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# Detection of New *arr-4* and *arr-5* Gene Cassettes in Clinical *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* Strains from Brazil[▽]

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**New *arr* alleles emerged in class 1 integrons from a clinical *Pseudomonas aeruginosa* strain (*arr-4*) and four *Klebsiella pneumoniae* strains (*arr-5*) in Brazil/American continent. *arr-4* was preceded by *aacA7-catB3*, whereas *arr-5* was the unique cassette. The putative proteins shared 75% (Arr-5) and 78% (Arr-4) identities with Arr-2.**

Rifampin resistance is a notable global health problem concern (11), since it is the front line drug for treating tuberculosis (4) and preventing meningococcal diseases (13). More than 90% of rifampin resistance is due to mutational alterations in an 81-bp rifampin resistance determining region of the *rpoB* gene (9). However, resistance may also arise by horizontal acquisition of *arr* genes, which code for ADP-ribosyltransferases responsible for the drug inactivation. The *arr-1* gene was first described in the *Mycobacterium smegmatis* chromosome (10). Subsequently, *arr-2* and *arr-3* alleles were described as gene cassettes in class 1 integrons present in gram-negative isolates from Europe and Asia (1, 6, 8, 14), showing high-level rifampin MICs ranging from 32 to >256 μg/ml.

We report here the identification and characterization of two new *arr* alleles, *arr-4* and *arr-5*, and the emergence of this class of gene in the American continent as gene cassettes from class 1 integrons present in clinical *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* strains.

One *P. aeruginosa* (PS1111) and four *K. pneumoniae* (K56, K224, K688, and K830) isolates recovered from different inpatients of a Brazilian hospital (Rio de Janeiro city) in 2005 tested positive for class 1 integron signatures (3). DNA macrorestriction using SpeI, followed by pulsed-field gel electrophoresis, was performed as previously described (3) and demonstrated four distinct profiles among the *K. pneumoniae* strains. Sequence analysis of the class 1 integron variable regions showed the presence of a 453-bp open reading frame, encoding a predicted protein of 150 amino acid residues in all integrons. These sequences from the *K. pneumoniae* strains and from isolate PS1111 shared 78% amino acid identity, and 75 and 78% amino acid identity, respectively, with ADP-ribosyltransferase Arr-2 (Fig. 1). Therefore, these open reading frames were named *arr-4* (PS1111) and *arr-5* (the *K. pneumoniae* strains). The genes were assigned to be parts of cassettes due to the presence of *attC* sites. The *arr-4* gene cassette was preceded by a *aacA7-catB3* array, which confer resistance to aminoglycosides and chloranphenicol, respectively, and *arr-5* was the unique cassette inserted in all *K. pneumoniae* integrons. The *arr-4* gene cassette is 516 bp long and presented an *attC* site only 22 bp in length due to the loss of 2L and 2R simple sites (Fig. 2). The *arr-5* gene cassette is 555 bp, presenting a classical *attC* of 61 bp, encompassing the core site sequences that make up the left-hand and right-hand consensus sites composed of 1L, 2L, 1R, and 2R simple sites (Fig. 2) (12).

*arr-2* and *arr-3* gene cassettes share 99% nucleotide identity and, moreover, present identical *attC* site sequences. Considering that a gene cassette is characterized by a particular *attC* site sequence (12), it can be assumed that they were inappropriately designated as distinct cassettes, and it is more likely that *arr*-3 is a variant of the canonical *arr-2*, diverging by only two point mutations. Conversely, the *arr-4* and *arr-5* genes had 75 and 72% nucleotide identities, respectively, with *arr-2* and were associated with unique *attC* sites, which presented no similarity to that from *arr-2*, characterizing three distinct *arr* gene cassettes. These findings altogether suggested that *arr-2*, *arr-4*, and *arr-5* gene cassettes have evolved independently from each other.

The antimicrobial susceptibility was tested for β-lactams, aminoglycosides, and ciprofloxacin by the disk diffusion method (2), and MICs of imipenem and rifampin were determined by the E-test method. The *K. pneumoniae* strains were determined to be multiresistant, except to carbapenems. PS1111 was resistant only to ciprofloxacin and ticarcillin-clavulanic acid. The MIC of rifampin for K830 and PS1111 was increased (>32 μg/ml), as observed previously in *arr-2*-carrying strains (5), whereas the rifampin MICs for K56, K224, and K688 ranged from 8 to 16 μg/ml. However, for the *K. pneumoniae* ATCC 10031 control strain the rifampin MIC was 6 μg/ml. PCRs were carried out with primers flanking both *arr-4* and *arr-5* gene cassettes in order to obtain the entire *arr* coding regions to be cloned. *arr-4*,

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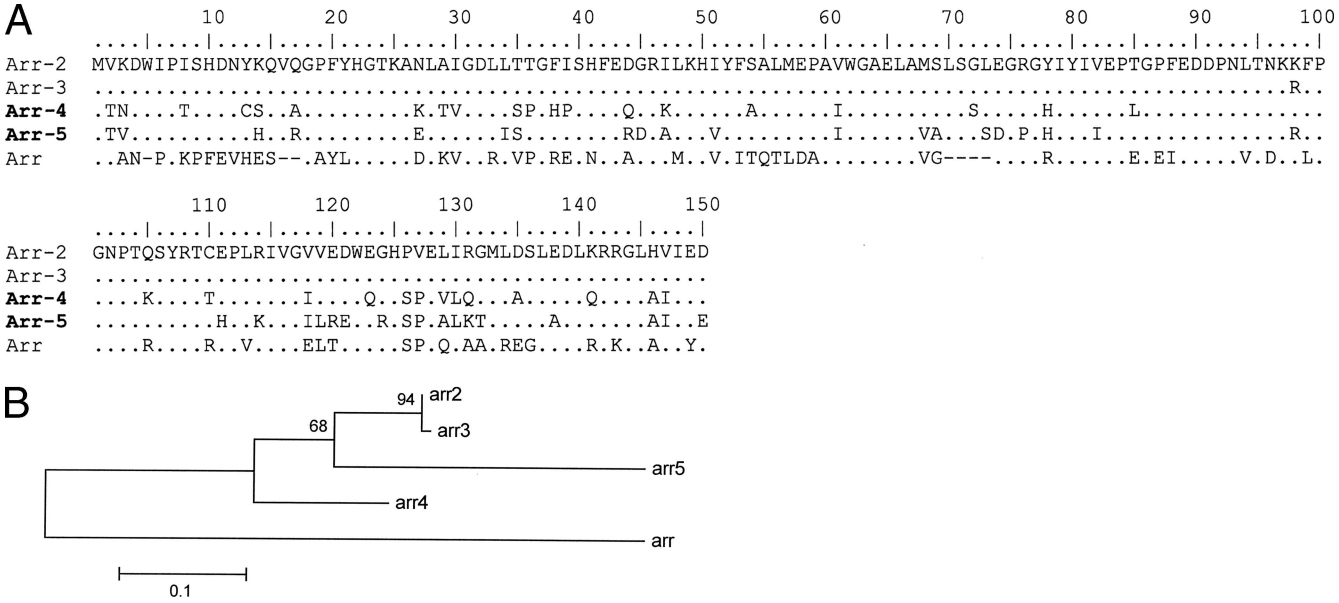


FIG. 1. (A) Arr amino acid sequence alignment. The GenBank accession numbers for the putative proteins included in the comparison are as follows: Arr (AAC05822), Arr-2 (AAC64366), Arr-3 (AAP20922), Arr-4 (EF660562), and Arr-5 (EF660563). The sequences described here are in boldface. Identical amino acid residues are represented by dots. (B) Neighbor-joining genetic tree constructed with Arr predicted proteins (MEGA3).

which was the last cassette, was amplified with primers annealing at the 3′ end of *catB3* gene cassette (CATB3, F, 5′-CACTGGAGAAGATCAAAGCG-3′) and at the 3′CS (for 3′ conserved segment) from class 1 integrons (INB, 5′-GGGCAGACTTGACCTGAT-3′). *arr-5*, the unique cassette inserted in the integron variable region, was obtained with primers targeting the 5′CS (INF, 5′-GGCATCCAAG CAGCAAG-3′) and 3′CS (INB) from class 1 integrons. The resulted products were cloned into pGEM-T Easy vector according to the manufacturer's instructions (Promega). The ligation reaction was used to transform the rifampin-sensitive *Escherichia coli* DH5α, and transformants were selected on Luria-Bertani agar plates containing 100 μg of ampicillin and 50 μg of rifampin/ml. PCR and sequence analysis showed that all selected transformants harbored *arr-4* or *arr-5* genes. A fresh inoculum of transformants was prepared at a concentration of 0.5 on the McFarland scale ($1.5 \times 10^8$ CFU/ml) and delivered onto Mueller-Hinton agar plates containing 100 μg of ampicillin/ml. The MICs were determined by the E-test method as the lowest drug concentration graded in the strip in which no growth was observed after 20 h of incubation at 37°C. The MICs of rifampin increased from 8 to >256 μg/ml for DH5α when transformed with both *arr-4* and *arr-5* genes. Therefore, the high-level rifampin MICs observed in transformed DH5α *E. coli* were due to *arr-4* and *arr-5* genes, proving the functionality of these new alleles in other bacterial species.

Sequence analysis revealed that the integron from PS1111 presented the strong version of cassette promoter (Pc), which is the most active configuration, whereas the *K. pneumoniae* integrons had the uncommon Pc configuration characterized by the weak Pc version, followed by the putative active second promoter (P2), usually responsible for 90% of the total promoter activity (7). These findings characterize the conditions for the expression of *arr-4* and *arr-5* genes found in these class 1 integrons.

Clinical *P. aeruginosa* and *K. pneumoniae* strains carrying new *arr* gene cassettes compose an ideal scenario for dissemination of these resistance determinants by horizontal gene transfer. In fact, the potential spread of *arr* gene is emphasized by our results, which showed four *K. pneumoniae* lineages carrying the same integron arrangement, composed by *arr-5* gene cassette, circulating at the same moment in a unique clinical setting.

**Nucleotide sequence accession numbers.** The nucleotide sequences of integrons harboring *arr-4* and *arr-5* have been submitted to GenBank under accession numbers EF660562 and EF660563, respectively.

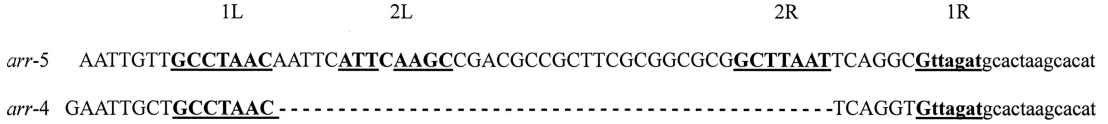


FIG. 2. *arr-4* and *arr-5 attC* sites. The core sites representing 1L, 2L, 1R, and 2R simple sites are in boldface and underlined (12). Dashes indicate the partial deletion of *arr-4 attC* site. Bases in lowercase are part of the next cassette.

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