bacilli.

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