

Molecular diversity and conjugal transferability of class 2 integrons among *Escherichia coli* isolates from food, animal and human sources

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ABSTRACT

Integrans are genetic platforms able to excise, integrate and express antibiotic resistance gene cassettes (GCs). Here we investigated the complete genetic organisation, genetic environment, location and conjugative transferability of a collection of class 2 integrans carried by *Escherichia coli* strains from different sources (poultry/pork meat, animals and humans). PCR cartography was conducted to determine the genetic arrangement of the integrans, their physical linkage to Tn7 and chromosomal insertion at the *attTn7* site. Clonal relatedness of specific isolates was determined by MLST and DOP-PCR (degenerate oligonucleotide primers PCR). Transferability of class 2 integrans was tested by conjugation. The resulting transconjugants were characterised by antimicrobial resistance genotyping, S1-PFGE and replicon typing. Although a limited diversity of GCs was shown, a high percentage of novel structures was identified owing to the integration of insertion sequence (IS) elements at different sites (IS3/IS4/IS5/IS21 families). Insertion of IS10 in the *attI2* site of a class 2 integran, between Pc2B and Pc2C promoters, was likely mediated by a site-specific transposition event. Chromosomal insertion of integrans at *attTn7* was confirmed in 80% of the isolates. Conjugation experiments demonstrated that 29% of class 2 integrans could be mobilised to *E. coli* CHS26, demonstrating that they can be located in conjugative/mobilisable elements at a low frequency. Reported structures evidence how class 2 integrans have evolved by the activity of integran integrases and the invasion of ISs. Since most of them are chromosomally located, dispersion is predominantly vertical, although conjugation events also contribute to the spread of class 2 integrans among bacterial communities.

1. Introduction

Escherichia coli is a Gram-negative member of the Enterobacteriaceae family that has a nearly ubiquitous distribution among humans, animals and the open environment. Although it naturally colonises the intestines of a wide range of animals, *E. coli* can also act as an opportunistic pathogen associated with community-acquired and nosocomial infections. The extensive use, and misuse, of antimicrobials both in clinical and farm animal settings has led to the selection and global spread of multidrug-resistant *E. coli* isolates [1,2].

Integrans are genetic platforms able to excise, integrate and express multiple gene cassettes (GCs), which are mobile genetic elements (MGEs) containing a promoterless coding sequence and a recombination site (*attC*). The basic structure of an integran includes the integrase gene (*intI*), a proximal recombination site (*attI*), and a promoter (P_c) that drives the expression of the GC [3]. Integrans are classified either as sedentary chromosomal integrans, located in the chromosome of different bacterial species and usually carrying multiple GCs encoding a wide range of functions, or as mobile integrans, embedded within MGEs such as transposons or conjugative elements [4]. Mobile integrans comprise five different classes based on the sequence of the integrase gene and usually harbour GCs conferring resistance to antibiotics [5]. Class 1 to 3 integrans differentially contribute to the global threat of antimicrobial resistance owing to their capability to capture and spread antibiotic resistance GCs (intragenomic gene transfer) and to be transferred via MGEs to other bacteria of the same or even different species (horizontal gene transfer) [3–5].

Class 1 and class 2 integrons are the most frequently detected integrons in Enterobacteriaceae. However, compared with class 1 integrons, class 2 integrons are less diverse in GC content, less prevalent and also less studied [6,7]. Class 2 integrons usually carry in their variable region (VR) the *dfrA1–sat2–aadA1–orfX* array (which confers resistance to trimethoprim, streptothricin and streptomycin/spectinomycin), followed by three additional genes (*ybfA*, *ybfB* and *ybgA*) embedded in the Tn7 transposition module (*tnsABCDE*). *orfX*, also known as *ybeA*, is considered a pseudo-GC of unknown function. The *intI2* gene is frequently interrupted by a premature stop codon, encoding a non-functional integrase. Most class 2 integrons are associated with Tn7 derivatives. The Tn7 transposon uses two alternative transposition pathways mediated by different target selecting proteins, TnsD and TnsE [8]. TnsD recognises a unique site located within the 3'-end of the essential *glmS* gene in the bacterial chromosome (*attTn7*), whilst TnsE directs Tn7 transposition to conjugative/mobilisable plasmids [9].

Insertion sequence (IS) elements are small DNA segments that act as autonomous transposable elements. They can disrupt and inactivate a gene, affect the expression of neighbouring genes and/or promote genome rearrangements. By interacting with other types of genetic platforms, such as integrons, ISs may form large assemblies with higher-order biological properties [10].

Here we investigated the complete genetic organisation, genetic environment, location and conjugative transferability of a collection of class 2 integrons carried by *E. coli* strains isolated from different sources in Spain, Tunisia and Mexico.

2. Materials and methods

2.1. Bacterial collection

A total of 35 *E. coli* isolates carrying class 2 integrons from different sources [meat products, $n = 15$ (poultry, $n = 14$; pork, $n = 1$); chickens, $n = 6$; pets, $n = 5$; humans, $n = 5$; wild animals, $n = 4$] and origins (Spain, Mexico and Tunisia) were selected and were included in the present work. *Escherichia coli* from wild animals (gull, stork, genet and roe deer), pets (dogs and turtles) and chickens were recovered from faeces and were processed as described previously [11–13] (Supplementary Table S1). Human-source strains were isolated from haemocultures ($n = 2$) or from healthy donor faeces ($n = 3$). Carriage of class 2 integrons was confirmed by amplification of an internal fragment of the class 2 integron integrase gene (*intI2*) [14].

2.2. PCR cartography

PCR cartography was performed with different primer combinations to fully characterise the VR of the class 2 integrons and the presence of the *tns* genes [7,15–18]. In integrons lacking the *orfX* pseudo-GC, the *ybfA–ybfB–ybgA* region and the transposition module, the 3'-ends of classic and unusual class 1 integrons were screened to discard potential hybrids of class 2/class 1 integrons [19,20]. Newly designed primers for amplification and sequencing were used to determine the genetic organisation of novel structures.

The integron location in the Tn7 transposon was confirmed by PCR [7,14] as well as the common chromosomal insertion of Tn7 adjacent to the housekeeping gene *glmS*

[21]. The internal region of the integrase gene *intI2* was sequenced for all isolates of this collection [12].

All of the primers used in this study for PCR amplification are illustrated and described in Fig. 1.

2.3. Conjugation experiments and characterisation of the resulting transconjugants

Conjugation experiments were carried out in all strains harbouring the *dfrA1* GC ($n = 31$) using rifampicin-resistant *E. coli* CSH26 (lactose-negative) as recipient strain and a donor:recipient ratio of 1:4 [22]. Transconjugants were selected on MacConkey agar supplemented with trimethoprim (30 mg/L) and rifampicin (100 mg/L) and were subjected to antibiotic susceptibility testing by disk diffusion according to the Clinical and Laboratory Standards Institute (CLSI) [23] and PCR analysis to confirm the presence of *intI2*.

The number and size of plasmids both in original and transconjugant strains were analysed by genomic DNA digestion with *S1* nuclease followed by pulsed-field gel electrophoresis (PFGE) [24]. Plasmid incompatibility (Inc) groups were determined by PCR-based replicon typing (PBRT) using genomic DNA as previously described [25].

On the basis of the phenotypic resistance profile exhibited by transconjugants, the presence of genes encoding resistance to sulfonamides (*sul1*, *sul2* and *sul3*), tetracycline [*tet(A)* and *tet(B)*] and chloramphenicol (*cmIA*, *floR* and *catB*) was

screened by PCR and subsequent sequencing [11]. In transconjugants positive for any of the genes that may be found in integrons, the linkage was screened by PCR.

2.4. Molecular typing

The clonal relatedness of specific bacterial isolates was determined by multilocus sequence typing (MLST) and DOP-PCR (degenerate oligonucleotide primers PCR) [26]. Sequence types (STs) were determined by analysing the nucleotide sequences of the PCR products from seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) and comparing them with those deposited in the MLST database (<https://enterobase.warwick.ac.uk/species/index/ecoli>).

2.5. Nucleotide sequence accession numbers

Nucleotide sequences of the novel class 2 integrons reported in this study owing to integration of ISs at different sites were submitted to the European Molecular Biology Laboratory (EMBL) database and were given the accession nos. **LT898484** (C3309 strain; Fig. 2, structure I), **LT898485** (C7210 strain; Fig. 2, structure G), **LT898486** (C7577 strain; Fig. 2, structure F) and **LT898487** (pn424 strain; Fig. 2, structure E).

3. Results and discussion

3.1. Class 2 integrons are evolving by integration of insertion sequence elements

The current study focused on the full molecular characterisation of 35 class 2 integrons carried by *E. coli* strains recovered from different sources and geographical origins (Supplementary Table S1). In contrast to the wide variety of

GCs reported among class 1 integrons and even among class 2 integrons from specific sources, such as wastewater environments [19], only three genetic arrangements regarding antibiotic resistance GCs were identified in the present study: *dfrA1-sat2-aadA1*; *estX-sat2-aadA1*; and *sat2-aadA1* (Fig. 2). Noteworthy, only one GC in these arrays, *estX*, encodes a putative esterase but it has not yet been established whether it inactivates any antibiotic [27]. This limited diversity in GCs is likely due to the premature stop codon at amino acid 179 of *intI2* exhibited by all of the integrons from the studied collection, which avoids the capture and excision of GCs by the class 2 integron integrase, and also to the fact that *IntI1* shows limited cross-specificity to excise some GCs found in class 2 integrons [28]. The less common arrays found in the VR of four *E. coli* strains recovered from chicken meat samples (*estX-sat2-aadA1*, *n* = 2; *sat2-aadA1*, *n* = 2) have been previously reported in Australia, Japan, Germany and China [6,19,22,28] and shared the feature of conserving *sat2* and *aadA1* GCs. The worldwide distribution of these structures, the absence of *dfrA1* and the preferential integration of GCs at the first position (*attI* × *attC* recombination) support the hypothesis of some authors who suggest that trimethoprim GCs could be acquired later in Tn7 evolution, likely by the *trans* action of class 1 integron integrase co-harboured in a single isolate with a class 2 integron [28,29]. Probably, the strong selective pressure, together with the well-known less efficient ability of the integrase to excise the first GC (*attI* × *attC* recombination) [28], once inserted *dfrA1* helped to maintain it, leading to what is currently considered the classic class 2 integron arrangement (*intI2-dfrA1-sat2-aadA1-orfX-ybfA-ybfB-ybgA-tnsE-tnsD-tnsC-tnsB-tnsA*).

Although we did not observe a high diversity of GCs among the studied collection, an unexpected finding was the identification of many novel structures due to the integration of a variety of IS elements. Twenty nine of the class 2 integrons were found to carry ISs in diverse sites (Fig. 2): (i) between *intl2* and *ybfA* genes (structures I and J); (ii) disrupting *ybfA* and *ybfB* genes (structures E, F, G and H); or (iii) disrupting *tns* genes (structures K, L and M). In contrast to the so-called IS1111-*attC* group elements, which have been shown to specifically target the *attC* sites of GCs and usually appear degenerated [30], the ISs identified in this work associated with class 2 integrons belong to a variety of families (IS3, IS4, IS5 and IS21), all of them were intact, with their respective inverted repeats (IRR and IRL) still identifiable, and they did not target *attC* recombination sites.

The novel structure reported in this study containing a complete copy of an ISSen4-element (IS3 family, subgroup IS407), inserted 55 bp downstream of the *orfX* pseudo-GC, was identified in two *E. coli* strains from Mexico. Both were assigned to ST57 and showed indistinguishable DOP-PCR patterns, but they were collected over different periods of time from distinct sources (pork meat, 2010; dog faeces, 2012; Supplementary Table S1). The fact that this integron structure, chromosomally integrated (*attTn7*), was found in two *E. coli* strains isolated from a specific geographic area and assigned to identical STs could suggest potential clonal dissemination. Moreover, the distribution of this novel integron configuration is not likely to be due to ISSen4 insertion into a 'hotspot' in the class 2 integron, as no marked target-site specificity has been previously identified for this element. However, further genomic analysis would be necessary to demonstrate the hypothesis of clonal spread. Since integrons are generally linked to highly efficient

MGEs, which facilitate their intracellular and intercellular mobility, clonal similarities might be coincidental.

To our knowledge, just a few ISs have been previously reported between the *intI2* and the *tns* genes of class 2 integrons: IS1 and IS630 elements located between *intI2* and the *attI2* recombination site in an *E. coli* and a *Shigella flexneri*, respectively [31,32]; IS26 disrupting *orfX* in an *Acinetobacter baumannii* [7]; and one intact IS911 interrupting the *sat1* GC in a *Shigella sonnei* strain [33]. Integration of IS elements within the VR of class 2 integrons may both suppress the expression of GCs or provide them with an additional capability to move [10]. IS26, IS1 and ISEc1, for instance, have been frequently associated with mobilisation of adjacent β -lactamase genes [34–36]. In addition, ISs could also reinforce the expression of other GCs or genes located in the integron. Regarding this latter aspect, the IS10-carrying integron detected here (structure J) might be of special relevance since the transposable element was inserted in the *attI2* site, between Pc2B and Pc2C promoters, likely blocking the expression of the GC (Fig. 3). The *attI2* appeared reconstructed immediately after the 9-bp duplication of the target site 5'-CGGTAAGCA-3', the same as the one described for an equal class 2 integron identified in Portugal (accession no. **LN827615.2**) [37]. This target sequence was almost identical to the consensus site originally described for IS10 (5'-NGCTNAGCN-3'), which led us to think that the IS10 integration could be mediated by a site-specific transposition event. Moreover, previous studies reported the existence of outward-facing promoters at both ends of the IS10 element [38,39] and, as demonstrated by different authors, IS10 insertions confer resistance to fluoroquinolones or chloramphenicol by transcriptional activation of adjacent genes

[40,41]. Furthermore, two class 1 integrons carrying this transposable element at different positions have been described and are registered in the INTEGRALL database (accession nos. **AY600086** and **FJ587511**) [42,43]. Interestingly, in one of these class 1 integrons (accession number **AY600086**), IS10 was located upstream of the *bla*_{OXA-2} cassette and it conferred higher resistance to ampicillin than another derivative lacking IS10 [minimum inhibitory concentration (MIC) = 250 µg/mL vs. 150 µg/mL]. The authors suggested that this difference in ampicillin resistance could be due to the positional effect of the IS10 insertion, which probably enhances *bla*_{OXA-2} transcription [42]. All of these observations encourage further investigation regarding the potential regulatory effects of IS10 integration in the expression of the GCs carried by class 1 and class 2 integrons.

3.2. Class 2 integrons embedded in mosaic structures of the Tn7 module

Another atypical class 2 integron construction detected in this study (Fig. 2; structure D) was characterised by the absence of the *orfX* pseudo-GC, the *ybfA–ybfB–ybgA* group of genes and the Tn7 transposition module (*tnsABCDE*). Although novel rearrangements and the occurrence of rarely reported hybrid class 2/class 1 integrons were tested, none of the PCRs were successful [19,20]. Despite the absence of the *tns* genes, positive amplification with primers Tn7LR/IntI2-F and subsequent sequencing of the obtained product demonstrated the association between this integron and the Tn7 transposon. Furthermore, it was found to be transferable by conjugation, which suggests its location on a conjugative/mobilisable element [e.g. plasmid or integrative and conjugative element (ICE)]. Complete deletion of the Tn7 transposition module may promote the stabilisation of this class 2

integron within the conjugative/mobilisable element, contributing to its spread among different bacterial populations via this specific genetic platform.

In addition, as previously reported [7], different mosaic structures of the *tns* module were detected among studied class 2 integrons (Fig. 2; structures B, C and D). However, most of the isolates (31/35; 89%) harboured all Tn7 transposition genes (*tnsA*, *tnsB*, *tnsC*, *tnsD* and *tnsE*). Interestingly, three strains showed complete IS elements disrupting *tnsD* (Fig. 2; structures L and M) or *tnsE* (Fig. 2; structure K), a finding that has been occasionally reported [44]. The IS elements in these strains belong to three different families, suggesting a non-specific target site for these *tns* genes. Since proteins encoded by the invaded genes are directly involved in the transposition capability and target selection pathways (*attTn7* vs. non-*attTn7* sites) of class 2 integrons, alterations in these regions may define a high degree of host specificity, evidencing an unseen role of ISs for modulating targets of Tn7 transposition.

3.3. Vertical and lateral inheritance of class 2 integrons

Chromosomal insertion of class 2 integrons at the well-known *attTn7* site was confirmed in 80% of the *E. coli* isolates by successfully amplifying and sequencing the right end of Tn7 and the 3'-end of *glmS*. Conjugation experiments demonstrated that 9 (29%) of 31 class 2 integrons could be transferred to *E. coli* CHS26, supporting that they can be inserted within Tn7 on conjugative/mobilisable elements at a lower frequency than in the chromosomes [9] (Table 1; Supplementary Table S1). Increased conjugation rates (ca. 10^{-5}) were shown for isolates in which no band was observed after Tn7LR-*glmS* amplification in the original strain compared with

those harbouring a copy of the integron at the chromosomal *attTn7* site (10^{-6} – 10^{-8}). In these latter cases, conjugal transfer might be explained by the occurrence of a TnsE-mediated transposition event during the process of conjugal replication, or the carriage of a second copy of a class 2 integron by the original isolate (one in the chromosome, another in a plasmid), as previously reported [45]. In that case, both integrons might have an identical structure, since all of the positive PCRs tested showed a single size amplicon.

Although in the present study donors predominantly showed a multidrug-resistant phenotype and carriage of multiple plasmids, *S1*-PFGE revealed that the majority of transconjugants harboured one plasmid with sizes ranging from 30 kb to 100 kb (IncI1, $n = 3$; IncF, $n = 1$; non-typeable, $n = 3$) and a much more susceptible antimicrobial pattern (Table 1). In fact, 56% ($n = 5$) of the transconjugants demonstrated to be resistant only to trimethoprim and streptomycin, in relation to the transfer of class 2 integrons. The remaining four transconjugants also mediated resistance to sulfonamides (*sul2*, $n = 2$; *sul1*, $n = 1$), tetracycline [*tet(A)*, $n = 2$] and/or chloramphenicol (*cmIA*, $n = 1$; *floR*, $n = 1$).

In summary, the present data show that the genetic organisation of class 2 integrons is highly conserved among *E. coli* from different ecosystems and geographic areas. Novel structures are mainly due to the insertion of IS elements, the activity of a functional integrase or by homologous recombination. Since most of the class 2 integrons are chromosomally located, dispersion of these elements appears to be predominantly vertical, although conjugative transfer may efficiently favour the spread of these genetic platforms to novel species, as shown by the presence of

conjugative/mobilisable plasmids of different incompatibility groups harbouring such integrons. Co-location of other resistance genes in the same plasmid, such as those encoding sulfonamide, chloramphenicol or tetracycline resistance, and the insertion of class 2 integrons within defective Tn7 transposons lacking some of the transposition genes, likely contribute to the persistence and dissemination of class 2 integrons.

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References

- [1] Chantziaras I, Boyen F, Callens B, Dewulf J. Correlation between veterinary antimicrobial use and antimicrobial resistance in food producing animals: a report on seven countries. *J Antimicrob Chemother* 2014;69:827-34.
- [2] Shallcross LJ, Davies DS. Antibiotic overuse: a key driver of antimicrobial resistance. *Br J Gen Pract*. 2014;64:604-5.
- [3] Deng Y, Bao X, Ji L, Chen L, Liu J, Miao J, Chen D, Bian H, Li Y, Yu G. Resistance integrons: class 1, 2 and 3 integrons. *Ann Clin Microbiol Antimicrob* 2015;14:45.
- [4] Escudero JA, Loot C, Nivina A, Mazel D. The Integron: adaptation on demand. *Microbiol Spectr* 2015;3:MDNA3-0019-2014.
- [5] Rowe-Magnus DA, Guerout AM, Mazel D. Bacterial resistance evolution by recruitment of super-integron gene cassettes. *Mol Microbiol* 2002;43:1657-69.
- [6] Barlow RS, Gobius KS. Diverse class 2 integrons in bacteria from beef cattle sources. *J Antimicrob Chemother* 2006;58:1133-8.
- [7] Ramírez MS, Piñeiro S, Argentinian Integron Study Group, Centrón D. Novel insights about class 2 integrons from experimental and genomic epidemiology. *Antimicrob Agents Chemother* 2010;54:699-706.
- [8] Parks AR, Peters JE. Tn7 elements: Engendering diversity from chromosomes to episomes. *Plasmid* 2009;61:1-14.
- [9] Wolkow CA, DeBoy RT, Craig NL. Conjugating plasmids are preferred targets for Tn7. *Genes Dev* 1996;10:2145-57.
- [10] Siguier P, Gourgouyere E, Chandler M. Bacterial insertion sequences: their genomic impact and diversity. *FEMS Microbiol Rev* 2014;38:865-891.

- [11] Alcalá L, Alonso CA, Simón C, González-Esteban C, Orós J, Rezusta A, Ortega C, Torres C. Wild birds, frequent carriers of extended-spectrum β -lactamase (ESBL) producing *Escherichia coli* of CTX-M and SHV-12 types. *Microb Ecol* 2016;72:861-9.
- [12] Cortés-Cortés G, Lozano-Zarain P, Torres C, Castañeda M, Sánchez GM, Alonso CA, López-Pliego L, Mayen MG, Martínez-Laguna Y, Rocha-Gracia RC. Detection and molecular characterization of *Escherichia coli* strains producers of extended-spectrum and CMY-2 type beta-lactamases, isolated from turtles in Mexico. *Vector Borne Zoonotic Dis* 2016;16:595-603.
- [13] Maamar E, Hammami S, Alonso CA, Dakhli N, Abbassi MS, Ferjani S, Hamzaoui Z, Saidani M, Torres C, Boutiba-Ben Boubaker I. High prevalence of extended-spectrum and plasmidic AmpC beta-lactamase-producing *Escherichia coli* from poultry in Tunisia. *Int J Food Microbiol* 2016;231:69-75.
- [14] Orman BE, Piñeiro SA, Arduino S, Galas M, Melano R, Caffer MI, Sordelli DO, Centrón D. Evolution of multiresistance in non-typhoid *Salmonella* serovans from 1984 to 1998 in Argentina. *Antimicrob Agents Chemother* 2002;46:3963-70.
- [15] White PA, McIver CJ, Rawlinson WD. Integrons and gene cassettes in the *Enterobacteriaceae*. *Antimicrob Agents Chemother* 2001;45:2658-61.
- [16] Navia MM, Ruiz J, Sánchez-Céspedes J, Vila J. Detection of dihydrofolate reductase genes by PCR and RFLP. *Diagn Microbiol Infect Dis* 2003;46:295-8.
- [17] Ramírez MS, Quiroga C, Centrón D. Novel rearrangement of a class 2 integron in two non-epidemiologically related isolates of *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2005;49:5179-81.

- [18] Quiroga MP, Andres P, Petroni A, Soler Bistué AJ, Guerriero L, Vargas LJ, Zorreguieta A, Tokumoto M, Quiroga C, Tolmasky ME, Galas M, Centrón D. Complex class 1 integrons with diverse variable regions including *aac(6')*-*Ib-cr*, and a novel allele, *qnrB10*, associated with *ISCR1* in clinical enterobacterial isolates from Argentina. *Antimicrob Agents Chemother* 2007;51:4466-70.
- [19] Xia R, Ren Y, Guo X, Xu H. Molecular diversity of class 2 integrons in antibiotic-resistant gram-negative bacteria found in wastewater environments in China. *Ecotoxicology* 2013;22:402-14.
- [20] Ploy MC, Denis F, Courvalin P, Lambert T. Molecular characterization of integrons in *Acinetobacter baumannii*: Description of a hybrid class 2 integron. *Antimicrob Agents Chemother* 2000;44:2684-8.
- [21] Gay NJ, Tybulewicz VL, Walker JE. Insertion of transposon Tn7 into the *Escherichia coli glmS* transcriptional terminator. *Biochem J* 1986;234:111-7.
- [22] Kadlec K, Schwarz S. Analysis and distribution of class 1 and class 2 integrons and associated gene cassettes among *Escherichia coli* isolates from swine, horses, cats and dogs collected in the BfT-GermVet monitoring study. *J Antimicrob Chemother* 2008;62:469-73.
- [23] Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing. Twenty fifth informational supplement. CLSI document M100-S25. Wayne, PA: National Committee for Clinical Laboratory Standards, 2015.
- [24] Schink AK, Kadlec K, Schwarz S. Analysis of *bla*CTX-M-carrying plasmids from *Escherichia coli* isolates collected in the BfT-Germ Vet study. *Appl Environ Microbiol* 2011;77:7142-6.

- [25] Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfal EJ. Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods* 2005;63:219-28.
- [26] Limansky AS, Viale A. Can composition and structural features of oligonucleotides contribute to their wide-scale applicability as random PCR primers in mapping bacterial genome diversity? *J Microbiol Methods* 2002;50:291-7.
- [27] Partridge SR, Hall RM. Correctly identifying the streptothricin resistance gene cassette. *J Clin Microbiol* 2005;43:4298-300.
- [28] Hansson K, Sundström L, Pelletier A, Roy PH. IntI2 Integron Integrase in Tn7. *J Bacteriol* 2002;184:1712-21.
- [29] Ahmed AM, Nakano H, Shimamoto T. Molecular characterization of integrons in non-typhoid *Salmonella* serovars isolated in Japan: description of an unusual class 2 integron. *J Antimicrob Chemother* 2005;55:371-4.
- [30] Tetu SG, Holmes AJ. A family of insertion sequences that impacts integrons by specific targeting of gene cassette recombination sites, the IS1111-attC group. *J Bacteriol* 2008;190:4959-70.
- [31] Briski L, Mazel D. Erythromycin esterase gene *ere(A)* is located in a functional gene cassette in an unusual class 2 integron. *Antimicrob Agents Chemother* 2003;47:3326-31.
- [32] Dubois V, Parizano MP, Arpin C, Coulange L, Beziau MC, Quentin C. High genetic stability of integrons in clinical isolates of *Shigella* spp. of worldwide origin. *Antimicrob Agents Chemother* 2007;51:1333-40.

- [33] Sow AG, Diallo MH, Gatet M, Denis F, Aïdara-Kane A, Ploy MC. Description of an unusual class 2 integron in *Shigella sonnei* isolates in Senegal (sub-Saharan Africa). *J Antimicrob Chemother* 2008;62:843-4.
- [34] Beyrouthy R, Robin F, Delmas J, Gibold L, Dalmasso G, Dabboussi F, Hamzé M, Bonnet R. IS1R-mediated plasticity of IncL/M plasmids leads to the insertion of *bla*OXA-48 into *Escherichia coli* chromosome. *Antimicrob Agents Chemother* 2014;58:3785-90.
- [35] Smet A, Van Nieuwerburgh F, Vandekerckhove TT, Marte A, Deforce D, Butaye P, Haesebrouck F. Complete nucleotide sequence of CTX-M-15-plasmids from clinical *Escherichia coli* isolates: insertional events of transposons and insertion sequences. *PLoS One* 2010;5:e11202.
- [36] Zhang F, Wang X, Xie L, Zheng Q, Guo X, Han L, Sun J. A novel transposon, Tn6306, mediates the spread of *bla*IMI in Enterobacteriaceae in hospitals. *Int J Infect Dis* 2017;65:22-6.
- [37] Jones-Dias D, Manageiro V, Martins AP, Ferreira E, Caniça M. New class 2 integron In2-4 among IncI1-positive *Escherichia coli* isolates carrying ESBL and PMA β genes from food animals in Portugal. *Foodborn Pathog Dis* 2016;13:36-9.
- [38] Martínez-García E, Navarro-Llorens JM, Tormo A. Identification of an unknown promoter, OUTIIp, within the IS10R element. *J Bacteriol* 2003;185:2046-50.
- [39] Simons RW, Hoopes BC, McClure WR, Kleckner N. Three promoters near the termini of IS10: pIN, pOUT and pIII. 1983;34:673-82.
- [40] Olliver A, Valle M, Chaslus-Dancla E, Cloeckaert A. Overexpression of the multidrug efflux operon *acrEF* by insertional activation with IS1 or IS10

elements in *Salmonella enterica* serovar typhimurium DT204 *acrB* mutants selected with fluoroquinolones. *Antimicrob Agents Chemother* 2005;49:289-301.

[41] González-Prieto C, Agúndez L, Llosa M. Chloramphenicol selection of IS10 transposition in the *cat* promoter region of widely used cloning vectors. *PLoS One* 2015;10:e0138615.

[42] Szczepanowski R, Krahn I, Pühler A, Schlüter A. Different molecular rearrangements in the integron of the IncP-1 beta resistance plasmid pB10 isolated from a wastewater treatment plant result in elevated beta-lactam resistance levels. *Arch Microbiol* 2004;182:429-435.

[43] Vinué L, Sáenz Y, Rojo-Bezares B, Olarte I, Undabeitia E, Somalo S, Zarazaga M, Torres C. Genetic environment of *sul* genes and characterisation of integrons in *Escherichia coli* isolates of blood origin in a Spanish hospital. *Int J Antimicrob Agents* 2010;35:492-496.

[44] Young HK, Qumsieh MJ, McIntosh ML. Nucleotide sequence and genetic analysis of the type IB trimethoprim-resistant, Tn4132-encoded dihydrofolate reductase. *J Antimicrob Chemother* 1994;34:715-25.

[45] Rodríguez I, Martín MC, Mendoza MC, Rodicio MR. Class 1 and class 2 integrons in non-prevalent serovars of *Salmonella enterica*: structure and association with transposons and plasmids. *J Antimicrob Chemother* 2006;58:1124-32.

Fig. 1. Schematic representation of the PCR cartography of class 2 integrons performed in this study, using the classic Tn7:In2-7 as a model. Arrows represent the localisation and direction of each primer.

Fig. 2. Genetic diversity of class 2 integron structures found among the studied *Escherichia coli* collection ($n = 35$). Arrows represent the open reading frames. * The asterisk in *intI2* shows the typical non-sense mutation. The black vertical bars represent the *attI2* and $\Delta attI2$ sites, and coloured bars represent the *attC* sites of the gene cassettes.

Fig. 3. Insertion of IS10 in the *attI2* site, between Pc2B and Pc2C promoters, of the class 2 integron harboured by *Escherichia coli* strain C4748. The -35 and -10 elements of the putative Pc promoters (Pc2D, Pc2A, Pc2B and Pc2C) are indicated and written in bold. The target site, duplicated after IS10 transposition, is shown in italics and underlined. The *intI2* start codon appears boxed and written in bold.

Table 1

Phenotypic and genotypic characteristics of the nine original *Escherichia coli* strains and their corresponding transconjugants harbouring class 2 integrons

Isolate	Source	Resistance phenotype	Class 2 integron structure ^a	Tn7 insertion in the <i>attTn7</i> chromosomal site	Plasmids			Transfer by conjugation of class 2 integrons	Class 2 integron-carrying transconjugants				
					<i>n</i>	Size (kb)	Replicon types		Conjugation frequency	Resistance phenotype	Plasmid replicon type	Plasmid size (kb)	Resistance genes outside the class 2 integron
C2575	Dog	AMP, CTX, CAZ, CIP, NAL, STR, SUL, SXT, TMP, TET	A	–	4	140, 100, 75, 40	FIB, F, I1	+	3.5×10^{-5}	STR, TMP	NT	ND	
C4746	Chicken meat	AMP, CTX, CAZ, CIP, NAL, STR, SUL, SXT, TMP, TET	A	–	4	140, 90, 75, 60	FIB, F, I1	+	4.8×10^{-5}	STR ¹ , TMP	NT	60	
C7218	Turtle	AMP, CTX, CAZ, STR, FOX, CHL, TOB, SUL, SXT, TMP, TET	D	–	2	130, 100	FIB, F	+	6.1×10^{-5}	CHL, STR, SUL, SXT, TMP, TET	NT	100	<i>floR</i> , <i>sul2</i> , <i>tet(A)</i>

C8432	Chicken	AMP, CTX, CIP ^l , NAL, STR, SUL, SXT, TMP, TET	C	-	3	200, 90, 70	FIB, F	+	1.9×10^{-5}	STR, SUL, SXT, TMP	F	70	<i>sul1</i>
C8461	Chicken	AMP, CTX, NAL, STR, SUL, SXT, TMP, TET	C	-	2	190, 90	FIB, F	+	9.4×10^{-7}	STR, TMP	NT	ND	
C3309	Pork meat	AMP, AMC, CTX, CAZ, FOX, CIP, NAL, STR, CHL, SUL, SXT, TMP, TET	I	+	3	125, 95, 80	FIB, F, I1	+	6.6×10^{-8}	CHL, STR, TMP	I1	95	<i>cmIA</i>
pn436	Chicken meat	STR, SUL, SXT, TMP	A	+	4	120, 90, 50, 20	F	+	2.7×10^{-6}	STR, TMP	NT	30	
C7577	Roe deer	AMP, CTX, CAZ, NAL, STR, TMP, TET	F	+	2	90, 20	P, I1	+	9.5×10^{-6}	STR, TMP	I1	30	
C6901	Chicken	AMP, CTX, NAL, STR, SUL, SXT, TMP, TET	A	+	4	95, 85, 65, 40	F, I1	+	7.2×10^{-6}	STR ^l , SUL, SXT, TMP, TET	I1	65	<i>sul2, tet(A)</i>

AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; CIP, ciprofloxacin; NAL, nalidixic acid; STR, streptomycin; SUL, sulfonamides; SXT, trimethoprim/sulfamethoxazole; TMP, trimethoprim; TET, tetracycline; FOX, ceftiofur; CHL, chloramphenicol; TOB, tobramycin; AMC, amoxicillin/clavulanic acid; NT, non-typeable; ND, not detected.

^a These structures correspond to those represented in Fig. 2.

^l Indicates intermediate resistance.

Supplementary Table S1

Characteristics of *Escherichia coli* strains carrying class 2 integrons included in this study ($n = 35$)

Strain no.	Source	Geographic origin	Year of isolation	Resistance phenotype	Class 2 integron structure ^a	Transfer by conjugation
C1949	Chicken meat	Spain	2009	AMP, CTX, CAZ, NAL, STR, TMP, TET	H	–
C4746	Chicken meat	Spain	2011	AMP, CTX, CAZ, CIP, NAL, STR, SUL, SXT, TMP, TET	A	+
C4748	Chicken meat	Spain	2011	AMP, CTX, CAZ, CIP, NAL, STR, SUL, SXT, TMP, TET	J	–
Pn425	Chicken meat	Spain	2008	AMP, AMC, STR, SUL, SXT, TMP, TET	Ñ	ND
Pn424	Chicken meat	Spain	2008	AMP, AMC, CAZ, FOX ^I , CIP, NAL, STR, SUL, SXT, TMP, TET	E	–
Pn430	Chicken meat	Spain	2008	AMP, AMC, CTX ^I , FOX ^I , CIP ^I , NAL, STR, TMP	N	ND
Pn432	Chicken meat	Spain	2008	AMP, AMC, CAZ, FOX ^I , STR, SUL, SXT, TMP, TET	N	ND
pn436	Chicken meat	Spain	2008	STR, SUL, SXT, TMP	A	+
Co1	Chicken meat	Spain	1998	AMP, CIP, NAL, STR, CHL, SUL, SXT, TMP, TET	A	–
Co6	Chicken meat	Spain	1998	AMP, NAL, STR, GEN, TOB, SUL, SXT, TMP	B	–
Co7	Chicken meat	Spain	1998	CIP, NAL, STR, GEN, SUL, SXT, TMP, TET	A	–
Co17	Chicken meat	Spain	1998	CIP, NAL, STR, CHL, SUL, SXT, TMP, TET	Ñ	ND
Co18	Chicken meat	Spain	1998	AMP, STR, GEN, SUL, SXT, TMP, TET	A	–
Co19	Chicken meat	Spain	1998	AMP, NAL, STR, CHL, SUL, SXT, TMP, TET	A	–
C3309	Pork meat	Mexico	2010	AMP, AMC, CTX, CAZ, FOX, CIP, NAL, STR, CHL, SUL, SXT, TMP, TET	I	+
C8461	Chicken	Tunisia	2014	AMP, CTX, NAL, STR, SUL, SXT, TMP, TET	C	+
C6901	Chicken	Tunisia	2013	AMP, CTX, NAL, STR, SUL, SXT, TMP, TET	A	+
C6924	Chicken	Tunisia	2013	AMP, CTX, NAL, STR, SUL, SXT, TMP, TET	A	–
C6932	Chicken	Tunisia	2013	AMP, CTX, NAL, STR, SUL, SXT, TMP, TET	A	–
C6935	Chicken	Tunisia	2013	AMP, CTX, CIP, NAL, STR, SUL, SXT, TMP, TET	A	–
C8432	Chicken	Tunisia	2014	AMP, CTX, CIP ^I , NAL, STR, SUL, SXT, TMP, TET	C	+
C2575	Pet (dog)	Spain	2009	AMP, CTX, CAZ, CIP, NAL, STR, SUL, SXT, TMP, TET	A	+
C2583	Pet (dog)	Spain	2009	AMP, CTX, CAZ, CIP, NAL, STR, GEN, SUL, SXT, TMP, TET	M	–

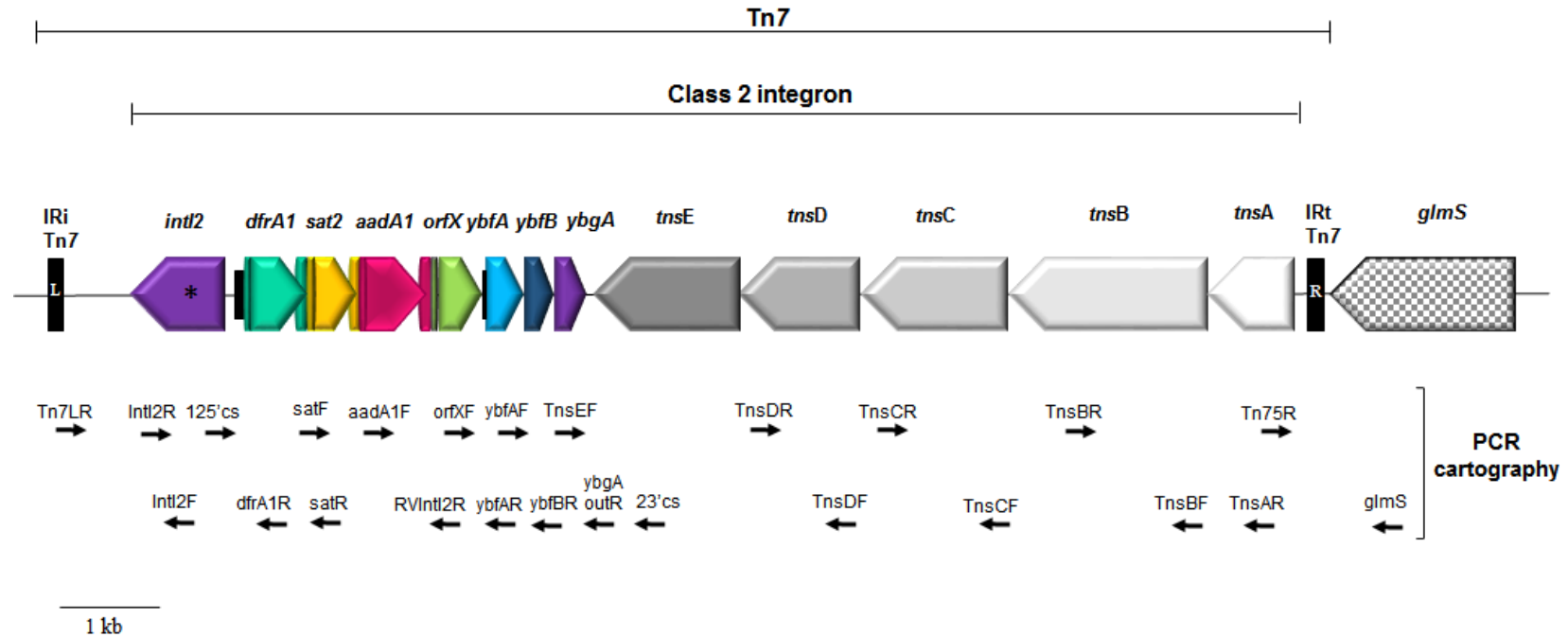
C7204	Pet (dog)	Mexico	2012	AMP, AMC, CTX, FOX, CIP, NAL, STR, GEN, AMK, TOB, SUL, SXT, TMP, TET	I	–
C7210	Pet (turtle)	Mexico	2012	AMP, CTX, CIP, NAL, STR, GEN, CHL, SUL, SXT, TMP, TET	G	–
C7218	Pet (turtle)	Mexico	2012	AMP, CTX, CAZ, FOX, STR, CHL, TOB, SUL, SXT, TMP, TET	D	+
C7577	Wild animal (roe deer)	Spain	2014	AMP, CTX, CAZ, NAL, STR, TMP, TET	F	+
C7373	Wild animal (genet)	Spain	2014	STR, TMP	A	–
C7377	Wild animal (stork)	Spain	2014	AMP, CAZ, CIP, NAL, STR, CHL, SUL, SXT, TMP, TET	K	–
C6477	Wild animal (seagull)	Spain	2013	AMP, AMC, CTX, FOX, CIP, NAL, TET	A	–
C1550	Human (faeces)	Mexico	2007	AMP, CTX, CAZ, FOX, CIP, NAL, STR ¹ , SUL, SXT, TMP	A	–
Pn205	Human (faeces)	Spain	2007	AMP, STR, CHL, SUL, SXT, TMP	A	–
Pn253	Human (faeces)	Spain	2007	AMP, STR, SUL, SXT, TMP, TET	L	–
Pn288	Human (blood)	Spain	2007	AMP, STR, SUL, SXT, TMP	A	–
Pn110	Human (blood)	Spain	2007	AMP, STR ¹ , SUL, SXT, TMP, TET	A	–

AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; NAL, nalidixic acid; STR, streptomycin; TMP, trimethoprim; TET, tetracycline; CIP, ciprofloxacin; SUL, sulfonamides; SXT, trimethoprim/sulfamethoxazole; AMC, amoxicillin/clavulanic acid; FOX, ceftazidime; CHL, chloramphenicol; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; ND, not determined.

^a These structures correspond to those represented in Fig. 2.

¹ Indicates intermediate resistance.

Fig.1.



Primer	Target	Sequence (5'-3')	Reference
Tn7LR	IR left of Tn7	GAAATGGAGTTTTTAAAGG	[7]
IntI2F	<i>intI2</i> gene	GCAAATGAAGTGCAACGC	[14]
IntI2R	<i>intI2</i> gene	ACACGCTTGCTAACGATG	[14]
125'CS	<i>intI2</i> gene	TTTTTGTCGCATATCCGTG	[7]
RV-IntI2	<i>intI2</i> gene downstream surrounding	CGGATCCCGGACGGCATGACGATT	[15]
dfrA1R	<i>dfrA1</i> gene cassette	TTAACCTTTTGCCAGATTT	[16]
satF	<i>sat2</i> gene cassette	TGAGCAGGTGGCGGAAAC	[17]
satR	<i>sat2</i> gene cassette	TCATCCTGTGCTCCCGAG	[17]
aadA1-F	<i>aadA1</i> gene cassette	TTGCTGGCCGTACATTTG	[18]
RVIntI2-R	<i>orfX</i> pseudo gene cassette	GATGCCATCGCAAGTACGAG	[15]
OrfX-F	<i>orfX</i> pseudo gene cassette	GCTTGCCCTGACAGATAACG	This study
ISSen4-R	ISSen4 element	CAACTGCCAAATACGACGG	This study
ybfA-R	<i>ybfA</i> gene	GTTATCTAGTTCGACATAGTC	[7]
ybfA-F	<i>ybfA</i> gene	GATGCCGAATGGATGGAT	This study

Primer	Target	Sequence (5'-3')	Reference
ybfB-R	<i>ybfB</i> gene	GCATGTGCACGTTCCGAC	[7]
IS100kyp-R	IS100 element (IS21 family)	GGTTTTCCCCACACCTGATG	This study
TnsEF	<i>ybgA</i> gene	AATGCCCTACTTTGACACGG	[17]
ybgA-out-R	<i>ybgA</i> gene downstream surrounding	TCCGTAGGGAAACACCTTGAG	This study
23'CS	<i>tnsE</i> gene	TGGGCTGAGAGATGGT	[7]
TnsDF	<i>tnsD</i> gene	GGGATTGTTAGTCCTAAGC	[17]
TnsDR	<i>tnsD</i> gene	CCGTCTAATTTGATAATCTTC	[17]
TnsCF	<i>tnsC</i> gene	GTTATCGTGATACGGGGG	[17]
TnsCR	<i>tnsC</i> gene	GCTATCCCAGTCGCTGGG	[17]
TnsBF	<i>tnsB</i> gene	CATGTGGTCCAAGAACATAAG	[17]
TnsBR	<i>tnsB</i> gene	GAGCAAGCATTTACAAAAGC	[17]
TnsAR	<i>tnsA</i> gene	GCTAACAGTACAAGAAGTTCC	[17]
Tn75R	<i>tnsA</i> gene	GACTCGTCCCGTCTTATGAG	[21]
GlmS	<i>glmS</i> gene	GGCGTCAGTTGTATGTCTT	[21]

Fig. 2.

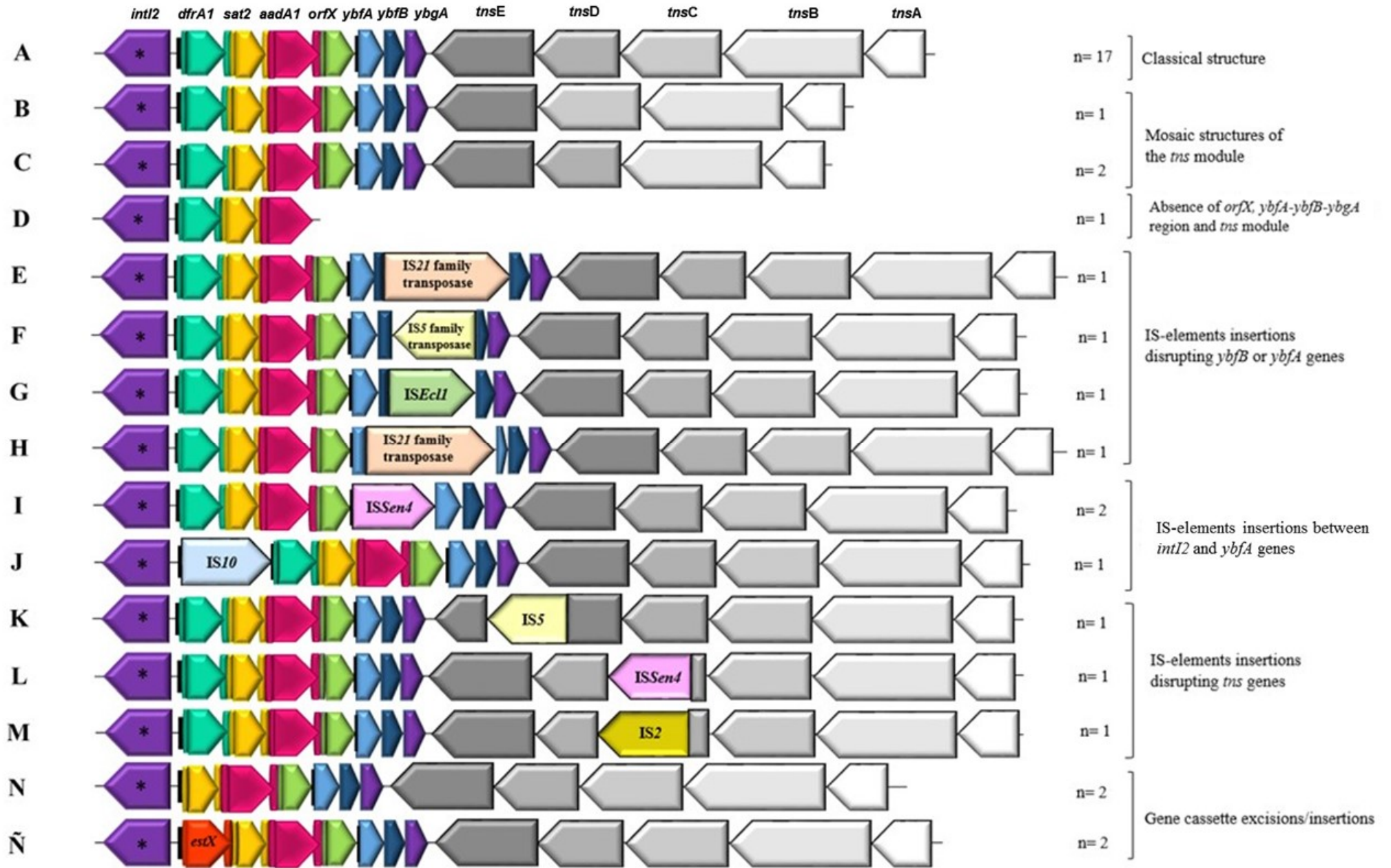


Fig. 3.

