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# Changes in Ciprofloxacin Resistance Levels in *Enterobacter aerogenes* Isolates Associated with Variable Expression of the *aac(6′)-Ib-cr* Gene

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**Two closely related *Enterobacter aerogenes* isolates presented a new identical *aac(6′)-Ib-cr* genetic environment, including IS*26*. One isolate showed lower MICs of ciprofloxacin, norfloxacin, tobramycin, and amikacin and decreased expression of *aac(6′)-Ib-cr*, which might be related to a 12-bp deletion causing a displacement of the −10 box upstream of the *aac(6′)-Ib-cr* gene.**

The *aac(6′)-Ib-cr* gene encodes an aminoglycoside 6′-acetyltransferase able to modify not only aminoglycosides but also quinolones with a piperazinyl substituent (such as ciprofloxacin and norfloxacin) by acetylation of the amino nitrogen on this chemical group (12). This gene has been detected in the variable regions of integrons and in several structures commonly associated with mobile genetic elements, especially the IS*26* insertion sequence (11, 13).

The aim of this work was to analyze the expression of the *aac(6′)-Ib-cr* gene in two clonally related *Enterobacter aerogenes* clinical isolates (C2653 and C2657) obtained from two patients in different wards of the same hospital (both isolates were recovered in 2009) and to correlate this expression with fluoroquinolone and aminoglycoside susceptibilities. In addition, other antimicrobial resistance mechanisms and plasmid content were investigated.

Both isolates showed a closely related XbaI-digested pulsed-field gel electrophoresis (PFGE) pattern (data not shown). Susceptibility testing to 23 antimicrobial agents was carried out by disk diffusion and agar dilution methods or Etest (5), and 34 genes conferring resistance to quinolones, β-lactams, carbapenems, aminoglycosides, tetracycline, chloramphenicol, sulfonamides, and trimethoprim were tested by PCR with subsequent sequencing of all amplicons obtained.

The C2653 isolate showed higher MIC values of ciprofloxacin and norfloxacin (but not levofloxacin), aminoglycosides, and carbapenems than the C2657 isolate did (Table 1). Both isolates carried the $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1b}}$, $bla_{\text{OXA-1}}$, *aph(3)-Ia*, *sul1*, *sul3*, and *dfrA12* genes. The presence of class 1 and class 2 integrons was determined, the characterization of their variable regions were analyzed (14), and a new gene cassette array, which was deposited in GenBank under accession number JF729199, was identified in both isolates (Table 1). The amino acid change D86Y was identified in the quinolone resistance-determining region (QRDR) of the GyrA protein, while the wild sequence was demonstrated in the ParC protein, as determined by PCR and sequencing of the corresponding genes in both isolates (3, 15).

Mutations in *omp35* and *omp36* porin genes of the two *E. aerogenes* isolates were analyzed by PCR and sequencing (6) and outer membrane proteins (OMPs) were obtained and visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (9). The carbapenem-resistant isolate C2653 did not express the two major porins. A deletion of the nucleotides TT at positions 71 and 72 of the *omp36* porin gene was detected, while *omp35* gene presented the wild sequence. Since these characteristics were not identified in the carbapenem-susceptible C2657 isolate, porin alteration might be responsible for the high MIC of carbapenems in the C2653 isolate.

Plasmids from the two *E. aerogenes* isolates were extracted, and genetic transfer of the *aac(6′)-Ib-cr* gene was carried out by transformation into *Escherichia coli* DH10B. Conjugation and transformation assays were carried out using aminoglycosides, quinolones, and β-lactams for selection. A transformant of *E. aerogenes* isolate C2653 could be obtained (selected on Mueller-Hinton agar plates supplemented with tobramycin [6 μg/ml]), but conjugation and transformation assays from the C2657 isolate were unsuccessful. Resistance phenotypes and genotypes of the donor, transformant, and recipient isolates are shown in Table 1. The acquisition of the *aac(6′)-Ib-cr* gene from isolate C2653 by the transformant was associated with an increase in the MICs of quinolones (except levofloxacin) and aminoglycosides.

The plasmids of donor and transformant isolates were classified according to their incompatibility group using the PCR-based replicon typing method (7), and their number and size was determined by a PFGE assay with the total DNA digested by S1 nuclease (1). Eight plasmid addiction systems were studied by PCR as previously described (10). The plasmid locations of the *aac(6′)-Ib-cr*, $bla_{\text{CTX-M-15}}$, and *intI1* genes were analyzed by transferring S1 DNA digested PFGE gels onto nylon membranes by Southern blotting and hybridized with specific probes. Hybridization was performed by using the digoxigenin (DIG) high prime DNA labeling and detection starter kit I (Roche Applied Science, Barcelona, Spain). The chromosomal location of the *aac(6′)-Ib-cr* gene was also determined by Southern hybridization following genomic DNA digestion with *I-Ceu*I nuclease as previously described (8).

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**TABLE 1** Resistance phenotype and genotypes of *E. aerogenes* donor isolates, the *E. coli* recipient strain, and the transformant strain

| Strain[a] | MIC (mg/liter)[b] | | | | | | | | | | | | | | Additional resistance[b,c] | Resistance genes[d] | Amino acid change in GyrA QRDR[e] | ParC QRDR | Class 1 integron variable region |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | NAL | CIP | LEV | NOR | TOB | AMK | KAN | GEN | AMP | CTX | ERT | IMP | MER | DOR | | | | | |
| C2657 | 1,024 | 1 | 2 | 8 | 1 | 2 | >256 | 0.5 | >256 | 128 | 0.5 | 1 | 0.5 | 0.5 | AMC, CAZ, FOX, AZT, STR (I), SUL, SXT, CHL, FFM (I) | *aac(6′)-Ib-cr*, $bla_{CTX-M-15}$, $bla_{TEM-1b}$, $bla_{OXA-1}$, *aph(3)-Ia*, *sul1*, *sul3*, *dfrA12* | D86Y | Wild | *dfrA12-orf-ΔaadA2-IS6100*[f] |
| C2653 | 512 | 8 | 2 | 64 | 64 | 32 | >256 | 2 | >256 | 256 | >32 | >32 | 2 | 4 | AMC, CAZ, FOX, AZT, IMP (I), ERT, STR (I), SUL (I), SXT (I), FFM (I) | *aac(6′)-Ib-cr*, $bla_{CTX-M-15}$, $bla_{TEM-1b}$, $bla_{OXA-1}$, *aph(3)-Ia*, *sul1*, *sul3*, *dfrA12* | D86Y | Wild | *dfrA12-orf-ΔaadA2-IS6100* |
| Tf-C2653 | 1 | 0.004 | 0.004 | 0.03 | 16 | 8 | 256 | 0.5 | >256 | 128 | 0.06 | 0.5 | 0.125 | 0.1 | AMC, CAZ (I), STR (I) | *aac(6′)-Ib-cr*, $bla_{CTX-M-15}$, $bla_{TEM-1b}$, $bla_{OXA-1}$, *aph(3)-Ia*, *dfrA12* | | | *dfrA12-orf-ΔaadA2-IS6100* |
| *E. coli* DH10B (recipient strain) | 0.25 | 0.002 | 0.004 | 0.007 | 0.125 | 0.03 | 1 | 0.125 | 4 | 0.03 | 0.03 | 0.25 | 0.03 | 0.05 | | | | | |

[a] Strain Tf-C2653 is a transformant of isolate C2653.

[b] NAL, nalidixic acid; CIP, ciprofloxacin; LEV, levofloxacin; NOR, norfloxacin; TOB, tobramycin; AMK, amikacin; KAN, kanamycin; GEN, gentamicin; AMP, ampicillin; CTX, cefotaxime; ERT, ertapenem; IMP, imipenem; MER, meropenem; DOR, doripenem; AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; FOX, cefoxitin; AZT, aztreonam; STR, streptomycin; SUL, sulfamethoxazole; SXT, trimethoprim-sulfamethoxazole; CHL, chloramphenicol; FFM, fosfomycin.

[c] (I), intermediate resistance.

[d] The resistance genes screened were *qnrA*, *qnrB*, *qnrS*, *aac(6′)-Ib-cr*, *qepA*, *oqxAB*, $bla_{CTX-M}$, $bla_{OXA}$, $bla_{SHV}$, $bla_{TEM}$, $bla_{VIM}$, $bla_{IMP}$, $bla_{SPM}$, $bla_{GIM}$, $bla_{SIM}$, $bla_{ACC}$, $bla_{CIT}$, $bla_{DHA}$, $bla_{EBC}$, $bla_{FOX}$, $bla_{MOX}$, *aac*(3)-I, *aac*(3)-II, *aac*(3)-III, *aac*(3)-IV, *aph*(3′)-Ia, *aph*(3′)-II, *aadA1* or *aadA2*, *aadA5*, *ant*(2′), *dfrA1* to *dfrA17*, *dfrB1*, *dfrB2*, and *dfrB3*.

[e] QRDR, quinolone resistance-determining region.

[f] The variable region of this integron is new and has been deposited in GenBank with accession number JF729199.

**TABLE 2** Plasmid characterization and plasmid location of *aac(6′)-Ib-cr*, $bla_{CTX-M-15}$, and *intI1* genes

| Strain[a] | Detected plasmid | | No. of plasmids detected | Size (kb) of plasmid detected | S1-PFGE-hybridization[c] | | |
|---|---|---|---|---|---|---|---|
| | Replicon type[b] | Addiction systems | | | Plasmid size (kb) | Replicon type[b] | Detected genes |
| C2653 | IncR, ColE | pemK/I | 3 | 9, 120, 200 | 200 | ND | *aac(6′)-Ib-cr*, *intI1*, $bla_{CTX-M-15}$ |
| Tf-C2653 | ND | pemK/I | 1 | 200 | 200 | ND | *aac(6′)-Ib-cr*, *intI1*, $bla_{CTX-M-15}$ |
| C2657 | IncR | pemK/I | 2 | 9, 200 | 200 | ND | *aac(6′)-Ib-cr*, *intI1*, $bla_{CTX-M-15}$ |

[a] Strain Tf-C2653 is a transformant of isolate C2653.

[b] ND, could not be determined.

[c] Total DNA was digested by S1 nuclease, and then PFGE and hybridization were performed.

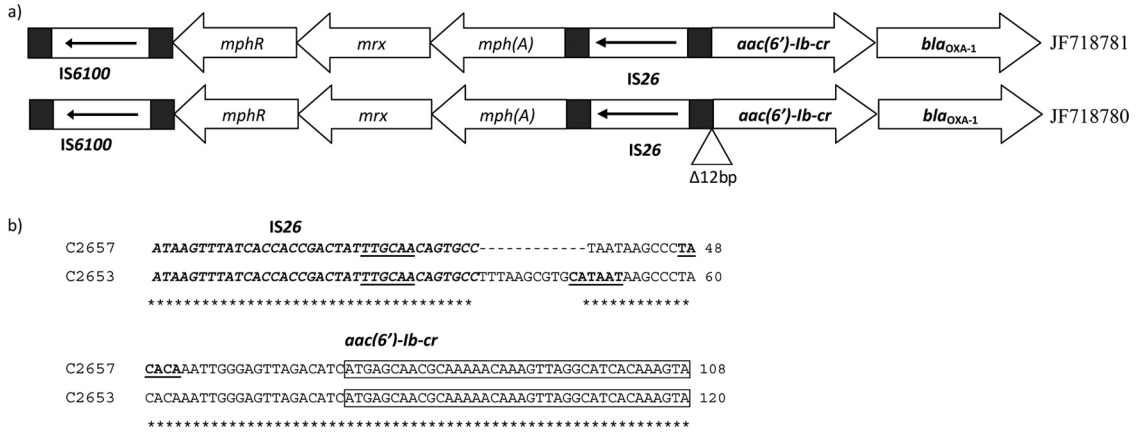


**FIG 1** Genetic environment of the *aac*(6′)-*Ib-cr* gene of *E. aerogenes* isolates. (a) Complete structure surrounding the *aac*(6′)-*Ib-cr* gene with their GenBank accession numbers. *mphR-mrx-mph*(A) is a macrolide inactivation gene cluster. (b) Alignment of the nucleotide sequence between the IS*26* insertion sequence and the *aac*(6′)-*Ib-cr* gene of the two *E. aerogenes* isolates. The IS*26* sequence is shown in italic type, the *aac*(6′)-*Ib-cr* gene is boxed, and the putative −10 and −35 boxes are underlined. Gaps introduced to maximize alignment are indicated by dashes.

Only a plasmidic location of the *aac*(6′)-*Ib-cr* gene was observed. Results of plasmid characterization are shown in Table 2. Both *E. aerogenes* isolates carried a high-molecular-weight plasmid of approximately 200 kb that carried the *aac*(6′)-*Ib-cr*, *intI1*, and $bla_{\text{CTX-M-15}}$ genes, and it was acquired by the Tf-C2653 isolate (Tf stands for transformant).

Determination of the genetic environment of the *aac*(6′)-*Ib-cr* gene was performed by cloning. The total DNA of *E. aerogenes* C2657 was extracted with QIAmp DNA minikit (Qiagen Science Inc., MD) and partially digested by Sau3AI restriction enzyme for 10 min at 37°C. The digestion products were ligated in the BamHI site of the pUC19 cloning vector, and they were introduced into *E. coli* DH10B by electroporation. The transformants harboring the recombinant plasmids were selected on Mueller-Hinton agar supplemented with ticarcillin (100 μg/ml) and tobramycin (6 μg/ml). Finally, inserts were analyzed by PCR and sequencing with M13 universal primers. PCR mapping and a primer walking sequencing method were used to elucidate a larger surrounding region of the *aac*(6′)-*Ib-cr* gene in the two *E. aerogenes* isolates based on the results obtained after cloning and the previously reported structures (16). The genetic environments of the *aac*(6′)-*Ib-cr* gene detected in the two isolates were the same except for a deletion of 12 bp between the IS*26* insertion sequence and the *aac*(6′)-*Ib-cr* gene observed in *E. aerogenes* C2657. These surrounding regions of *aac*(6′)-*Ib-cr* gene in both isolates had not been previously reported and were deposited in GenBank with accession numbers JF718780 and JF718781 (Fig. 1). The level of expression of the *aac*(6′)-*Ib-cr* gene was determined by real-time PCR (RTi-PCR) in the two *E. aerogenes* isolates following a methodology described before (4). Primers used for RTi-PCR were aac6-RTi-F (F stands for forward) (5′-TGCATCACAACTGGGCAAAGGCT-3′) and aac6-RTi-R (R stands for reverse) (5′-ACACGGCTGGACCATA TGGGGT-3′) for the *aac*(6′)-*Ib-cr* gene and rrsKp-F (5′-CAGGC GGTCTGTCAGTCGGAT-3′) and rrsKp-R (5′-CGCACCTGAG CGTCAGTCTTTG-3′) for the *rrs* gene. The amplicon sizes of the *aac*(6′)-*Ib-cr* and *rrs* genes were 191 and 184 bp, respectively. The *rrs* gene was used as an internal reference to normalize the relative amount of RNA. The expression of the *aac*(6′)-*Ib-cr* gene was 365 times higher in *E. aerogenes* C2653 than in *E. aerogenes* C2657, reflecting the almost complete absence of expression of the gene in the latter strain. The genetic environment of *aac*(6′)-*Ib-cr* was identical in both isolates, but in the case of isolate C2567, a deletion of 12 bp was identified between IS*26* and the *aac*(6′)-*Ib-cr* gene (Fig. 1).

These results totally agree with the fact that the level of resistance to ciprofloxacin, norfloxacin, tobramycin, and amikacin of *E. aerogenes* C2653 was clearly higher than that of isolate C2657. As expected, the level of resistance to levofloxacin, which does not have a piperazinyl nitrogen, was the same in both isolates, confirming that fluoroquinolones lacking that piperazinyl group are not affected by the AAC(6′)-Ib-cr enzymatic variant. It is important to point out that some authors had suggested that there was probably a −10 box, which could be involved in the expression of the *aac*(6′)-*Ib-cr* gene located downstream of IS*26* (2, 11). Assuming that there is a −10 box, the −35 box would be located 17 bp upstream (TTGCAA), which was detected in both isolates. In that case, the −10 box of strain C2657 could be displaced 12 bp (TAC ACA) and is probably involved in the lack of expression of *aac*(6′)-*Ib-cr* gene.

**Nucleotide sequence accession numbers.** Nucleotide sequence data reported in this paper are available in the GenBank nucleotide database under accession numbers JF729199, JF718780 (*E. aerogenes* C2657), and JF718781 (*E. aerogenes* C2653).

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