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 blaKPC-2-carrying In0 and the blaIMP-30-carrying In1069 coexists in a single isolate of Escherichia coli. In0 contains a Pch1 promoter and a truncated aacA4'-3 gene cassette (GCaacA4'-3), as well as a blaKPC-2-containing region of Tn6296 integrated between Pch1 and GCaacA4'-3. In1069 carries GCblaIMP-30 and GCaacA4'-3 in this order. The other five integrons, In893 and In1287 to In1290, are genetically related to In1069, and all possess a core GCaacA4'-3. The integration or excision of one or more individual gene cassettes, such as GCblaIMP-30, GCaadA16, GCcatB3, GCarr3 and GCDfrA27, upstream or downstream of GCaacA4'-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, blaKPC-2, blaIMP-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].
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 Tn402 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 (qacEA1)-sulphonamide resistance (sul1)-orfs region occurs immediately downstream of the GC array [1–3]. Eventually, class 1 integrons manifest as a prototype structure organized in order of IRi, a 5′-conserved segment (5′-CS: intI1-attI), a central variable region (the GC array), a 3′-conserved segment (3′-CS: qacEA1-sul1-orfs-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 Tn402, 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 blaKPC-2-carrying unusual In0 and the blaIMP-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 accordance with operation manual.

Integron cloning and sequencing

For cloning of In0 and In1069, the blaKPC-2 and blaIMP-30-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 BanHI, and ligated into the cloning vector pMD19-T. This was transformed into host bacteria Escherichia coli TOP10, which were screened for blaKPC-2 or blaIMP-30 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
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 determined to harbor class 1 integrons In0 and In1069, In893, In1287 to In1290 were deposited in GenBank under accession numbers KP870110, KM589497, KX434463, KX371912, and KX387648 to KX387650, respectively.

Clinical bacterial isolates containing integrons

| Table 1 Oligonucleotide primers used in this study |
|-----------------------------------------------|
| Target | Primer | Primer sequence (5′-3′) | Amplicon length (bp) | Reference |
|--------|--------|-------------------------|---------------------|-----------|
| bla<sub>KPC</sub> | KPC-F | GTATCGCCGTACTTCTGCG | 637 | [26] |
|        | KPC-R | GGTGCTGTTTCCCTTACGCT |          |          |
| bla<sub>IMP</sub> | IMP-F | GGAATAGGTGCGTATTCTTC | 232 | [26] |
|        | IMP-R | GGTGAGTTTCCCTTACGCT |          |          |
| bla<sub>NDM</sub> | NDM-F | GGTGAGTTCGGACGTCTGCTT | 621 | [26] |
|        | NDM-R | GGAATGCGTACATACGGATC |          |          |
| bla<sub>VIM</sub> | VIM-F | GATGCGTTTCTGTTGCTCAT | 390 | [26] |
|        | VIM-R | GGAATGCGTACATACGGATC |          |          |
| bla<sub>OXA-23</sub> | OXA-23-F | GATCGGATTGGAGAACCAAGA | 501 | [27] |
|        | OXA-23-R | ATTTGCTACCCTTACGCT |          |          |
| bla<sub>OXA-48</sub> | OXA-48-F | TTGTTGCCGATCGATTCG | 744 | [28] |
|        | OXA-48-R | GAGACTTTTTGTTGATACG |          |          |
| bla<sub>OXA-58</sub> | OXA-58-F | AAGATCGGGCTTCTGCTCAT | 599 | [27] |
|        | OXA-58-R | CAGGTGCTTCTTCTGTTCG |          |          |
| aac<sub>A4</sub> | aacA4-F | TATGAGTGCTAAATCGAT | 395 | [23] |
|        | aacA4-R | CCGCTTCTTGCTACG |          |          |
| int<sub>1</sub> | int1-F | GCTGAAAGGTCTGTCATAC | 515 | This study |
|        | int1-R | GTTCTTCTACGCGAGGT |          |          |
| tni<sub>R</sub> | tniR-F | CGAGGTTTGCGTGTCG | 375 | This study |
|        | tniR-R | ATCGCCACCTTGGCTTCC |          |          |
| int<sub>1</sub> to tni<sub>R</sub> | int1-F | GCTGAAAGGTCTGTCATAC | Variable | This study |
|        | tniR-R | ACCTGATCGTTGGAAG |          |          |

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Plus meropenem was ineffective. Carbenapenem-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 bacteraemia. *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 *blaKPC* and *blaIMP*, but none of the other tested carbapenemase
genes blaNDM, blaVIM, blaOXA-23, blaOXA-48, or blaOXA-58, in strain EC6335. Further cloning and sequencing disclosed that EC6335 harbored two novel class 1 integrons: an unusual blaKPC-2-carrying In0EC6335 and a blalMP-30-carrying In1069. In0EC6335 and In1069 (see Additional file 2) were present in two different BamH1-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 ceph- alosporin, 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 carbapene- mase genes, blakPC-2 and blalMP-30, while In0-TOP10 and In1069-TOP10 carry only one gene, blakPC-2 or blalMP-30, respectively.

Discussion
Genetic futures of novel In0EC6335
In0EC6335 differs dramatically from the prototype In0pVS1 (accession number U49101) from the Pseudomonas aeruginosa plasmid pVS1, and they only overlap each other by a majority region of 5′-CS (Fig. 1). In0pVS1 has a weak PcW promoter and an unoccupied attl site with no gene cassettes, thereby representing an ancestor of more com- plex integrons [21]. In0pVS1 possesses an intact 5′-CS, whereas its 3′-CS is disrupted by the insertion of IS1326, leading to the truncation of tniABQC of Tn402 into tniA-tnibDI [21].
In0EC6335 contains a strong PcH1 promoter and a truncated GCaacA4'-3 composed of ΔaacA4'-3 and attCaacA4; a blaKPC-2-containing region of Tn6296 has been integrated between the PcH1 promoter and the truncated GCaacA4'-3 remnant are a blaTEM-1-containing region of Tn6296 and an ISKpn27-containing region from Tn6296 (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 In0EC6335 has a 36 bp deletion at its 5′ end. It is most likely that In0EC6335 originates from a GCaacA4'-3-carrying integron that has recombined with Tn6296, which is one of the major mobile platforms of blaKPC genes in China [22–24], and Tn3 as a major mobile platform of blaTEM-1 [22–24].

**Inferred evolution of In1069 and related integrons in Enterobacteriaceae**

In1069 carries two GCs, GCblaIMP-30 (carbapenem resistance) and GCaacA4'-3, in this order, and its intI1 gene is truncated because of the connection of IS26 at its 3′ end. We also determined the sequences of four genetically related integrons, In893 and In1287 to In1290, from three different Enterobacteriaceae species: *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* (Fig. 2). In1069 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 Tn402), and possess a core GCaacA4'-3 in which the attC site has an unusual 9 bp deletion (CCCTTCCAT) (Fig. 2). Five more GCs, including GCblaIMP-30, GCaadA16 (aminoglycoside resistance), GCCcatB3 (phenicol resistance), GCarr3, and GCdfrA27 (rifampin resistance), are also present in these integrons. The integration/excision of one or more of these GCs upstream/downstream of the GCaacA4'-3 core generates various organizations of GC arrays, which are mediated by the IntI1-based, attC-recognizing site-specific recombination system [25]. In893 seems to represent the most primitive form of these integrons. In893, In1069, and In1287 carry one or two different resistance markers, while In1288 to In1290 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 In1069-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, respectively. (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
All the authors read and approved the final manuscript.

Consent for publication
Not applicable.

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

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