Characterization of the novel In1059 harbouring VIM gene cassette

Dongguo Wang¹*, Jinhong Yang²†, Meiyu Fang³†, Wei He⁴†, Ying Zhang⁴, Caixia Liu² and Dongsheng Zhou⁵,1*

Abstract

Background: VIM-type enzyme encodes the most widely acquired metallo-β-lactamases in Gram-negative bacteria. To obtain current epidemiological data for integrons from enterobacteria in hospital, the study characterizes the genetic structure in In1059 by comparison with In846 integrons harbouring VIM gene and other class 1 integrons including In37, In62, In843 and In1021 with the aim of identifying the putative mechanisms involved integron mobilization and infer evolution of relevant integrons.

Methods: Six of 69 recombinant plasmids from clinical strains were found to be class 1 integrons by digestion with BamHI, drug susceptibility testing, conjugation experiments, PCR amplification, integron cloning and sequencing, genome comparison, and detection of carbapenemase activity.

Results: The sequences of the six recombinant plasmids encoding In1021, In843, In846, In37, In62, and the novel In1059 integron had approximate lengths of ~4.8-, 4.1-, 5.1-, 5.3-, 5.3- and 6.6- kb, respectively. The genetic structures of these integrons were mapped and characterized, and the carbapenemase activities of their parental strains were assessed.

Conclusions: Our results suggest that the six variable integron structures and regular variations that exist in the gene cassettes provide a putative mechanism for the integron changes. Our study has also shown that the genetic features in the integrons named above fall within a scheme involving the stepwise and parallel evolution of class 1 integron variation likely under antibiotic selection pressure in clinical settings.

Keywords: Novel integron, In1059, Plasmid digestion, Cloning, Mobilization inference, Evolution
which occur in single-stranded sequences and activate the folded bottom strand [11, 12]. Because of their linkage with transposons or being plasmid encoded, class 1 integrons can capture genetic structures, express gene cassettes, and facilitate their own mobility, but they are incapable of self-mobilization [5, 13].

Integrons usually contain three functional components: an integrase gene (intI), a primary recombination site (attI), and an outward-orientated promoter (PC) [14]. Cassette integrations occur mainly at the attI site and this ensures the expression of the captured cassettes under the control of the PC promoter [7, 15]. For most class 1 integrons, the 5′-CS regions are similar and include intI and attI genes; however, differences exist in the 3′-CS sequences and the three open reading frames (ORFs) [16].

VIM-type metallo-β-lactamases were first isolated from Pseudomonas aeruginosa and other Gram-negative nonfermenting bacteria in Europe [17–20]. These enzymes were subsequently observed worldwide in Gram-negative nonfermentative bacteria and in Enterobacteriaceae. Class 1 integrons harbouring VIM-type gene cassettes have spread among various Gram-negative pathogens [21]. Therefore, this study aimed to characterize the genetic structures of the novel In1059 integron by comparison with the In846 integron and other structural class 1 integrons from enterobacteriaceae in terms of the VIM-type gene cassettes. From this analysis, we have also identified the described mechanism underlying integron mobilization, revealing the relations amongst structures of class 1 integrons including novel In1059 and other integrons in the study.

Methods
Clinical bacterial isolates and drug susceptibility testing
In total, 69 non-redundant multidrug-resistant enterobacteria strains, including non-typhoidal Salmonella Nsa243 and Nsa217 (harbouring In37 and In1021), Enterobacter cloacae Ecl175 (In843), Klebsiella pneumoniae Kpn761 and Kp3349 (In62 and novel In1059), and Enterobacter aerogenes Eae634 (In846), were recovered from hospitalized patients with clinical infections. The strains were assessed for integrons at the Taizhou Municipal Hospital of China (July 2013 to July 2015). Bacterial species was identified by 16S rRNA gene sequencing [22]. All the above integron-harbouring isolates, plus Escherichia coli TOP10 (Invitrogen, USA), were used in the study. E. coli TOP10 was used as the host for the cloning experiments and for susceptibility testing experiments.

The minimum inhibitory concentration (MIC) values of 12 antimicrobial agents, including cephalosporins (cefazolin, cefazidime and ceftriaxone), aminoglycosides (netilmicin, tobramycin and amikacin), carbapenems (ertapenem, meropenem and imipenem), and quinolones (norfloxacin, ofloxacin and ciprofloxacin), were determined using drug susceptibility plates and the Microscan broth dilution method (Microscan, Washington, USA). The MICs were interpreted according to the Clinical and Laboratory Standards Institute guidelines [23].

Plasmid digestion, integron cloning and DNA sequencing
Plasmids from six integron-harbouring isolates were collected using an AxyPrep Plasmid Miniprep kit (Axygen Biosciences, Beijing, China) according to the manufacturer’s instructions and according to Wang et al., 2014 [24]. The plasmids were digested with BamHI (TaKaRa, Dalian, China), and the six integron-harbouring recombinant plasmids were electrophoretically resolved to generate genetic maps.

To characterize the recombinant plasmid-encoding integrons from the above six isolates, we digested the plasmids with BamHI and ligated the relevant fragments to the pMD19-T cloning vector (TaKaRa, Dalian, China), and then transformed the ligation mixture into E. coli TOP10 host bacteria. Colonies that were aacA4-positive were isolated and the inserts within the recombinant pMD19-T vectors were sequenced using the primers specified in Table 1 and the following PCR conditions: 3 min at 94 °C, 30 cycles of 1 min each at 94 °C, 50–59 °C and 72 °C, followed by 10 min at 72 °C. The total reaction volume was 25 μL, and the eluent volume was 10 μL. After amplification, the PCR products were separated by gel electrophoresis on a 0.6% agarose gel run at 90 V for 90 min in 0.5 × TBE buffer. Next, the plasmid DNAs or fragments of different sizes that harboured integrons were recovered, and the initial positions of the relevant genes in the recombinant plasmids were determined according to a previously established method for estimating plasmid DNA sizes [25]. After that, the integron sequences involving different genes were obtained, and the genetic structures were mapped and characterized.

Sequence annotation and genome comparison
ORFs were predicted with RAST (http://rast.nmpdr.org/) and further annotated using BLASTP and BLASTN programs (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against UniProtKB/Swiss-Prot (http://web.expasy.org/docs/swiss-prot_guideline.html) and National Center for Biotechnology NR databases (https://www.ncbi.nlm.nih.gov/). Database annotation of drug-resistance genes, mobile elements and other genes was based on CARD (http://arpcard.mcmaster.ca), the β-lactamase (http://www.ncbi.nlm.nih.gov/pathogens/submit_beta_lactamase) database, ISfinder (https://www-is.biotoul.fr/), and INTEGRALL (http://integrall.bio.ua.pt/). Sequence comparisons were performed with BLASTN and CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Gene organisation diagrams were drawn with Inkscape (https://inkscape.org).
Carbapenemase activity detection

The activities of A, B and D carbapenemase classes in the bacterial cell extracts for the above mentioned six isolates were determined using a modified CarbaNP test [26]. Overnight bacterial cell cultures in Mueller-Hinton (MH) broth were each diluted 1:100 into 3 mL of fresh MH broth, and the bacteria were grown at 37 °C with shaking at 200 rpm to reach OD600s of 1.0 to 1.4. When required, ampicillin was used (200 μg/mL). Bacterial cells were harvested from 2 mL of each culture, and each pellet from the individual cultures was washed twice with 20 mM Tris- HCl (pH 7.8). Each cell pellet was resuspended in 500 μL of 20 mM Tris-HCl (pH 7.8), lysed by sonication and spun at 10000×g for 5 min. Each 50 μL supernatant (containing the enzymatic bacterial suspension fraction) was mixed with 50 μL of the following substrates (I to V), 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: Table 1 Primers used for PCR amplification and sequencing

| Primer | Sequence (5′-3′) | Length (bp) | Reference |
|--------|------------------|-------------|-----------|
| 5′-CS  | F GGCATCCAAAGCAGCAAGC | Variable | [40] |
| 3′-CS  | R AACGAGACTGACCTGAT | | |
| Int1   | F AGCACCCTGGCTAGAAAGAACAG | 3500 | This study |
|        | R GTATAATGCTTGATGATGCTG | | |
| intt1  | F GGGTCAAGGGATCTGGATTTCG | 1250 | [33] |
|        | R ACATGGCTGTAATATCATCAGTCG | | |
| sul1   | F ATGGTGACGGGTGGCATTCTGA | 900 | [34] |
|        | R TCTGGCTCCAAATCTAGTACCGATC | | |
| qaeEΔ1 | F ATCCGATAGTGGCGAAGT | 600 | [35] |
|        | R CAGCTTTTGCCCATGAAGC | | |
| aadA   | F ACATCATCCGTGGGCGTATC | | This study |
|        | R TTTTGGCCGATACCTGGTGTA | | |
| tnpA   | F GCATGGCAGATCCTGGTATAGG | 920 | [36] |
|        | R CTCGGGAGATGCTGGCCTACT | | |
| aad B  | F TCACAGCCCAAATACACGAGG | 857 | This study |
|        | R TGCCTCCACAAACTCAATT | | |
| aacA4  | F TGGCAACACGCAACGCATTCC | 800 | [37] |
|        | R TTAAGCATCAGCTGGTGATT | | |
| btaOXA | F ATGAAAAACACAAATACATACACCTCAGC | 900 | This study |
|        | R GTGTGTTGGAATGTTGCAGCATT | | |
| btaKPC | F TGTACGTATAGCCTGCTTC | 1000 | [31] |
|        | R CTCAATGCTACGATTCACAA | | |
| btaTEM | F CTGCTATTTTGTCATCC | 1061 | [24] |
|        | R CTCAGTATGGGGCTGTC | | |
| btaVIM | F CAGCACCAGCGTGGACATA | 1512 | This study |
|        | R TCACAGTAAACCAGCAATCA | | |
| btaXAP | F GCAGCAGCAGCGTGGACATA | 1394 | This study |
|        | R GCTTTTCCTCCCAACTCCC | | |
| tniC   | F GCTCTGTGTTGAGTGGTG | 993 | This study |
|        | R GGATCCCTCCGCCGTTG | | |
| dfr    | F GTGAAATAATCATCAGAGTG | 750 | [38] |
|        | R TTAACCCCTTTCAGCGATTTG | | |
| am-3   | F GTGACTTGTGATAACCACG | 450 | This study |
|        | R ACACGTGATGACCAAGGTTCC | | |
| catB   | F CCGAAGATTGCCAAGAGGTTG | 980 | [39] |
|        | R AGTGTGTCAGGGTACGCGG | | |
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, 0.8 mg/μL tazobactam, and 3 mM EDTA (pH 7.8), and 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).

Nucleotide sequence accession numbers
In1021, In843, In846, In37, In62 sequences, along with In1059, and In1021 sequences, were deposited in GenBank under the accession numbers KR338349, KR338350, KR338351, KR338352, KJ716225 and KM589496, respectively.

Results and discussion
Integron cloning experiments and antibiotic susceptibility testing
Six of 69 isolates, which resistance to aminoglycoside, quinolone, cephalosporin, and carbapenem antibiotics, from the clinical patients met the requirements of this study. Isolates Kpn761, Eae634, and Kp3349 were collected from blood culture, head wound secretion, and sputum, respectively, from ICU patients. Isolate Ecl175 came from a blood culture from a respiratory medical patient. Isolates Nsa217 and Nsa243 were collected from patient blood cultures in the hospital’s Infectious Disease Unit. Following BamHI digestion and ligation to a PMD19-T cloning vector, the recombinant plasmids were transformed into the competent cells, E. coli TOP10, by heat shock conversion, then, achieved the positive transformants which were selected by blue-white spot experiments, and sequenced. The six integron-containing recombinant plasmids transformed into E. coli TOP10 cells were used for the integron cloning conjugation experiments. The susceptibility test results are listed in Table 2. Concurrently, the six recombinant plasmids were electrophoresed to estimate their sizes (Fig. 1). The susceptibility test results indicated that the conjugation experiments were successful and that the resultant antibiotic resistance was caused by plasmid-mediated genes. The electrophoresis results following BamHI digestion indicated that the sizes of In1021, In843, In846, In37, In62 and the novel In1059 integron were ~4.8 kb, 4.2 kb, 5.1 kb, 5.3 kb, 5.3 kb and 6.6 kb each in length, respectively (Fig. 1).

| Strains   | Plasmids   | Cephalosporins | Carbapenems | Aminoglycosides | Fluoroquinolones |
|-----------|------------|----------------|--------------|-----------------|-----------------|
|           |            | CZ            | CAZ          | CTX             | EPM             | MPM             | IPM             | NET | TOB | AK | NOR | OFL | CIP |
| Nsa217    | In37       | 256/R         | 128/R        | 128/R           | 16/R            | 16/R            | 8/R             | 64/R | 32/R | 128/R | 0.10/S | 0.05/S | 0.25/S |
| In37-TOP10|            | 16/R          | 8/R          | 8/R             | 8/R             | 4/R             | 16/R            | 16/R | 32/R | 0.05/S | 0.003/S | 0.125/S |
| TOP10     |            | 1/S           | 0.5/S        | 0.5/S           | 0.5/S           | 0.5/S           | 0.25/S          | 2/S  | 0.025/S | 1/S | 0.05/S | 0.003/S | 0.125/S |
| Ecl175    | In843      | 2/S           | 1/S          | 1/S             | 0.5/S           | 0.5/S           | 0.25/S          | 512/R | 128/R | 512/R | 0.10/S | 0.05/S | 0.25/S |
| In843-TOP10|            | 1/S           | 0.5/S        | 0.5/S           | 0.25/S          | 0.25/S          | 0.125/S         | 128/R | 64/R  | 128/R | 0.05/S | 0.003/S | 0.125/S |
| TOP10     |            | 1/S           | 0.5/S        | 0.5/S           | 0.5/S           | 0.5/S           | 0.25/S          | 64/R | 32/R  | 64/R  | 0.05/S | 0.003/S | 0.125/S |
| Kpn761    | In62       | 2/S           | 1/S          | 1/S             | 0.5/S           | 0.5/S           | 0.25/S          | 128/R | 64/R  | 128/R | 0.10/S | 0.05/S | 0.25/S |
| In62-TOP10|            | 1/S           | 0.5/S        | 0.5/S           | 0.25/S          | 0.25/S          | 0.125/S         | 64/R | 32/R  | 32/R  | 0.05/S | 0.003/S | 0.125/S |
| TOP10     |            | 1/S           | 0.5/S        | 0.5/S           | 0.5/S           | 0.5/S           | 0.25/S          | 2/S  | 0.025/S | 1/S | 0.05/S | 0.003/S | 0.125/S |
| Nsa217    | In1021     | 2/S           | 1/S          | 1/S             | 0.5/S           | 0.5/S           | 0.25/S          | 256/R | 128/R | 256/R | 0.10/S | 0.05/S | 0.25/S |
| In1021-TOP10|            | 1/S           | 0.5/S        | 0.5/S           | 0.25/S          | 0.25/S          | 0.125/S         | 128/R | 64/R  | 128/R | 0.05/S | 0.003/S | 0.125/S |
| TOP10     |            | 1/S           | 0.5/S        | 0.5/S           | 0.5/S           | 0.5/S           | 0.25/S          | 2/S  | 0.025/S | 1/S | 0.05/S | 0.003/S | 0.125/S |
| Eae634    | In846      | 256/R         | 128/R        | 128/R           | 64/R            | 64/R            | 64/R            | 128/R | 64/R  | 128/R | 0.10/S | 0.05/S | 0.25/S |
| In846-TOP10|            | 64/R          | 16/R         | 16/R            | 16/R            | 16/R            | 16/R            | 64/R | 32/R  | 64/R  | 0.05/S | 0.003/S | 0.125/S |
| TOP10     |            | 1/S           | 0.5/S        | 0.5/S           | 0.5/S           | 0.5/S           | 0.25/S          | 2/S  | 0.025/S | 1/S | 0.05/S | 0.003/S | 0.125/S |
| KP3349    | In1059     | 256/R         | 128/R        | 128/R           | 64/R            | 64/R            | 64/R            | 128/R | 64/R  | 128/R | 0.10/S | 0.05/S | 0.25/S |
| In1059-TOP10|            | 32/R          | 16/R         | 16/R            | 16/R            | 16/R            | 16/R            | 32/R | 16/R  | 16/R  | 0.05/S | 0.003/S | 0.125/S |
| TOP10     |            | 1/S           | 0.5/S        | 0.5/S           | 0.5/S           | 0.5/S           | 0.25/S          | 2/S  | 0.025/S | 1/S | 0.05/S | 0.003/S | 0.125/S |

Abbreviations: CZ Cefazolin, CAZ Ceftazidime, EPM Ertapenem, MPM Meropenem, IPM Imipenem, NET Netilmicin, TOB Tobramycin, AK Amikacin, NOR Norfloxacin, OFL Ofloxacin, CIP Ciprofloxacin
Table 3: Comparison of novel In1059 with other integrons in the study

| Integron | In1059 | In846 | In843 | In37 | In62 | In1021 |
|----------|--------|-------|-------|------|------|--------|
| Pc variant | PcH1 | PcH1 | None | None | PcH1 | PcH1 |
| *-35*: tggaca | *-35*: tggaca | *-35*: tggaca | | | | |
| *-35*: taact | *-10*: taact | | | | | |
| P2 promoter | Absent | Absent | Absent | Absent | Absent | Absent |
| Pint1 | Yes | Yes | Yes | Yes | Yes | Yes |
| *-10*: agtcta | *-10*: agtcta | *-10*: agggcg | | | | |
| *-35*: cagcaa | *-35*: cagcaa | | | | | |
| 19 bp ORF11 duplication | No | No | No | No | No | No |
| intI1 | Int1R32 H30 | Int1R32 H30 | Remnant of intI1 | Remnant of intI1 | Int1R32 H30 | Int1R32 H30 |
| 1010 bp-length | 1014 bp-length | 4 bp-length | 63 bp-length | 63 bp-length | 1014 bp-length | 1014 bp-length |
| 63 bp-length | 63 bp-length | 4 bp-length | 63 bp-length | 63 bp-length | | |
| Array of gene cassettes | GCblaVIM-1 | GCblaVIM-1 | GCaacA4cr | GCaacA4cr | GCaacA4cr | GCaacA4cr |
| | (L;R) | (L;R) | (L;R) | (L;R) | (L;R) | (L;R) |
| attC | attCblaVIM(L;R); attCgaacA4cr (L; Del); attCcatB3 (L;R); attCamo (L; Del) | attCblaVIM(L;R); attCgaacA4cr (L; Del); attCcatB3 (L;R); attCamo (L; Del) | attCgaacA4cr (L;R); attCamo (L; Del) | attCgaacA4cr (L;R); attCamo (L; Del) | attCgaacA4cr (L;R); attCamo (L; Del) | attCgaacA4cr (L;R); attCamo (L; Del) |
| 3'sCS | qacEΔ1-sulI | qacEΔ1-sulI | qacEΔ1-sulI | qacEΔ1-sulI | qacEΔ1-sulI | qacEΔ1-sulI |

Abbreviations: attC (L; R) left- and right-hand parts of attC in gene cassettes, attC (L; Del) normal left- and deleted right-hand parts of attC in gene cassettes, suggesting no functional gene cassette, attC (L; R) mutated mutated left- and mutated right-hand parts of attC in gene cassettes, GC abbreviation of gene cassette.
while the existence of GC\textit{bla}_{OXA-1} indicates that this integron differs in its biochemistry to In843, In62 and In1021. In37 and In843 differ from In62, In846, In1021 and In1059 class 1 integrons in their 5\textquotesingle-CSs (remnant of intI1), and both lack Pc variant (Table 3 and Additional file 1). In62 appears to have the basic pattern identified in the six class 1 integrons, all of which commonly contain GC\textit{aacA4cr}. But only GC\textit{CaacA4cr} exists in In62. Notably, one \textit{aadA16} and four gene cassettes in the In843 gene are inserted into IS26 in two parts, before reintegrating with the \textit{qacE}\textsuperscript{109} (109 nucleotide remanants of \textit{qacE}) gene (Table 3 and Additional file 1). Unusually, some fragments such as \textit{qacE}\textsuperscript{109} or \textit{qacE}\textsuperscript{100} combined with GC\textit{CaadA16} in In843 and In1021, G\textit{Carr}-3 in In1021 and In846, and GC\textit{CaadB} in In1059 have never been recovered from any class 1 integron to date, and these may have "pseudo-gene cassette" functions (Table 3 and Additional file 1). Indeed, \textit{qacE}\textsuperscript{101} was discovered in class 1 integrons [27], and no \textit{qacE}\textsuperscript{100} or \textit{qacE}\textsuperscript{109} elements have previously been found. In the novel In1059 integron, the GC\textit{CaacA4} gene coding sequence displays a previously unseen duplication and an unusual 9 bp deletion (cccttcCAT) in its \textit{attC}, thereby inactivating it (Table 3 and Additional file 1). The duplication extends the sequence to 576-bp in length instead of the usual 555-bp. The resulting gene, which is a novel allele, has been assigned aac\textit{A4}\textsuperscript{37} (Fig. 3 and Table 3 and Additional file 1), and the fragment itself appears to be embedded within the start of GC\textit{CaadA16}, resulting in a change in its right-hand part of \textit{attC} site (e.g. the motif locates upstream of the coding sequence), and the remaining part of the \textit{attC} site in GC\textit{CaadA16} seems to be a hybrid between the usual one in GC\textit{CaadA16} and the usual one in GC\textit{CaacA4} (\textit{attC}_{\textit{caadA16}/\textit{caacA4}}, Table 3 and Additional file 1); furthermore, the GC\textit{CaadB} sequence lacks a right-hand \textit{attC} part making it look like a pseudo-gene cassette (Table 3 and Additional file 1). However, GC\textit{bla}_{VIM-5} appears to be usual \textit{attC} and normal gene cassette functional in novel In1059 integron.

Sequence comparisons of InV117 [28] (part of Tn1696, GenBank accession number DQ310703), In1059 and In37 reveal high levels of sequence homology among them (Fig.
2B-a), and In37 in particular shares high sequence identity with In1059. Because the Tn1696 module (including InV117) has the potential to transpose, this suggests that In37 and In1059 should also potentially be mobile elements. Compared with In70 [20] (part of Tn7017, GenBank accession number KJ571202), In1059 and In843 share the most sequence homology with In70 (Fig. 2B-b), and this is especially so for novel In1059. These results provide new insight about the structure of In1059, which coupled with the known plasticity of the In70 genetic context in the recombination plasmids, means that this might have mediated mobilization of the blaVIM-1-containing In70 integron platform [21].

**Mobilization and evolutionary inferences for In0 to In1059**

Generally, In0 could emerge from excision of all cassettes of an integron. If In0 captures GCaadB on attI site likely under antibiotic pressure, structure A might be transferred to structure B, maybe becoming a novel integron (Fig. 3I); then, likely under similar conditions, if joining with GCaadA16, structure B might become structure C, too (Fig. 3I); Also, if joined by GCaadA4'-3, structure C might turn into structure D, perhaps involving a novel integron as well; meanwhile, if inserted into GCBlaVIM-5 on attII site and IS26 on site between GCaadB and qacE1, then, replaced sulA with sulI and

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**Fig. 3** Genetic structures and proposed mobilization and evolution of In0 to In1059 and the analysis of intI1 and attC in class 1 integrons from this study. I, genetic structures and putative mobilization. A, In0; B, A + GCaadB; C, B + GCaadA16; D, C + GCaadA4'-3; E, In1059 = D + GCBlaVIM-5 + IS26 + (sulI + orf5) (replacing sulA); II, Sequences and structures of attI1 (59-be, 59-base elements) and attC. II-a, the attI1 (59-be) site positions are denoted by the bold black line. Open bars indicate potential IntI1 recognition sites, and arrows under the 7-bp core sites indicate relative orientations. The 7-bp core site sequences are shown in bold type, and the strong and weak IntI1-binding sites and direct repeats, DR1 and DR2, are indicated in accordance with the literature [29]. The simple site locations are denoted by an open bar. The “aacaaga(a)” sequences in the black boxes are reminiscent of the spacer in attI1 and occur between the two gene cassettes; recombination or excision of gene cassettes occurs frequently at these sites as indicated in panel I, process C to D. II-b, the attC sequences of class 1 integrons. The bold black letters denote the 7-bp core site, while the numbers are the nucleotide positions (left, negative, right, positive) of the recombination cross-over points, as indicated by a vertical arrow where the recombination or excision of gene cassettes regularly occurs, as shown in panel I. The GenBank accession numbers are shown on the left-hand side of the diagram.
orf5, structure D will convert into structure E, likely under the right conditions, just as novel In1059 assigned in the study (Fig. 3 and Table 3 and Additional file 1). According to Collis et al., 1998 [29], the recombination or excision of gene cassettes from class 1 integrons frequently occurs at sites of vertical arrow (Fig. 3II-a) between the “ataataaa[a]” array and its next nucleotide in attI, and sites of vertical arrow (Fig. 3II-b) between “g” and “t” of “gatgatg” array in attC; however, the study appears slightly mutant sequences in terms of the crossover point of attI and attC (Fig. 3II-a and b). Integrons are associated with MDR in Gram-negative bacteria [30, 31, 32] and have been determined as the primary source of drug-resistance genes, and they are also suspected to be reservoirs of drug-resistance genes in patients with bacterial infections [20].

Conclusions
Antibiotic overuse makes it likely that more and more MDR strains will appear in clinical isolates, and most of these strains will contain class 1 integrons. The features of the integrons described above denote a scheme involving the stepwise and parallel evolution of class 1 integron variation likely under antibiotic selection pressure in clinical settings.

Additional files

Additional file 1: Analysis for integrons: In1059, In843, In846, In37 and In62. (XLS 48 kb)

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

Authors’ contributions
Conceived and designed the experiments: DW. Performed the experiments: all authors. Analyzed the data: DW and DZ. Contributed to the writing of the manuscript: DW. Read and approved the final manuscript: all authors.

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

Consent for publication
Not applicable.

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 the institutions indicated. The study was carried out in accordance with the approved guidelines of the Ethics Committee of our Hospital, China.

Author details
1. Department of Clinical Laboratory Medicine, Taizhou Municipal Hospital affiliated with Taizhou University and the Institute of Molecular Diagnostics of Taizhou University, 381 Zhongshan Eastern Road, Taizhou, Zhejiang 318000, China.
2. Department of Clinical Laboratory Medicine, the Second Affiliated Hospital and Yuying Children’s Hospital of Wenzhou Medical University, 109 Xuanyue Western Road, Wenzhou, Zhejiang 325002, China.
3. Department of Clinical Laboratory Medicine, Hangzhou First People’s Hospital of Nanjing Medical University, 261 Huansha Road, Hangzhou, Zhejiang 310006, China.
4. Department of Clinical Laboratory Medicine, Wenzhou Hospital of Integrated Traditional Chinese and Western Medicine, 75 Jinxiu Road, Wenzhou, Zhejiang 325001, China.
5. State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, 20 Fengtai Eastern Avenue, Beijing 100071, China.

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