Class 1 Integron-Borne Gene Cassettes in Multidrug-Resistant Yersinia enterocolitica Strains of Different Phenotypic and Genetic Types

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Seventy nine strains of Yersinia enterocolitica resistant to one or more antimicrobials were analyzed for integrons. Only class 1 sulI integrases containing aadA1a (28 strains), aadA1a-dfr1-sat1 (2 strains), and dfr1-aadA1a (1 strain) gene cassettes were found. The first two types were found in clinical isolates belonging to serotype O:3, biotypes 2 to 4, and eight combined ribotypes, and the third was found in the reference strain, CECT4054 (O:8). All screened resistance markers were found in strains with and without integrons (except for chloramphenicol resistance, encoded by catA1 gene, which was only present in strains with integrons), but in different resistance profiles (R profiles). A profile (ampicillin, streptomycin, sulfadiazine, and trimethoprim resistance, encoded by the tem1, aadA1, sulI, and dfr1 genes, respectively) was found in strains, with and without integrons. Integrons and some of the resistance genes are located on plasmids with sizes ranging between 65 and 140 kb. This is the first report of class 1 integrins in Y. enterocolitica.

An efficient route of acquisition and dissemination of antimicrobial resistance determinants is through mobile elements, including plasmids, transposons, and gene cassettes in integrons (4, 5, 9–12, 14, 16–19). The essential components of an integron include the integrase gene (int), the attachment site (attl), and the promoter (4, 5, 11, 17, 18). Class 1 integrons are the integrons most frequently found among clinical isolates, and their structure consists of two conserved segments (5′- and 3′-CS) and an internal variable region (VR) that contains gene cassettes encoding antimicrobial resistance determinants. Like the other classes of integrons, their 5′-CS contains the intI1 and attI loci, while their 3′-CS is specific and usually contains qacEΔ1 and sulI genes, which confer resistance to quaternary ammonium compounds and sulfonamides, respectively (4, 5, 11, 17). The occurrence of integrons among bacteria is currently under investigation, and several studies report a widespread distribution of these elements among pathogenic species (5, 7, 9–12, 14, 16–19), but no investigation about the zoonotic organism Yersinia enterocolitica has been reported to date. Y. enterocolitica is an enteroinvasive bacteria, mainly associated with enterocolitis and less frequently with a wide variety of clinical and immunologic manifestations (1, 3, 8). The purpose of this study was to explore the presence of integrons and the resistance genes therein in Y. enterocolitica. In addition, resistance profiles (R profiles) in strains with and without integrons, the locations of integrons on plasmids, and the spread of integrons among phenotypic and genetic types of Y. enterocolitica were studied. For this purpose, a set of 7S clinical isolates collected in the Principality of Asturias (PA), Spain, and reference strains from the Colección Española de Cultivos Tipo (CECT500, -559, -4054, and -4055) were analyzed. These isolates were selected because they had been previously characterized by serotyping, biotyping, riboprobing, and PCR ribotyping and clustered into different types and lineages in a previous work (13).

Detection of integrons and resistance genes therein. In a first step, the set of Y. enterocolitica was assayed for presence of integrons by PCR. For this, we used degenerated primers targeted to conserved regions of intI1, intI2, and intI3 genes (19), aliquots of broth cultures as template DNA, and PCR conditions previously described (9). The PCR products were digested with Hinfl to determine the integrase class (19). The results showed that 30 isolates and the reference strain CECT4054 contained intI1 integrase genes (Fig. 1), but no intI2 or intI3 was detected. In a second step, the integron-positive isolates were tested by PCR with qacEΔ1-sulI and 5′-CS/3′-CS (9, 12) primers. The size (680 bp) of the resulting amplicon with the first primer pair confirmed the presence of the qacEΔ1-sulI genes. The sizes (about 1,000, 1,600, and 1,900 bp) of the amplicons generated with the second primer pair showed the presence of three different inserted regions. Each clinical isolate generated amplicons of a single size: 1,000 bp for 28 isolates, 1,900 bp for 2 isolates, and 1,600 bp for CECT4054. In a third step, amplicons of 1,000 and 1,900 bp (from two isolates each) and the single amplicon of 1,600 bp were analyzed by sequencing as described elsewhere (9, 10). The sequencing results showed that all of them carried the aadA1a gene cassette (AJ009820), alone (1,000 bp) or in combination with dfr1 (1,600 bp) or dfr1-sat1 (1,900 bp) (accession no. AF382145 and AY140652, respectively). To confirm that all 1,000-bp amplicons contained the aadA1a gene (integron In2), two tests were performed: (i) nested PCR with, as a template, DNA aliquots of 1,000-bp amplicons and aadA intragenic primers (18), and (ii) PvuI digestion of the 1,000-bp amplicons, because the aadA1a and aadA2 sequences differ in that only the former contains a PvuI restriction site (9). The results confirm the presence of the aadA1a (In2) in those isolates generating 1,000-bp amplicons (figure not shown). The aadA1a gene encoded an aminoglycoside adenyltransferase [AAD(3′)] and conferred resistance to streptomycin and spec-
resistance to trimethoprim, and the sat1 gene resistant dihydrofolate reductase (DHFRa) and conferred tinomycin, the dfr1 gene encoded streptomycin acetyltransferase (SAT-1) and conferred resistance to streptomycin. These findings support the fact that only resistance to streptomycin, sulfonamides, and trimethoprim could be directly related to integrons in Y. enterocolitica.

Associations between antimicrobial resistance patterns and class 1 sat1 integrons. Antimicrobial susceptibility of the clinical and control strains was tested according to the National Committee for Clinical Laboratory Standards (15) by a disk diffusion technique with commercial disks (bioMérieux and Oxoid, Madrid, Spain). The panel of antimicrobials tested is compiled in the footnotes to Table 1. No strain was susceptible to all antimicrobials, although all isolates were susceptible to amoxicillin/clavulanic acid and cefotaxime, and >95% were susceptible to gentamicin and kanamycin. Chloramphenicol resistance was only found among strains with integrons, whereas resistance to all other agents was displayed by strains with and without integrons, but at different frequencies and in different combinations (Table 1). Strains were grouped into R profile patterns, and only one R pattern was common to strains with and without integrons (Table 1). Non-integron-borne resistance genes were also screened by PCR as described in references 9 and 10. The following genes were found: tem1-like (coding for ampicillin resistance through a β-lactamase), catA1 (coding for chloramphenicol resistance through an acetyltransferase), aphA1 (coding for kanamycin-neomycin resistance through a phosphotransferase), aac(3’)-II (coding for gentamicin resistance through an acetyltransferase), and tet(A) (coding for tetracycline resistance through an efflux system protein). It is noticeable that, among the clinical isolates, all of those containing integron In2, but none of those that were integron free, showed resistance to chloramphenicol and contained the catA1 gene. In the nalidixic acid-resistant isolates, mutations in the DNA gyrase were screened by amplification-restriction of the gyrA and parC genes (6). Only mutations involving codon Asp-87 or Ser-83 of the gyrA gene were detected. The distribution of resistance genes among strains carrying integrons is shown in Table 1.

Plasmid profiles and plasmid location of the integrons. Plasmids were extracted from all Y. enterocolitica isolates containing integrons, 10 isolates without integrons, and the control strains, transferred to nylon membranes, and hybridized with specific probes for the intI1, aadA1a, catA1, tem1, and virF genes as described in references 2 and 10. Diverse plasmids with sizes ranging from 3 up to 150 kb, grouped into different plasmid profiles, were detected, but the intI1 and aadA1a probes mapped only in plasmids larger than 60 kb (Fig. 2). It should be mentioned that failures in the extraction and visualization of the large plasmids were frequent. For this reason, the number of isolates carrying large plasmids and the number of plasmid profiles initially observed may not be accurate. Hybridization experiments with integron probes support that integrons with the three gene cassette configurations are located on large plasmids of different size: about 140, 130, and 65 kb for dfr1-aadA1a, aadA1a, and aadA1a-dfr1-sat1, respectively. The second integrons may be also located on the chromosome of some clinical isolates, because intI1 and aadA1a probes did not hybridize with their plasmids. The catA1 probe mapped on integron-containing plasmids larger than 120 kb while the tem1 probe gave a positive signal with the chromosomal rests (Fig. 2). Plasmids of about 70 kb (the size of the typical Y. enterocolitica pYV virulence plasmid) (2) hybridized with the virF probe, and sometimes hybridized with the intI1 probe, but not with aadA1a and catA1 probes, and they were detected in strains with and without integron. Three features—plasmid size, integron-borne gene cassettes, and mapping of catA1 gene—were used to initially differentiate three types of R plasmids (Fig. 2 and Table 1), which were labeled pUO-Ye-R1 to -R3 (for plasmid University of Oviedo—Y. enterocolitica resistance).

To ascertain whether the integron-borne plasmids were self-transferable, eight strains were tested by conjugation on liquid medium using Escherichia coli K12 J53 as a recipient (10). These strains were as follows: CECT4054, containing pUO-Ye-R1; 17457/94 and 26719/95, both containing pUO-Ye-R2; and 8276/95, 36349/95, 37533/95; 37824/95, and 7036/93, all containing pUO-Ye-R3 (Table 1). Only pUO-Ye-R1 (carrying the integron-borne dfr1-aadA1a gene cassette configuration) was

FIG. 1. Analysis of integrons in representative Y. enterocolitica clinical isolates and integron control strains. (A) Amplification products generated with generic integrase primers. (B) Hinfl digestion of amplicons of panel A. (C) Amplification products generated with 5′-CS3′-CS primers. Lanes: 1, 2, and 3, representative Y. enterocolitica strains containing integron-borne aadA1a (LSP 8276/95), dfr1-aadA1a (CECT4054), and aadA1a-dfr1-sat1 (LSP 17457/94) gene cassettes, respectively; C1 and C2, class 1 and 2 integron controls generated by Salmonella enterica serotype Enteritidis LSP 49/00 and Proteus mirabilis 1/01, respectively; Sw, Se, and St, S. enterica serotypes Wien LSP 153/96, Enteritidis LSP 49/00, and Typhimurium LSP 14/92, respectively, used as class 1 integron control strains; L, 100-bp ladder (Gibco, BRL) used as the molecular size standard.
Strains: AMP (9), AMP STM (1), SUL SXT (3), AMP STM SUL (14), STM SUL SXT (1).

Antimicrobial agents were tested at the following concentrations: ampicillin (AMP), 1000 μg/ml; gentamicin (GEN), 10 μg/ml; chloramphenicol (CHL), 30 μg/ml; streptomycin (STM), 25 μg/ml; sulfa-trimethoprim (SUL/TEM), 20 μg/ml; tetracycline (TET), 30 μg/ml; and nalidixic acid (NAL), 5 μg/ml.

Integron-borne resistance is boldfaced.

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<tr>
<th>Type of Strain</th>
<th>Resistance Gene(s) in Class I Integron</th>
<th>Resistance Gene(s) in Class 2 Integron</th>
<th>Resistance Gene(s) in Class 3 Integron</th>
<th>Resistance Gene(s) in Class 4 Integron</th>
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<td>P. mirabilis</td>
<td>ampicillin resistance (ampC)</td>
<td>sulfa-trimethoprim resistance (sul)</td>
<td>tetracycline resistance (tet)</td>
<td>chloramphenicol resistance (aphA1)</td>
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<td>S. enterica</td>
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TABLE 1. Relevant features of A. aerogenes strains carrying integrons.
self-transferable, and the E. coli transconjugants (verified by three tests: random amplified polymorphic DNA analysis, lactose fermentation, and indole production) expressed resistance to chloramphenicol, kanamycin, streptomycin, sulfadiazine, tetracycline, and trimethoprim and were amplified with the \textit{catA1-aphA1}, \textit{aadA1-sul1}, \textit{dfr1}, and \textit{intI1} primers.

**Spread of class 1 sul1 integrons among genetic types of Y. enterocolitica.** It has been commented above that the set of strains under study had been previously typed by different procedures (13). The 30 clinical isolates carrying integrons belonged to a single serotype (O:3), three biotypes (BT2 to BT4), and eight combined ribotypes (CRTs I1,2,3,5,6, II, VIII, and X). The reference strain CECT4054 belonged to O:8, BT2, and CRT XIII25. It is noteworthy that riboprobing grouped Y. enterocolitica into three well-differentiated clusters or lineages. Isolates containing the integron-borne \textit{aadA1a} gene cassette (In2), located on plasmids (pUO-Ye-R3) and presumably on the chromosome, generated different CRTs. One of these ribotypes (CRT I1) was also generated by two isolates containing the integron-borne \textit{aadA1a-dfr1-sat1} gene cassette configuration located on pUO-Ye-R2. Moreover, all clinical isolates containing integrons fell into the major cluster, while CECT4054, containing the integron-borne \textit{dfr1-aadA1a} gene cassette configuration on the plasmid pUO-Ye-R1, fell into another cluster (13). These findings showed that class 1 integrons, with three types of gene cassette configurations, are plasmid located and spread among different serological, biochemical, and genetic types of Y. enterocolitica. Further work needs to be done to determine the relative arrangement of the integrons with other resistance genes as well as the genetic relationships between the resistance plasmids of Y. enterocolitica.

It is also noticeable that \textit{aadA1a} is a gene cassette frequently found in class 1 integrons, a fact that could be related to its location on the transposon Tn21 (4, 11, 12). This gene cassette has frequently been found, alone or in association with others (including the array 5′-CS-dfr1-aadA1a-3′-CS), among serotypes of \textit{Salmonella} in our laboratory (9) and among different gram-negative bacteria in other laboratories (4, 5, 12, 14, 16–19). However, this is the first time that we found the 5′-CS-aadA1a-dfr1-sat1-3′-CS in class 1 integrons. Remarkably, these three gene cassettes in a different order (5′-CS-dfr1-sat1-aadA1a-3′-CS) have been frequently reported in class 2 integrons associated with Tn7 in other species (16, 19).

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