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Genetic characterization of novel class 1 Integrons In0, In1069 and In1287 to In1290, and the inference of In1069-associated integron evolution in *Enterobacteriaceae*

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Abstract

Background: This study aims to characterize genetically related class 1 integrons In1069, In893 and In1287 to In1290, and to further propose a scheme of stepwise integration or excision of individual gene cassettes (GCs) to generation of these integron variations.

Methods: Six of 139 non-redundant *Enterobacteriaceae* strains were studied by bacterial antimicrobial susceptibility testing, detection of carbapenemase activity, and integron sequencing and sequence comparison.

Results: Six novel class 1 integrons, In0, In1069, and In1287 to In1290, together with the previously characterized In893, were determined from the above strains. An unusual bla_{KPC-2}-carrying In0 and the bla_{IMP-30}-carrying In1069 coexists in a single isolate of *Escherichia coli*. In0 contains a PcH1 promoter and a truncated *aacA4′-3* gene cassette (GC*aacA4′-3*), as well as a bla_{KPC-2}-containing region of Tn*6296* integrated between PcH1 and GC*aacA4′-3*. In1069 carries GCbla_{IMP-30} and GC*aacA4′-3* in this order. The other five integrons, In893 and In1287 to In1290, are genetically related to In1069, and all possess a core GC*aacA4′-3*. The integration or excision of one or more individual gene cassettes, such as GCbla_{IMP-30}, GC*aadA16*, GC*catB3*, GC*arr3* and GC*dfrA27*, upstream or downstream of GC*aacA4′-3* generates various gene cassettes arrays among these five integrons.

Conclusions: These findings provide the insight into stepwise and parallel evolution of In1069-associated integron variations likely under antibiotic selection pressure in clinical settings.

Keywords: Class 1 integron, Gene cassette, bla_{KPC-2}, bla_{IMP-30}, *Enterobacteriaceae*

Background

Integrons are genetic elements containing a site-specific recombination system capable of integrating, exchanging, and expressing gene cassettes (GCs). Each GC is composed of an exogenous and often promoterless gene together with a recombination site, *attC* [1–5]. The recombination system possesses an integrase gene, *intI*, needed for site-specific recombination, an adjacent recombination site, *attI*, recognized by the *IntI* integrase, and a promoter, Pc, located upstream of *attI* and necessary for efficient transcription and expression of GCs [1–5]. The *attC* site is also recognized by *IntI*, and recombination between *attC* and *attI* leads to the addition and exchange of GCs and further generation of a multi-GC array within the integron structure [1–5].

Based on the amino acid sequences of *IntI* integrase, integrons can be divided into different classes, with those carrying *intI1* defined as class 1, *intI2* as class 2, *intI3* as class 3, and so on. Class 1 integrons are the most common type present among *Enterobacteriaceae* isolates [6–8].

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Class 1 integrons constitute a substantial reservoir of resistance genes that confer a selective advantage upon strong selection pressure imposed by human use of antimicrobial compounds, leading to the horizontal transfer of integron-carrying resistance markers from the community to hospitals and the development of multidrug resistance (MDR) among *Enterobacteriaceae*, independent of species or isolate origin [6–8].

The ancestors of class 1 integrons are not considered to be mobile elements, and the connection of class 1 integrons with Tn*402* with a complete *tniABQC* transposition module generates a hybrid structure flanked by the 25 bp terminal inverted repeat initial (IRi) and inverted repeat terminal (IRt), making class 1 integrons capable of self-mobility [1–3]. The capture and formation of a quaternary ammonium compound resistance (*qacEΔ1*)-sulphonamide resistance (*sul1*)-*orf5* region occurs immediately downstream of the GC array [1–3]. Eventually, class 1integrons manifest as a prototype structure organized in order of IRi, a 5′-conserved segment (5′-CS: *intI-attI*), a central variable region (the GC array), a 3′-conserved segment (3′-CS: *qacEΔ1-sul1-orf5-tniABQC*), and IRt [1–3]. Most class 1 integrons from clinical contexts carry modifications at their 5′ and 3′ ends, especially partial or complete deletions of the *tniABQC* module of Tn*402*, which impairs their mobility [4, 5]. These integrons are often inserted within mobile DNA elements such as plasmids and transposons, facilitating their rapid spread in the community and within hospitals [4, 5].

This work presents the sequences of six novel class 1 integrons, In0, In1069 and In1287 to In1290, together with the previously characterized In893. These integrons were obtained from clinical *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* isolates. The $bla_{\text{KPC-2}}$-carrying unusual In0 and the $bla_{\text{IMP-30}}$-carrying In1069 coexist in a single *Escherichia coli* isolate. The detailed genetic characterization of genetically related integrons In1069, In893 and In1287 to In1290 denotes a scheme of stepwise integration or excision of individual GCs to generate these integron variations.

Methods

Bacterial isolates and identification

A total of 139 non-redundant *Enterobacteriaceae* strains, including *Escherichia coli* EC6335, *Escherichia coli* EC4212, *Klebsiella pneumoniae* KP1262, *Enterobacter cloacae* ECL2236, *Escherichia coli* EC7328 and *Klebsiella pneumoniae* KP5325, were recovered from hospitalized patients with nosocomial infections in a teaching hospital of Taizhou, China, from January 2014 to September 2015. Bacterial species were identified by 16S rRNA gene sequencing [9]. All DNA markers listed in Table 1 were screened by PCR amplification (ThermoFisher scientific, USA) using the listed primers, followed by amplicon sequencing on an ABI 3730 Sequencer (ThermoFisher scientific, USA). PCR was run for 3 min at 94 °C followed by 30 cycles of 1 min of denaturing at 94 °C and annealing at 50 to 59 °C according to various primers, with a final elongation of 10 min at 72 °C on Life Veriti® PCR machine (Invitrogen, USA). The total reaction volume was 20 μL containing 4 μL 5 × PCR buffer, 0.4 μL of 10 mM dNTPs, 1 μL each of 10 μM primers and 0.2 μL Polymerase, with nuclease-free water filled up to 20 μL. PCR amplification and amplicon sequencing were run in according with operation manual.

Integron cloning and sequencing

For cloning of In0 and In1069, the bla_{KPC}- and bla_{IMP}-positive strain EC6335 was identified by PCR. Plasmid DNA was then isolated from this strain using the AxyPrep Plasmid Miniprep kit (Axygen, USA), digested with *Bam*HI, and ligated into the cloning vector pMD19-T. This was transformed into host bacteria *Escherichia coli* TOP10, which were screened for bla_{KPC}- or bla_{IMP}-positivity. Inserts within recombinant pMD19-T vectors from the above transformants were sequenced using the primer walking method. For cloning of In893 and In1287 to In1290, the strains EC4212, KP1262, ECL2236, EC7328, and KP5325, each co-harboring the three genes *aacA4*, *intI1*, and *tniR*, were identified by PCR, then DNA fragments were amplified from these strains using the primer pair *intI1*-F/*tniR*-R2 and subsequently sequenced as above.

Sequence annotation and comparison

Open reading frames were predicted using RAST 2.0 [10] combined with BLASTP/BLASTN [11] searches against the UniProtKB/Swiss-Prot database [12] and the RefSeq database [13]. Annotation of resistance genes, mobile elements, and other features was carried out using the online databases including CARD [14], ResFinder [15], ISfinder [16] and INTEGRALL [17]. Multiple and pairwise sequence comparisons were performed using MUSCLE 3.8.31 [18] and BLASTN, respectively. Gene organization diagrams were drawn in Inkscape 0.48.1 (https://inkscape.org).

Detection of carbapenemase activity

The activity of class A/B/D carbapenemases in bacterial cell extracts was determined via a modified Carba NP test [19]. Overnight bacterial cell cultures in MH broth were diluted 1: 100 into 3 mL of fresh MH broth, and bacteria were allowed to grow at 37 °C with shaking at 200 rpm to reach an OD600 of 1.0 to 1.4. If required, ampicillin was used at 200 μg/mL. Bacterial cells were harvested from 2 mL of the above culture, and washed twice with 20 mM Tris-HCl (pH 7.8). Cell pellets were

Table 1 Oligonucleotide primers used in this study

Target	Primer	Primer sequence (5′-3′)	Amplicon length (bp)	Reference
bla_{KPC}	KPC-F	GTATCGCCGTCTAGTTCTGC	637	[26]
	KPC-R	GGTCGTGTTTCCCTTTAGCC		
bla_{IMP}	IMP-F	GGAATAGAGTGGCTTAAYTCTC	232	[26]
	IMP-R	GGTTTAAYAAAACAACCACC		
bla_{NDM}	NDM-F	GGTTTGGCGATCTGGTTTTC	621	[26]
	NDM-R	CGGAATGGCTCATCACGATC		
bla_{VIM}	VIM-F	GATGGTGTTTGGTCGCATA	390	[26]
	VIM-R	CGAATGCGCAGCACCAG		
bla_{OXA-23}	OXA-23-F	GATCGGATTGGAGAACCAGA	501	[27]
	OXA-23-R	ATTTCTGACCGCATTTCCAT		
bla_{OXA-48}	OXA-48-F	TTGGTGGCATCGATTATCGG	744	[28]
	OXA-48-R	GAGCACTTCTTTTGTGATGGC		
bla_{OXA-58}	OXA-58-F	AAGTATTGGGGCTTGTGCTG	599	[27]
	OXA-58-R	CCCCTCTGCGCTCTACATAC		
aacA4	aacA4-F	TATGAGTGGCTAAATCGAT	395	[23]
	aacA4-R	CCCGCTTTCTCGTAGCA		
intI1	intI1-F	GCTGAAAGGTCTGGTCATAC	515	This study
	intI1-R	GTTCTTCTACGGCAAGGTG		
tniR	tniR-F	CCAGGGTTGGCTGCTTGC	375	This study
	tniR-R1	ATCGCCCACCTTGGTTTCC		
intI1 to *tniR*	intI1-F	GCTGAAAGGTCTGGTCATAC	Variable	This study
	tniR-R2	ACGCTGATCGTGTGGAAG		

resuspended in 500 μL of 20 mM Tris-HCl (pH 7.8), and lysed by sonication, followed by centrifugation at 10,000×*g* at 4 °C for 5 min. A total of 50 μL of the supernatant (the enzymatic bacterial suspension) was separately mixed with 50 μL each of substrates I to V: 50 μL supernatant was added to 50 μL substrate I, then separately 50 μL supernatant was added to 50 μL substrate II, and so on; followed by incubation at 37 °C for a maximum of 2 h. Substrate I: 0.054% phenol red plus 0.1 mM ZnSO4 (pH 7.8); substrate II: 0.054% phenol red plus 0.1 mM ZnSO4 (pH 7.8), and 0.6 mg/μL imipenem; substrate III: 0.054% phenol red plus 0.1 mM ZnSO4 (pH 7.8), 0.6 mg/μL imipenem, and 0.8 mg/μL tazobactam; substrate IV: 0.054% phenol red plus 0.1 mM ZnSO4 (pH 7.8), 0.6 mg/μL imipenem, and 3 mM EDTA (pH 7.8); substrate V: 0.054% phenol red plus 0.1 mM ZnSO4 (pH 7.8), 0.6 mg/μL imipenem, 0.8 mg/μL tazobactam, and 3 mM EDTA (pH 7.8).

Bacterial antimicrobial susceptibility testing

Bacterial antimicrobial susceptibility was tested by the MicroScan broth dilution method (MicroScan, USA) and interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [20].

Nucleotide sequence accession numbers

The sequences of In0, In1069, In893, and In1287 to In1290 were deposited in GenBank under accession numbers KP870110, KM589497, KX434463, KX371912, and KX387648 to KX387650, respectively.

Results

Clinical bacterial isolates containing integrons

Escherichia coli EC6335, *Escherichia coli* EC4212, *Klebsiella pneumoniae* KP1262, *Enterobacter cloacae* ECL2236, *Escherichia coli* EC7328, and *Klebsiella pneumoniae* KP5325 were determind to harbor class 1 intergrons In0 and In1069, In893, In1287, In1288, In1289, and In1290 (see Additional file 1), respetively. Modular stuctures and sequence comprasion of these integrons and related reference sequences were showed in Figs. 1 and 2. These strains were isolated from diffrent patients from the single hospital.

A 53-year-old male with vomiting, high fever, and septic shock was admitted in the hospital in August 2015. The patient had underlying diabetes, and complained of an infected leg wound. Large doses of vasoactive drugs were needed to maintain his blood pressure, but empirical intravenous antimicrobial administration of teicoplanin

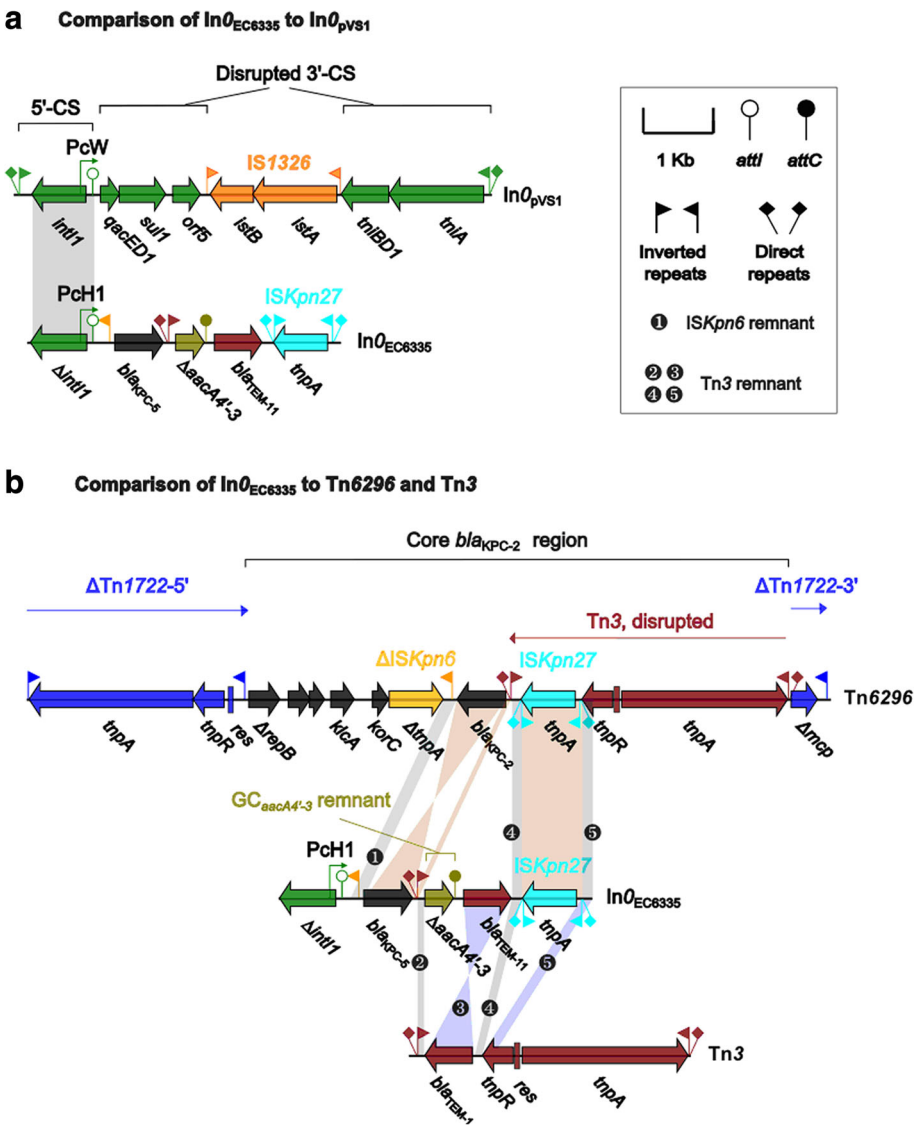

Fig. 1 Genetic structure of $In0_{EC6335}$ and comparison with related regions. Genes are denoted by arrows and colored according to gene function classification. Shaded areas denote regions of homology (>95% nucleotide identity). (**a**) Comparison of In0EC6335 to InpVS1; (**b**) Comparison of In0EC6335 to Tn6296 and Tn3

plus meropenem was ineffective. Carbapenem-nonsusceptible *Escherichia coli* EC6335 was subsequently isolated from the wound secretions. The patient was switched to intravenous administration of levofloxacin plus fluconazole based on antimicrobial susceptibility test results. His symptoms associated with infection progressively improved and he was discharged after 10 days of antimicrobial treatment.

Klebsiella pneumoniae KP1262 and *Escherichia coli* EC4212 were isolated from the blood specimens in the infection unit in July 2015. Both patients complained of suffering high fevers for two to three days, and were diagnosed to have bacteriaemia. *Enterobacter cloacae* ECL2236 was isolated from the sputum specimens in the respiration medicine unit in January 2014. *Escherichia coli* EC7328 was recovered from the urine specimens in the urinary surgery ward in September 2015. *Klebsiella pneumoniae* KP5325 was a cultivated from the sputum in the neurosurgery unit in May 2014. All of these three patients were treated by intravenous administration of ceftriaxone according to the antimicrobial susceptibility test results, and their symptoms associated with infections were gradually recovered after several days of treatments.

Coexistence of In0 and In1069 in Escherichia coli EC6335

PCR screening indicated the presence of *bla*KPC and *bla*IMP, but none of the other tested carbapenemase

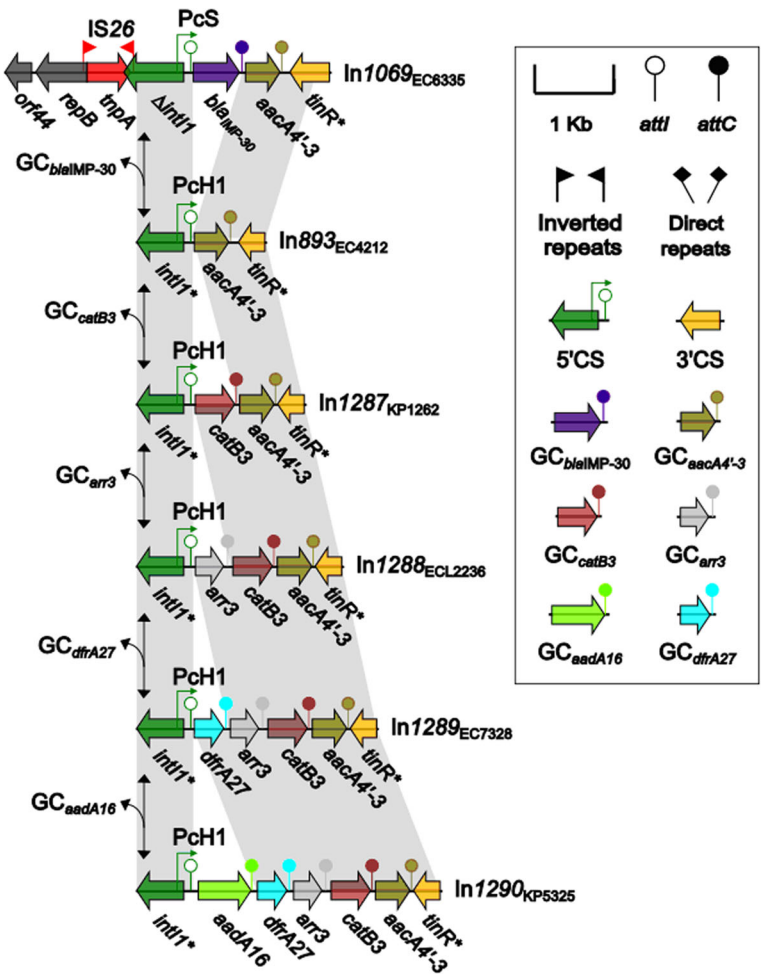

Fig. 2 Genetic structures and proposed evolutionary history of In1069 and related integrons. Genes are denoted by arrows and colored according to gene function classification. Shaded areas denote regions of homology (>95% nucleotide identity). Double-headed arrows indicate the addition or excision of relevant gene casstte. Asterisks denote the partially sequenced coding regions of relevant genes

genes *bla*NDM, *bla*VIM, *bla*OXA-23, *bla*OXA-48, or *bla*OXA-58, in strain EC6335. Further cloning and sequencing disclosed that EC6335 harbored two novel class 1 integrons: an unusual *bla*KPC-2-carrying In0EC6335 and a *bla*IMP-30-carrying In1069. In0EC6335 and In1069 (see Additional file 2) were present in two different *Bam*HI-digested EC6335 DNA fragments, which were independently cloned into pMD19-T and transferred into *Escherichia coli* TOP10, generating the *Escherichia coli* transformants In0-TOP10 and In1069-TOP10, respectively.

Strains In0-TOP10 and In1069-TOP10 have class A and B carbapenemase activities, respectively, while EC6335 appears to have class A + B activity (data not shown). All the above strains were resistant to the cephalosporin, carbapenem, and aminoglycoside drugs tested but remained susceptible to the fluoroquinolone drugs tested (Table 2). EC6335 displayed much higher levels of resistance to cephalosporins/carbapenems than In0-TOP10 and In1069-TOP10 (Table 2), which was consistent with the fact that EC6335 harbors two carbapenemase genes, blaKPC-2 and blaIMP-30, while In0-TOP10 and In1069-TOP10 carry only one gene, *bla*KPC-2 or *bla*IMP-30, respectively.

Discussion

Genetic futures of novel $In0_{EC6335}$

$In0_{EC6335}$ differs dramatically from the prototype $In0_{pVS1}$ (accession number U49101) from the *Pseudomonas aeruginosa* plasmid pVS1, and they only overlap each other by a majority region of 5′-CS (Fig. 1). $In0_{pVS1}$ has a weak PcW promoter and an unoccupied *attI* site with no gene cassettes, thereby representing an ancestor of more complex integrons [21]. $In0_{pVS1}$ possesses an intact 5′-CS, whereas its 3′-CS is disrupted by the insertion of IS*1326*, leading to the truncation of *tniABQC* of Tn*402* into *tniA-tniBD1* [21].

Table 2 Antimicrobial drug susceptibility profiles

Category	Antibiotics	MIC (mg/L)/antimicrobial susceptibility			
		EC6335 (In0+ In1069)	In0-TOP10 (In0)	In1069-TOP10 (In1069)	TOP10
Cephalosporins	Cefazolin	512/R	16/R	256/R	1/S
	Ceftazidime	256/R	4/R	128/R	0.5/S
	Ceftriaxone	256/R	8/R	128/R	0.5/S
Carbapenems	Ertapenem	16R	8/R	8/R	0.5/S
	Meropenem	16/R	8/R	8/R	0.5/S
	Imipenem	8/R	4/R	4/R	0.25/S
Aminoglycosides	Netilmicin	256/R	64/R	16/R	2/S
	Tobramycin	128/R	32/R	16/R	0.025/S
	Amikacin	512/R	128/R	32/R	1/S
Fluoroquinolones	Norfloxacin	0.10/S	0.10/S	0.05/S	0.05/S
	Ofloxacin	0.005/S	0.005/S	0.003/S	0.003/S
	Ciprofloxacin	0.25/S	0.25/S	0.125/S	0.0125/S

S sensitive; *R* resistant

In*0*$_{EC6335}$ contains a strong PcH1 promoter and a truncated GC*aacA4'-3* composed of *ΔaacA4'-3* and *att-C*$_{aacA4}$; a *bla*$_{KPC-2}$-containing region of Tn*6296* has been integrated between the PcH1 promoter and the truncated GC*aacA4'-3*, and located downstream of the GC*aacA4'-3* remnant are a *bla*$_{TEM-1}$-containing region of Tn*6296* and an IS*Kpn27-* containing region from Tn*6296* (Fig. 1). *AacA4'-3* (555 bp in length; aminoglycoside resistance) is a derivative of the reference *aacA4* gene (accession number AF034958), and encodes the variations Asn5Thr and Leu102Ser compared with *aacA4*. The *ΔaacA4'-3* gene of In*0*$_{EC6335}$ has a 36 bp deletion at its 5′ end. It is most likely that In*0*$_{EC6335}$ originates from a GC*aacA4'-3*-carrying integron that has recombined with Tn*6296*, which is one of the major mobile platforms of *bla*$_{KPC}$ genes in China [22–24], and Tn*3* as a major mobile platform of *bla*$_{TEM-1}$ [22–24].

Inferred evolution of In1069 and related integrons in Enterobacteriae

In*1069* carries two GCs, GC*bla*$_{IMP-30}$ (carbapenem resistance) and GC*aacA4'-3*, in this order, and its *intI1* gene is truncated because of the connection of IS*26* at its 3′ end. We also determined the sequences of four genetically related integrons, In*893* and In*1287* to In*1290*, from three different *Enterobacteriaceae* species: *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* (Fig. 2). In*1069* and the other four integrons carry PcS and PcH1, which are both strong promoters known to drive GC expression [21].

These integrons appear to have conserved 5′-CS and 3′-CS *(tni of* Tn*402*), and possess a core GC*aacA4'-3* in which the *attC* site has an unusual 9 bp deletion (CCCTTCCAT) (Fig. 2). Five more GCs, including GC*bla*$_{IMP-30}$, GC*aadA16* (aminoglycoside resistance), GC*catB3* (phenicol resistance), *GCarr3*, and GC*dfrA27* (rifampin resistance), are also present in these integrons. The integration/excision of one or more of these GCs upstream/downstream of the GC*aacA4'-3* core generates various organizations of GC arrays, which are mediated by the *IntI*-based, *attC*-recognizing site-specific recombination system [25]. In*893* seems to represent the most primitive form of these integrons. In*893*, In*1069*, and In*1287* carry one or two different resistance markers, while In*1288* to In*1290* have evolved to capture the determinants for at least three different classes of antibiotics, mostly likely conferring MDR. These findings provide the insight into stepwise and parallel evolution of In*1069*-associated integron variations (Fig. 2).

Conclusions

Excessive use of antibiotics causes the spread of MDR *Enterobacteriaceae* strains in clinical settings, most of which harbor class 1 integrons. The characterization of novel class 1 integrons In0, In1069 and In1287 to In1290 denotes a step-by-step and parallel evolution scheme involving massive genetic changes in integron GC arrays under high levels of antibiotic selection pressure in clinical settings.

Additional files

Additional file 1: Sequence analysis for In0, In1069, In893, and In1287 to In1290, respetively. (ZIP 65 kb)

Additional file 2: Integron analysis for In0 and In1069. (XLS 33 kb)

Abbreviations
3′-CS: 3′-conserved segment; 5′-CS: 5′-conserved segment; GC: gene cassette; MDR: multidrug resistance; MIC: minimum inhibitory concentration; PCR: polymerase chain reaction

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Availability of data and materials
Please contact author for data requests.

Authors' contributions
Conception and design of the study: WD and ZD. Acquisition of data: All the authors. Analysis and interpretation of data: All the authors. Drafting the article: WD and ZD. Revising it critically for important intellectual content: All the authors. Final approval of the version to be submitted: All the authors. All the authors read and approved the final manuscript.

Ethics approval and consent to participate
The use of patient specimens and all related experimental protocols were approved by the Committee on Human Research at all the institutions involved in this study. The study was carried out in accordance with the approved guidelines of the Ethics Committee of Taizhou Municipal Hospital affiliated with Taizhou University.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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