RESEARCH NOTE

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Molecular characterization of InJR06, a class 1 integron located in a conjugative plasmid of *Salmonella enterica* ser. Typhimurium

Summary. The presence of class 1, 2, and 3 integrons was investigated in four pediatric isolates of *Salmonella enterica* ser. Typhimurium (*S*. Typhimurium). A class 1 integron was detected in one *S.* Typhimurium strain, the only one that also showed resistance to various aminoglycoside antibiotics. This integron, called InJR06, and the aminoglycoside resistance determinants were located in pS06, a large (≥ 55 kb) conjugative plasmid. A single mobile cassette (encoding the aminoglycoside adenylyltransferase ANT(3´´)-Ia) was detected in the variable region of InJR06, while the architecture of the *attI1* and *attC* sites was conserved. [**Int Microbiol** 2005; 8(4):287-290]

Key words: *Salmonella* Typhimurium · class 1 integron · aminoglycoside · *ant(3´´)-Ia* · gene cassette

Introduction

Integrons are genetic elements that can integrate gene cassettes, usually antibiotic resistance genes, by site-specifc recombination [28]. To date, ten integron classes have been identified and, among them, class 1, 2, and 3 integrons have been implicated in antibiotic resistance [5,23]. All integrons have a 5´ conserved segment (5´-CS), including an *intI* gene encoding an integrase and an *attI* recombination site, but they have distinct 3´ conserved segments (3´-CS). However, integrons most frequently found in resistant clinical isolates of members of the *Enterobacteriaceae* belong to class 1 [2,8,31]. In class 1 integrons, which is the only well-characterized group, a specific recombination site, *attI1*, is located next to the *intI1* gene and is recognized by the IntI1 integrase, and a promoter, Pc, which directs transcription of the cassette-borne genes, lies within the *intI1* gene [4,28].

The 3´ conserved segment of class 1 integrons includes *qacEΔ1*, a deletion derivate of the antiseptic resistance gene *qacE*, and the *sul1* gene, which encodes sulfonamide resistance [11,21]. The *attC* site (59-base element) is usually found associated with a single open reading frame in a structure termed a gene cassette. More than 70 different gene cassettes encoding proteins that confer resistance to many antibiotic families have been characterized within integrons so far; i.e., enzymes that inactivate antibiotics (β-lactams, aminoglycosides, chloramphenicol), metabolic by-passes (trimetoprim), and efflux systems (chloramphenicol) [12,22]. Most of the *attC* sites of integron-associated resistance gene cassettes identified to date share only slight homologies. Their lengths and sequences vary considerably (from 51 bp to 141 bp) and their sequence similarities are primarily restricted to their 7-bp boundaries, which correspond to the inverse core site (RYYYAAC) and the core site (G↓TTRRRY, where R is a purine, Y is a pyrimidine, and the arrow shows the recombination point) [3,29].

Integrons can be considered as natural cloning and expression systems; since their genetic flexibility allows numerous cassette rearrangements under selective pressure. The study of these assortments can lead to a better understanding of multidrug-resistant strain evolution [24]. Nontyphoid *Salmonella* infections are increasingly common, appear with variable geographical incidence, and are often resistant to multiple antibiotics [25]. The aim of our study was to investigate the presence of the three classes of inte-

grons in *S.* Typhimurium clinical isolates to characterize their gene cassette assortment.

Material and methods

Bacterial strains. Four *Salmonella enterica* serovar Typhimurium strains were isolated in 1997 from the stool and blood of children at the Ricardo Gutiérrez Hospital, Santa Fe, Argentina.

Susceptibility testing. Antibiotic susceptibility (of both the clinical isolates and the *Escherichia coli* transconjugants) was calculated by the disk diffusion method on Mueller-Hinton (MH) agar according to NCCLS (National Committee for Clinical Laboratory Standards, USA) recommendations [18]. Antimicrobial agents tested were ampicillin, cephalotin, cefotaxime, sulfisoxazole, tetracycline, chloramphenicol, nalidixic acid, gentamicin, amikacin, kanamycin, netilmicin, tobramycin, and streptomycin (Britania, Argentina). The minimum inhibitory concentration (MIC) of selected aminoglycosides was then determined by the agar dilution technique on MH agar plates with an inoculum of 10^4 CFU per spot according to NCCLS recommendations [17].

Plasmid content and conjugation. Plasmid DNA from *S.* Typhimurium S06 (pS06) was extracted and analyzed as described by Sambrook et al. [26]. Plasmid size was estimated using *Escherichia coli* V517 as a reference [15]. Conjugative mobilization of pS06 was attempted by liquid-medium mating [26] to *E. coli* CAG12177 (M. Berlyn, *E. coli* Genetic Stock Center). Selection was carried out on Luria-Bertani (LB) agar plates containing gentamicin (30 mg/l) and tetracycline (20 mg/l).

PCR amplification. Architecture of the class 1 integron was determined by PCR mapping using different primer combinations (see Fig. 1) [7]. PCR amplification of *intI2* and *intI3* was done employing specific primers [9].

Cloning and DNA sequencing. The PCR product was ligated into pGEM-T vector (pGEM-T easy vector system I, Promega, USA) and transformed into *E. coli* TOP10F' (Invitrogen, Holland). Bacteria harboring recombinant plasmids were selected on LB agar plate containing ampicillin (100 mg/l), IPTG (1 mM), and X-Gal (40 mg/l). Two recombinant plasmids were sequenced in both strands by the automated Sanger method [27]. Sequences were analyzed with the NCBI [http://www.ncbi.nlm.nih.gov/] tools.

Nucleotide sequence accession number. The nucleotide sequence data reported in this work are available from the EMBL database (EBI, European Bioinformatics Institute) under the accession number AJ496285.

Results and Discussion

One of the four *S.* Typhimurium strains (S06) showed decreased susceptibility to sulfisoxazole and the aminoglycosides gentamicin, kanamycin, tobramycin, and streptomycin, but not to β-lactams (Table 1). Gentamicin and amikacin MICs were 128 mg/l and 1 mg/l, respectively.

The *intI2* and *intI3* genes were not detected in any of these isolates. Typical elements of class 1 integron were detected by PCR only in S06: *intI1* from 5´-CS, *qacEΔ1* and *sul1* from 3´-CS, and only one fragment of 1 kb corresponding to the variable region. The map of this class 1 integron (called InJR06), obtained by a PCR-based strategy and sequencing, is shown in Fig. 1A.

A larger, 55-kb plasmid (pS06) was detected in S06 and transferred to a recipient *E. coli* strain by liquid-medium conjugation. The transconjugant (called CAG06) strain had reduced susceptibility to aminoglycosides and sulfisoxazole. As expected, fragments corresponding to *intI1*, *qacEΔ1*, and *sul1* genes were amplified by PCR in the CAG06 strain (Fig. 1B). These findings clearly show that InJR06-class 1 integron and the aminoglycoside-resistant determinants are associated and are located in pS06 conjugative plasmid.

Table 1. Antibiotic susceptibility of the strains

Antibiotics (μg)	*Salmonella* Typhimurium Clinical isolate (S06)	*Escherichia coli* CAG 12177 Recipient strain	*Escherichia coli* CAG06 Transconjugant
Ampicillin (10)	24 (S)[a]	21 (S)	21 (S)
Cephalotin (30)	24 (S)	20 (S)	21 (S)
Streptomycin (10)	9 (R)	21 (S)	10 (R)
Gentamicin (10)	7 (R)	22 (S)	6 (R)
Kanamycin (30)	17 (I)	22 (S)	18 (S)
Amikacin (30)	21 (S)	21 (S)	22 (S)
Tobramycin (10)	13 (I)	20 (S)	14 (I)
Netilmicin (30)	17 (S)	25 (S)	22 (S)
Sulfisoxazole (300)	6 (R)	23 (S)	6 (R)
Tetracycline (30)	21 (S)	6 (R)	6 (R)

[a]The inhibition halo in mm is shown and interpretation of results is in accordance with NCCLS recommendations. S, sensible; I, intermediate; R, resistant.

The 1-kb variable region PCR product was cloned and sequenced. Sequence analysis of the 1009-bp fragment revealed two different regions: (i) a 5´ segment, of approximately 100 bp, where the *attI1* site included into the 5´-CS of class 1 integrons is identified (Fig. 2A), and (ii) an open reading frame that is 100% identical to the *ant(3´´)-Ia* gene (also called *aadA1a* gene) followed by an *attC* site (Fig. 2B). This *aadA1a*-integrated cassette encodes a 3´´ aminoglycoside adenylyltransferase (AadA1a protein) associated with streptomycin and spectinomycin resistance. Sequence analysis of the variable region showed that a recombination site (*attI1*) is present between nucleotides 46 and 103 (Fig. 2A). As described by Partridge et al. [20], the last 38 nucleotides correspond to the minimal size for the insertion of a new gene cassette. The core sites of the directed repeat regions (DR1 and DR2), the IntI1 binding site (simple site), and the recombination crossover point are shown in Fig. 2A.

The 60-bp *attC* site of *aadA1a* cassette has the typical configuration of two simple sites (LH and RH) separated by a central region, as shown in Fig. 2B [29]. Each simple site contains a pair of inversely oriented core sites whose consensus sequences are RYYYAAC and GTTRRRY. These core sites are designated 1L and 2L at the left-hand (LH) end and 2R and 1R at the right-hand (RH) end. The IntI1 integrase binds in these simple sites and recombination crossover occurs between the G and the first T in the 1R core site [29].

It is already known that pS06 is a conjugative plasmid carrying a class 1 integron containing a single *aadA1a* cas-

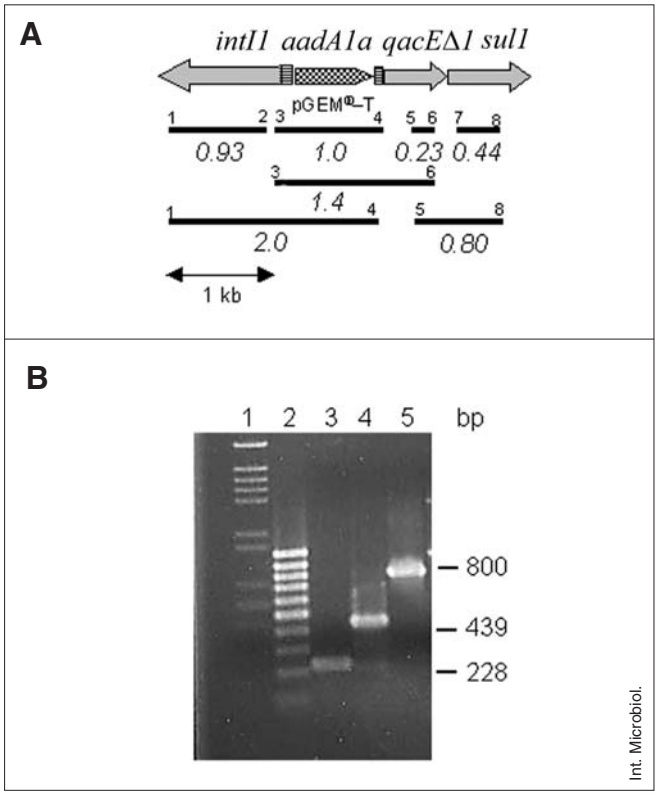

Fig. 1. (**A**) PCR mapping strategy and resulting structure of InJR06. Numbers in italics correspond to the approximate sizes (in kilobases) of the PCR products. Numbers at both ends of each PCR product indicate the pair of primers used for the different PCR amplifications (1 -I3, 2 -I5, 3 -5´CS, 4- 3´CS, 5- qacEΔ1F, 6- qacEΔ1B, 7- Sul1F, 8- Sul1B) [6]. Arrows indicate the direction of transcription. Cloned insert used for sequencing is indicated with the vector employed. (**B**) PCR identification of the genes present at the 3´CS fragment (*qacEΔ1* and *sul1*) of the InJR06 integron. Lane 1 φ29/*Hin*dIII molecular marker, lane 2 100 bp DNA ladder, lane 3 *qacEΔ1*, lane 4 *sul1*, lane 5 *qacEΔ1* + *sul1*.

sette. Although this cassette encodes a 263-amino-acid adenylyltransferase associated with both streptomycin and spectinomycin resistance, we found no data relating AadA enzymes with the resistance profile to other aminoglycosides. Therefore, according to the results described in this work, we assumed that pS06 harbors another aminoglycoside resistance mechanism. The *aadA1a* gene has been described previously as part of Tn*1331* or as a gene cassette of unusual class 1 integrons (bearing orf513 and a partial duplication of 3´-CS), both present in different *Enterobacteriaceae* [1,16,19]. However, these unusual class 1 integrons harbor the $bla_{CTX\text{-}M\text{-}2}$-β-lactamase gene in their architecture; therefore, InJR06 could be a precursor of them ($bla_{CTX\text{-}M\text{-}2}$ gene was not detected in S06; Di Conza J, PhD thesis).

The detection of typical *attI1* and *attC* sequences adjacent to the *aadA1a* gene suggests that a new gene cassette can be integrated by site-specific recombination into the variable region of these genetic elements. Thus, the accumulation of resistance genes by integrons is a plausible explanation for the emergence of multiple resistant strains, and their location in a conjugative plasmid can contribute to the widespread dissemination of antibiotic resistance. An increasing number of cases of uptake of resistant genes by integrons has been reported in the last few years [6,10,13,14,30]. In addition, PCR mapping of integrons can be a useful epidemiological tool to study the evolution of multiresistance plasmids and transposons.

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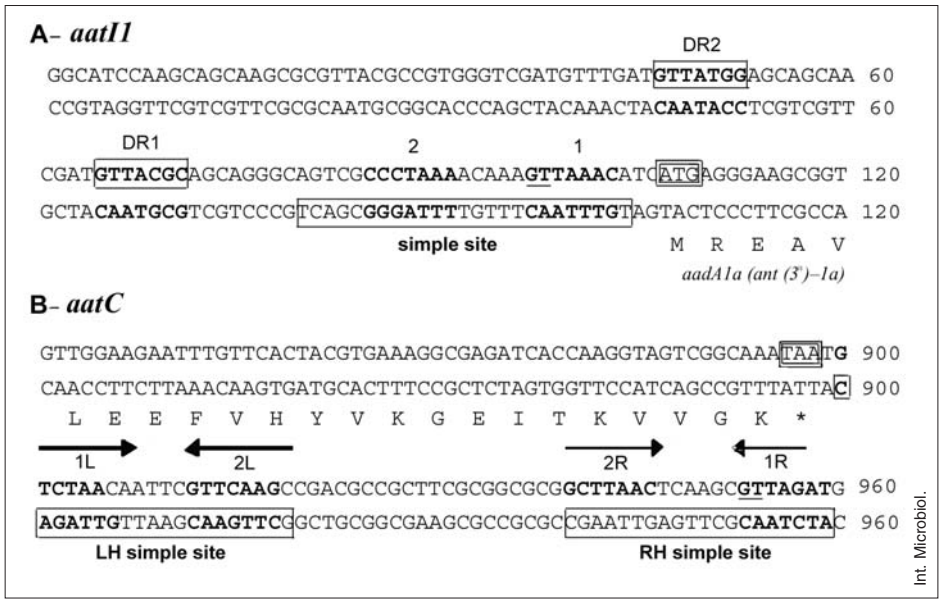

Fig. 2. Architecture of recombination sites adjacent to the *aadA1a* gene. A- *attI1* site: The core sites present into the directed repeat regions (DR1 and DR2) are labeled in boldface type. The *attI*1 simple site is boxed, with the 7-bp core sites labeled as described by Partridge et al. [16] in boldface type. The *attI*1 x 59-be recombination crossover point is underlined. The start codon of AadA1a protein is boxed with double line. B-*attC* or 59-be site: LH and RH simple sites are boxed and the core sites, labeled as described by Stokes et al. [25], are in boldface type, with their relative orientations indicated by the arrows above the sequence. The inverted repeat consensus sequences RYYYAAC and GTTRRRY are present in 1L and 1R domains respectively. The position of the recombination crossover point for a potential insertion of another gene cassette is underlined. The end codon of AadA1a protein is boxed with double line.

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Caracterización molecular de InJR06, un integron de clase 1 situado en un plásmido conjugativo de *Salmonella enterica* ser. Typhimurium

Resumen. Se investigó la presencia de integrones de clase 1, 2 y 3 en cuatro aislamientos pediátricos de *Salmonella enterica* ser. Typhimurium (*S.* Typhimurium). Un integrón de clase 1 se detectó en una cepa de *S.* Typhimurium, la única que además presentaba resistencia a varios antibióticos aminoglucósidos. Este integrón, llamado InJR06, y los determinantes de resistencia a aminoglucósidos se localizaron en pS06, un plásmido conjugativo de tamaño grande (≥ 55 kb). El análisis de la región variable de InJR06 mostró que un casete génico codifica la aminoglucósido adeniltransferasa ANT(3´´)-Ia y que la arquitectura de los sitios *attI1* y *attC* está conservada. [**Int Microbiol** 2005; 8(4):287-290]

Palabras clave: *Salmonella* Typhimurium · integrón de clase 1 · aminoglucósidos · *ant(3´´)-Ia* · casete génico

Caracterização molecular de InJR06, um integron de classe 1 situado em um plásmido conjugativo de *Salmonela enterica ser.* Typhimurium

Resumo. Se averiguou a presença de integrones de classe 1, 2 e 3 em quatro isolamentos pediátricos de *Salmonela enterica* ser. Typhimurium (*S.* Typhimurium). Um integrón de classe 1 se pôde detectar em uma cepa de *S.* Typhimurium, a única que além disso apresentava resistência a vários antibióticos aminoglucósidos. Este integrón, chamado InJR06, e os determinantes de resistência a aminoglucósidos se localizaram em pS06, um plásmido conjugativo de tamanho grande (≥ 55 kb). A análise da região variável de InJR06 mostrou que um cassete genético codifica a aminoglucósido adeniltransferasa ANT(3´´)-Ia e que a arquitetura dos lugares *attI1* e *attC* está conservada. [**Int Microbiol** 2005; 8(4):287-290]

Palavras chiave: *Salmonella Typhimurium* · integrón de classe 1 · aminoglucósidos · *ant(3´´)-Ia* · cassete genético