Molecular characteristics of class 1 and class 2 integrons and their relationships to antibiotic resistance in clinical isolates of *Shigella sonnei* and *Shigella flexneri*

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Objectives: To analyse the gene cassettes and determine the roles of class 1 and class 2 integrons in antibiotic-resistant strains of *Shigella sonnei* (*n* = 31) and *Shigella flexneri* (*n* = 33).

Methods: Various molecular techniques, including PCR and Southern-blotting analysis, were used to analyse various markers of class 1 and class 2 integrons in these 64 *S. sonnei* and *S. flexneri* isolates collected in Hangzhou, China. The gene cassette arrays in integrons were identified by DNA sequencing and/or restriction fragment length polymorphism. Two genomic DNA fragments, one containing *intI1* from a *S. flexneri* isolate that contains *intI1* but lacks 3'-conserved region and another containing *intI2* from a *S. sonnei* isolate, were cloned into pUC19 vectors and sequenced. The links between integron gene cassette arrays and antibiotic resistance were analysed.

Results: Class 2 integrons were present in 80.6% (25/31) of the *S. sonnei* isolates and 87.9% (29/33) of the *S. flexneri* isolates. All of these integron 2-positive isolates contained constant gene cassette arrays of *dfrA1 + sat1 + aadA1* which confer resistance to trimethoprim and streptomycin. It was demonstrated that the class 2 integron was located in the *Tn7* region inside the *attTn7* locus downstream of *glmS* in *Shigella*. Class 1 integrons were found in 9.4% (6/64) of *Shigella* spp. isolates. An atypical class 1 integron without a 3'-conserved segment on the *Shigella* chromosome, termed *Shigella* atypical class 1 integron (SAI), was present in 84.9% (28/33) of *S. flexneri* isolates. The SAI contained two gene cassettes, *blaOXA30* and *aadA1*; however, the SAI conferred resistance to ampicillin, but not to streptomycin, in *Escherichia coli* host. The *blaOXA30* and *aadA1* cassettes of the SAI seemed to be always coordinately excised or integrated.

Conclusions: Multiple and complex mechanisms involving mobile genetic elements in class 1 and class 2 integrons and antibiotic resistance have been developed in the evolution of *Shigella* strains.

Keywords: *Tn7*, *blaOXA30*, *dfrA1*, *aadA1*

Introduction

Shigellosis is a common diarrhoeal disease in developing as well as industrialized countries. In Asia, the incidence and deaths were estimated to be 91 million and 414,000 annually, respectively. Among *Shigella* species, *Shigella flexneri* is the most common serotype, followed by *Shigella sonnei*. Treatment with antibiotics has been effective in alleviating the dysenteric syndrome of shigellosis, reducing the duration of pathogen excretion to prevent disease transmission and lowering the risk of potential complications for the past several decades. However, at the same time, *Shigella* isolates have progressively acquired resistance to antibiotics, including ampicillin, streptomycin, trimethoprim/sulfamethoxazole and tetracycline. Determinants of antibiotic resistance in *Shigella* isolates are frequently borne within mobile genetic elements, including the R plasmids, transposons, integrons and genomic islands, on the bacterial genome. Mobile elements may facilitate the dissemination of resistance determinants among species, even genera.

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Table 1. Distribution of class 1 integrons, an atypical integron (the SAI) and class 2 integrons

<table>
<thead>
<tr>
<th>Shigella isolates</th>
<th>Class 1 integron</th>
<th>Atypical class 1 integron</th>
<th>Class 2 integron</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>intI1</td>
<td>inF–inB</td>
</tr>
<tr>
<td>S. sonnei</td>
<td>31</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>S. flexneri</td>
<td>33</td>
<td>31</td>
<td>2</td>
</tr>
<tr>
<td>2a</td>
<td>13</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>1a</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2b</td>
<td>9</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>4a</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>x variant</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>y variant</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

*intI1-positive, inF–inB-positive and 3’-CS-positive would be considered as class-1-integron-positive; intI1-positive and intI1-3IS1-positive would be considered as SAI-positive.

Integrons are gene-capture systems that harbour antibiotic resistance genes and may provide a flexible approach for bacteria to adapt to the pressure caused by antibiotics. The resistance to some antibiotics in Shigella species is associated with the presence of class 1 and class 2 integrons that contain resistance gene cassettes. The gene cassettes within class 1 integrons found on chromosome or plasmid in Shigella spp. often encode resistance to ampicillin (oxa-1), streptomycin (aadA) and/or trimethoprim (dfrA). Class 2 integrons borne on Tn7 are often present in S. sonnei isolates and their gene cassette arrays are usually constant, consisting of dfrA1, sat1 and aadA1. These genes confer resistance to trimethoprim, streptothricin and streptomycin, respectively.

The objectives of the present study were to analyse the molecular characteristics of class 1 and class 2 integrons, including their distribution and locations in the genome, and the link between gene cassettes and antibiotic resistance in S. flexneri and S. sonnei isolates collected from Hangzhou, China, during a period of 4 years (1998–2002). In addition, the characteristics of an atypical class 1 integron without 3’-conserved segment (3’-CS) and its link to antibiotic resistance in S. flexneri isolates were identified.

Materials and methods

Bacterial isolates

A total of 64 Shigella isolates (31 S. sonnei and 33 S. flexneri including 13 of serotype 2a, 1 of 1a, 9 of 2b, 2 of 4a, 2 of 6, 3 of x variant and 3 of y variant) were collected from Hangzhou First People’s Hospital, Yuhang Center for Disease Control and Prevention, and Lingai Center for Disease Control and Prevention, Hangzhou, Zhejiang Province, People’s Republic of China, from 1998 to 2002 (Table 1). Among the isolates, 28 S. sonnei and 31 S. flexneri isolates were collected from patients with sporadic diarrhoea, 3 S. sonnei isolates were collected from three patients in one outbreak, and 2 S. flexneri 2a isolates were collected from a patient and a well-water sample involved in another outbreak. The susceptibility of the isolates to ampicillin, streptomycin and trimethoprim was tested by the disc diffusion method on Mueller–Hinton agar, and the MICs of those antibiotics were determined by the agar dilution method, according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS; now the Clinical Laboratories Standards Institute). Escherichia coli ATCC 25922 was used as a quality control strain for both the disc diffusion method and the agar dilution method. The susceptibility data of Shigella isolates tested were accepted only when the zone diameter or MIC for E. coli ATCC 25922 tested in parallel was within the acceptable ranges given in the NCCLS guidelines.

PCR and Southern-blotting analysis

Class 1 integrons were analysed by PCR using three sets of primers as described previously: intI1L and intI1R,20 inF and inB21 and qacE-D1-F and sul1-B.21 The primers cover the 5’-conserved segment (5’-CS) (intI1), the cassette region and the 3’-CS of class 1 integrons, respectively (Table 2). The PCR products with primers inF and inB were sequenced to determine the gene cassettes of class 1 integrons. Class 2 integrons were examined by PCR with primers intI2L and intI2R specific for the intI2 gene (Table 2).22 Class 1 integron-containing S. sonnei isolate C101-1 and intI2-positive S. flexneri 2a isolate C101, both confirmed by sequencing their PCR products, were used as positive controls for PCR tests.

Genomic DNA and plasmid were isolated from four representative isolates that were positive for intI1 and negative for other gene cassette regions (inF–inB) as determined by PCR. The genomic DNA was then digested with BamHI and PstI. The restricted genomic DNA and the plasmid DNA were then electrophoresed in 1.0% agarose gels, transferred and fixed to nylon membrane, and hybridized to digoxigenin-labelled DNA probes specific for intI1 using the DIG high prime DNA labelling and detection kit (Roche) according to the manufacturer’s instructions. A 3.3 kb BamHI–PstI DNA fragment containing intI1 from genomic DNA of S. flexneri 2a isolate C101-1 was cloned into vector pUC19 (termed pUC19-C101) and sequenced (Table 3). An atypical class 1 integron without 3’-CS was identified in this cloned DNA fragment (Figure 1). A pair of primers, intI1ca-F and IS1ca-R (Table 2) that are specific for intI1 and IS1, were designed to amplify the variable region in all Shigella isolates, and the PCR products from one S. sonnei isolate and four S. flexneri isolates with serotypes 2a, 2b, x variant and y variant were sequenced directly. Another pair of primers, inv-oxaR and inv-aadL (Table 2)
specific for blaOXA30 and aadA1, was used to identify the gene cassette array (Figure 1).

To find the location of the class 2 integron in the Shigella genome, HindIII-digested genomic DNA and the plasmid DNA that were isolated from an intI2-positive S. sonnei isolate and an intI2-positive S. flexneri isolate were hybridized to a digoxigenin-labelled probe specific for intI2. A 10.7 kb HindIII-restricted genomic DNA fragment from S. sonnei isolate C202 was cloned into vector pUC19.
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![Diagram](https://academic.oup.com/jac/article-abstract/58/2/288/719679)

**Figure 1.** Physical map of the *Shigella* atypical class 1 integron (SAI), an atypical class 1 integron found in *S. flexneri* isolates, and its cassette excision patterns. Primers used in the study are shown by small open arrows.

For the inverse PCR analysis, two sets of divergent primers, inv-oxaL/inv-oxaR and inv-aadL/inv-aadR (Table 2) (Figure 1) specific for *blt*oxa<sub>30</sub> and *aadA1* cassettes of the atypical class 1 integron, were used to detect free circular cassettes in boiled overnight broth of *E. coli* strain DH5α containing pUC-C101. The amplified PCR products were sequenced to determine the recombination site.

*E. coli* strains and plasmids used in the conduct assay are listed in Table 3. The donor *E. coli* cells (strain UB3p) were constructed using the following steps: (i) Plasmid R388 was introduced into *E. coli* strain UB1637 (Str<sup>r</sup>) by conjugation. The R388 was a natural conjugal IncW plasmid harbouring a class 1 integron with a gene cassette array of *dfrB2* + *orfA* between 5<sup>′</sup>-CS and 3<sup>′</sup>-CS that confers resistance to trimethoprim through its *dfrB2* cassette. (ii) The R388-containing UB1637 cells were then sequentially transformed with p112 (Amp<sup>′</sup>), a plasmid expressing the *intI1* integrase gene, and pSU-C101 (Kan<sup>r</sup>) which was constructed by subcloning the *BamHI–PstI* fragment on pUC-C101 into pSU38 (Kan<sup>r</sup>).

Overnight cultures of 0.5 mL of donor cells (*E. coli* strain UB3p) and 0.5 mL of recipient cells (*E. coli* strain UB5201 [Nal<sup>r</sup>]) were mixed and pelleted. The mixture was resuspended in fresh Luria–Bertani (LB) broth and plated on LB agar, then incubated at 37 °C for 3 h. Dilutions of the mixed cultures were screened on LB media containing nalidixic acid (60 mg/L) and ampicillin (100 mg/L) for transconjugants. Nine transconjugants were randomly selected for identification of gene cassette arrays in the class 1 integrons on the recombination R388s by the RFLP analyses, using *XmnI* and *EcoRI*-restricted PCR products that were amplified with primers int<sup>−</sup>I and int<sup>+</sup>-CS, different cassettes or 3<sup>′</sup>-CS.

Identification of recombination patterns of gene cassettes

The inverse PCR technique and the conduct assay<sup>22</sup> were used to examine the recombination function of the gene cassettes on the atypical class 1 integron without 3<sup>′</sup>-CS carried in the *BamHI–PstI* fragment in pUC-C101.

The primers used in the present study were either derived from previous publications or designed by primer design software FastPCR (http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm) and Primer 3 online (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

(termed pUC-C202) (Table 3) and sequenced. A primer set, int<sup>−</sup>I<sub>2ca</sub>-F and int<sup>−</sup>I<sub>2ca</sub>-R (Table 2) specific for int<sup>−</sup>I<sub>2</sub> and *ybfA* in Tn<sup>7</sup> (Figure 2), was designed to amplify the gene cassettes of class 2 integrons in all *Shigella* isolates. The amplified products were digested by *EcoRI* to identify the gene cassette arrays using restriction fragment length polymorphism (RFLP) analysis. Three of these products from three *S. flexneri* isolates with serotypes 2a, 2b and x variant were directly sequenced.

Three primer sets were designed to identify the insert site of Tn<sup>7</sup> in *Shigella* spp. isolates according to the sequences of the right end of Tn<sup>7</sup>, and *glmS* known to be upstream of the insertion site of Tn<sup>7</sup> in *E. coli* (accession number V00620), and the *HindII*–restricted genomic DNA fragment. The primer set of *yfiA1*–F/Tn<sup>7</sup>L-R (Table 2) was used to amplify the joint fragment between IS<sup>4</sup> on the chromosomal backbone and the left end of Tn<sup>7</sup>, and the primer set of Tn<sup>7</sup>R–*F/glmS*–R (Table 2) was used to amplify the joint fragment between the right end of Tn<sup>7</sup> and *glmS* on the backbone, whereas the primer set of *yfiA1*–F/*glmS*–R was used to amplify the joint fragment between the IS<sup>4</sup> and *glmS* on the backbone that lacks Tn<sup>7</sup> insertion (Figure 2).

The primers used in the present study were either derived from previous publications or designed by primer design software FastPCR (http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm) and Primer 3 online (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Identification of recombination patterns of gene cassettes

The inverse PCR technique and the conduct assay<sup>22</sup> were used to examine the recombination function of the gene cassettes on the atypical class 1 integron without 3<sup>′</sup>-CS carried in the *BamHI–PstI* fragment in pUC-C101.
The sequences of the 3.3 kb BamHI–PstI DNA fragment containing the atypical class 1 integron from genomic DNA of S. flexneri isolate C101-1 and the 10.7 kb HindIII-restricted genomic DNA fragment containing a class 2 integron from S. sonnei isolate C202 were deposited in the GenBank database under accession numbers AY574195 and AY639870, respectively.

Results and discussion

Class 1 integrons and gene cassettes in Shigella spp.

Among the 31 S. sonnei isolates, 4 (12.9%) were found to be positive for all three markers of class 1 integrons, in which three kinds of gene cassette arrays, aadA2, dfrA17 + aadA5 and dfrA1 + aadA1a, were identified (Tables 1 and 4). Of the 33 S. flexneri isolates, 2 isolates (6.1%), 1 S. flexneri 6 and 1 S. flexneri y variant, were positive for all three markers of class 1 integrons and harboured the gene cassette arrays of dfrV and dfrA17 + aadA5, respectively (Tables 1 and 4). No empty integron [i.e. positive by PCR analysis for gene cassette region (inF–inB) but negative for gene cassettes] was found. All of these four gene cassette arrays found among Shigella spp. isolates in this study are also often present in clinical and environmental isolates of the Enterobacteriaceae.23–27 These findings suggest that the transfer of antibiotic resistance genes can occur through gene cassettes on class 1 integrons among Shigella spp. and other bacteria.

Atypical class 1 integron in Shigella spp.

For the three markers of class 1 integrons, we found that 29 (87.9%) S. flexneri isolates and 1 (3.2%) S. sonnei isolate were positive for intI1, but were negative for their gene cassette regions (inF–inB). Among the 29 intI1-positive isolates, only 3 S. flexneri isolates were positive for 3’-CS (Table 1), suggesting that these isolates might have an atypical class 1 integron without 3’-CS.

To find the location of the atypical integrons and their gene cassettes, four isolates that were positive for intI1 and negative

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Figure 2. Physical maps of the left and right ends of Tn7 containing a complete class 2 integron and its flank sequences from S. sonnei strain C202 (a), and flank sequences of glmS in S. flexneri 2a strains 301 (b) and 2457T (c) without Tn7 insert. Primers used in the study are showed by small open arrows.

Nucleotide sequence accession numbers

The sequences of the 3.3 kb BamHI–PstI DNA fragment containing the atypical class 1 integron from genomic DNA of S. flexneri 2a isolate C101-1 and the 10.7 kb HindIII-restricted genomic DNA fragment containing a class 2 integron from S. sonnei isolate C202 were deposited in the GenBank database under accession numbers AY574195 and AY639870, respectively.
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Table 4. Characteristics of class 1 integrons in *Shigella* spp.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Shigella</th>
<th>intI</th>
<th>gene cassettes (5′−3′)</th>
<th>3′-CS</th>
<th>disc diffusion</th>
<th>MIC (mg/L)</th>
<th>disc diffusion</th>
<th>MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C103-1</td>
<td><em>S. sonnei</em></td>
<td>+</td>
<td>aadA2</td>
<td>+</td>
<td>S</td>
<td>4</td>
<td>S</td>
<td>8</td>
</tr>
<tr>
<td>yh02-25</td>
<td><em>S. sonnei</em></td>
<td>+</td>
<td>aadA2</td>
<td>+</td>
<td>S</td>
<td>4</td>
<td>S</td>
<td>8</td>
</tr>
<tr>
<td>yh31</td>
<td><em>S. sonnei</em></td>
<td>+</td>
<td>dfrA17, aadA5</td>
<td>+</td>
<td>R</td>
<td>&gt;128</td>
<td>S</td>
<td>4</td>
</tr>
<tr>
<td>yh02-26</td>
<td><em>S. sonnei</em></td>
<td>+</td>
<td>dfrA1, aadA1a</td>
<td>+</td>
<td>R</td>
<td>&gt;128</td>
<td>S</td>
<td>4</td>
</tr>
<tr>
<td>yh24</td>
<td><em>S. flexneri</em> 6</td>
<td>+</td>
<td>dfrV</td>
<td>+</td>
<td>R</td>
<td>&gt;128</td>
<td>R</td>
<td>128</td>
</tr>
<tr>
<td>yh02-14</td>
<td><em>S. flexneri</em> y variant</td>
<td>+</td>
<td>dfrA17, aadA5</td>
<td>+</td>
<td>R</td>
<td>&gt;128</td>
<td>R</td>
<td>&gt;128</td>
</tr>
</tbody>
</table>

for gene cassette regions (inF−inB) (2 isolates of *S. flexneri* 2a, 1 *S. flexneri* 2b and 1 *S. sonnei*) were selected, and genomic DNA and plasmid DNA isolated from these isolates were analysed by Southern-blotting analysis with a digoxigenin-labelled probe specific for intI. A positive band of 3.3 kb was observed in *BamHI* and *PstI*-restricted genomic DNA isolated from all four isolates, but no signal was detected in plasmid DNA of these isolates, suggesting that the hypothesized atypical integron is only present in the chromosomes of these isolates. The 3.3 kb fragment from one of the four isolates, *S. flexneri* 2a isolate C101-1, was then cloned into vector pUC19 (termed pUC-C101) and sequenced. Analysis of the DNA sequence revealed that this atypical integron consists of intI, attL, two gene cassettes of *blaOXA30* and *aadA1*, and part of IS1 (Figure 1; see more sequence details in Figure S1 in Supplementary data available at JAC Online). Because the atypical integron is negative for class 1 integrons as determined by commonly used PCR, we termed it *Shigella* atypical class 1 integron (SAI). Due to the confusion in the nomenclature of the *aadA* family in the public nucleotide sequence database, we termed the *aadA1* in the SAI *aadA1SAI* in this paper.

To confirm the presence of the atypical integron in *S. flexneri* and *S. sonnei* isolates, we used PCR with primer set intI1ca-F/IS1ca-R to amplify the variable region of the atypical integron. The gene cassette arrays were then identified by PCR analysis with another primer set inv-oxaL/inv-aadL. Among the 29 *S. flexneri* and 1 *S. sonnei* isolates that were positive for intI and negative for cassette regions (inF−inB), 28 *S. flexneri* isolates and 1 *S. sonnei* isolate were positive in both PCRs. The amplified PCR fragment sizes were 2453 bp when primer set intI1ca-F and IS1ca-R was used and 456 bp when primer set inv-oxaL and inv-aadL was used. Of five nucleotide sequences of the PCR products with primer set intI1ca-F/IS1ca-R from one *S. sonnei* isolate was negative in both PCRs. No positive PCRs were observed in the six class 1 integron-containing isolates (2 *S. flexneri* and 4 *S. sonnei*), and the other intI negative isolates (2 *S. flexneri* and 26 *S. sonnei*) when primer set intI1ca-F/IS1ca-R was used (Table 1). These results indicate that the atypical integron was present in 84.9% of *S. flexneri* isolates and 3.2% of *S. sonnei* isolates in this study. The atypical integron and class 1 integron did not coexist in the isolates examined.

Using the Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST/), we found a DNA fragment on the chromosome of *S. flexneri* 2a strain YSH6000 with 99.4% sequence similarity to the SAI, which consists of the integrase gene intI1 and two antibiotic resistance genes, *oxa1* (100% identical to *blaOXA30*) and *aadA1*, followed by IS1 (accession number AF326777). In strain YSH6000, this DNA fragment and two contiguous determinants of resistance to chloramphenicol and tetracycline are located in a 16.7 kb IS1-flanked element, the *Shigella* resistance locus (SRL) which is located in a 66 kb pathogenicity island (PAI) on the bacterial chromosome. That region was designated the SRL PAI. Another similar fragment containing the recombinase gene and *blaOXA30* (accession number AF255921) was also found in *S. flexneri* 2a isolates collected from Hong Kong and Shanghai. However, integrons have not been annotated to these sequences. Identification of these integrons will be helpful to understand more precisely the role of integrons in the mechanism of horizontal transfer of resistance genes.

**Recombination patterns of SAI gene cassettes**

In the inverse PCR analysis, two clear bands with lengths of ~1200 and 800 bp were amplified from boiled overnight broth of pUC-C101-containing *E. coli* strain DH5α by primer sets of inv-oxaL/inv-aadL and inv-aadL/inv-aadR, respectively. The nucleotide sequence of the 1200 bp band indicated that the free circular molecule consisted of two cassettes of *blaOXA30* and *aadA1SAI*, which was caused by the recombination between *attL* and *attC* of the *aadA1SAI* cassette (Figures 1 and 3). However, a predicted band with a length of 296 bp, suggesting recombination between *attL* and *attC* of *blaOXA30* was not detected by the inverse PCR using primers inv-oxaL/inv-aadL. The sequence of the 800 bp in the inverse PCR using primers inv-aadL/inv-aadR revealed the existence of a free circular cassette of *aadA1SAI* produced by recombination between *attC* of the *blaOXA30* cassette and *attC* of the *aadA1SAI* cassette (Figures 1 and 3).
between 

E. coli

strains, suggesting that horizontal transfer of the 'CS, class 1 integrons containing the gene cassette array of with on plasmids in Class 2 integrons were detected in 80.6% (25/31) of the isolates and 87.9% (29/33) of the S. sonnei isolates. However, although the attC cassettes of the SAI are coordinately excised or integrated, attC of the blaOXA30 cassette and attC of the aadA1 cassette (b) were used to screen for transconjugants only these transconjugants from one transconjugant, there was one failed identification of a cassette array in one transconjugant in spite of the presence of the aadA1 cassette in the environment.

The above findings indicate that the blaOXA30 and aadA1 cassettes of the SAI are coordinated excised or integrated, although the attC site has been identified in each of the two cassettes based on sequence analysis. The reason for the recombination of the two linked cassettes remains unknown, but this pattern may partially explain the phenomenon of why almost all blaOXA30 cassettes found in integrons so far are linked to the various gene cassette arrays found in class 1 integrons, the class 2 integrons were present in all of the S. flexneri serotype 2a isolates (n = 13) and serotype 2b isolates (n = 9) examined (Table 1). Class 2 integrons were localized on chromosomes by Southern-blotting analysis of HindIII-restricted genomic DNA and plasmid DNA isolated from one S. sonnei isolate and one S. flexneri isolate that are both positive for intI2. A 10.7 kb HindIII-restricted genomic DNA fragment from S. sonnei isolate C202 was cloned into vector pUC19 (termed pUC-C202) (Table 2) and sequenced. The fragment contains partial fimbrial operon, IS4, and the left end of Tn7; it contained a complete class 2 integron, including intI2 and a gene cassette array of dfrA1 + sat + aadA1 + orfX that confer resistance to trimethoprim, streptothricin and streptomycin (Figure 2; see more sequence details in Figure S2 in Supplementary data available at JAC Online). To differentiate the aadA1 SAI, the aadA1 cassette in the class 2 integron was termed aadA1\(_{707}\). The identity between nucleotide acid sequences of aadA1 SAI and aadA1\(_{707}\) was 99.4% (787/792). Compared with aadA1 SAI, the valine codon (GTG) at the 4th amino acid residue was replaced by the alanine codon (GCG) and the glutamic acid codon (GAA) at the 235th amino acid residue was deleted in the sequence of aadA1\(_{707}\).

The 3361 bp DNA fragment over the gene cassette region in the class 2 integron was amplified by PCR in all intI2-positive Shigella spp. isolates using primer set intI2ca-F/intI2ca-R (Table 1). All PCR products shared the same EcoRI-RFLP pattern. In addition, three nucleotide sequences of the PCR products from three S. flexneri isolates with serotypes 2a, 2b and x variant were 100% identical to that of the gene cassette region of the class 2 integron on pUC-C202. These results suggest that all gene cassette arrays in these class 2 integrons were dfrA1 + sat + aadA1 + orfX. In contrast to the various gene cassette arrays found in class 1 integrons, the cassette arrays in class 2 integrons are usually constant, as observed in this and several other studies, which is believed to
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be due to the mutation of an internal stop codon within *intI2*. It has been shown that Tn7 can be inserted in a single orientation into a specific target site, *attTn7*, downstream of the *glmUS* operon on the *E. coli* chromosome to minute 82.\(^{25}\) We found here that IS4 presents downstream of *glmUS* on genomes of *S. flexneri* 2a strain 2457T (GenBank accession number AE014073) and strain 301 (GenBank accession number AE005674, in which IS4 was annotated *yi41*), and adjacent to the left end of Tn7 in the *HindIII* fragment from *S. sonnei* isolate C202 (Figure 2), suggesting that the Tn7 in *S. sonnei* isolate C202 may insert into *attTn7* downstream of *glmUS*. This hypothesis was supported by results from sequencing the right end of Tn7 and the 3′ end of *glmS* amplified from isolate C202 using primers Tn7R-F and glmS-R (nucleotide sequence data are shown in Figure S2 in Supplementary data available at JAC Online) (Figure 2).

The Tn7 insertion site in the rest of the *Shigella* spp. isolates was identified by PCR analysis using three primer sets, *yi41*-F/Tn7L-R, Tn7R-F/glmS-R and *yi41*-F/glmS-R. Tn7s were inserted at the same site, *attTn7*, in all 54 *intI2*-positive *Shigella* spp. isolates, since the PCRs were positive for primer sets *yi41*-F/Tn7L-R (610 bp) and Tn7R-F/glmS-R (560 bp), but negative for primer set *yi41*-F/glmS-R (too far to be amplified). In contrast, the remaining 10 *intI2*-negative isolates were negative in both PCRs with primer sets *yi41*-F/Tn7L-R and Tn7R-F/glmS-R. Of these 10 isolates, 6 *S. sonnei* isolates were positive in PCRs using the primer set *yi41*-F/glmS-R (472 bp), indicating no Tn7 insertion; however, 4 *S. flexneri* isolates (2 of serotype 6 and 2 of serotype y variant) were negative in the same PCRs.

To our knowledge, this is the first experimental identification of Tn7 insertion in *Shigella* spp. The location of Tn7 on *Shigella* chromosome can explain the phenomenon observed in an earlier study that co-transfer of streptomycin and trimethoprim resistance encoded by the class 2 integron in *S. sonnei* was not observed in conjugation experiments,\(^{13}\) since the class 2 integron was not located on a conjugative plasmid.

Resistance conferred by class 1 integrons, SAI and class 2 integrons

All 33 tested isolates (17 *S. sonnei* and 16 *S. flexneri*) harbouring class 2 integrons were resistant to both trimethoprim and streptomycin. However, of the six *S. sonnei* isolates without class 2 integrons, four isolates (two with class 1 integrons containing the *aadA2* cassette and two without class 1 integrons) were susceptible to trimethoprim and two isolates with class 1 integrons containing cassettes *dfv* or *dfv* + *aadA5* or *dfv* + *aadA1* were resistant to trimethoprim, whereas five isolates were susceptible to streptomycin, but two, one and one of them contained class 1 integrons with cassettes of *aadA2*, *dfv* + *aadA5* or *dfv* + *aadA1*, respectively, but one other isolate without class 1 integron was resistant to streptomycin (Table 4). Moreover, of four *S. flexneri* isolates without class 2 integrons, two isolates (one serotype 6 and one serotype y variant) containing class 1 integrons with cassettes of *dfv* or *dfv* + *aadA5* were resistant to both trimethoprim and streptomycin (Table 4), whereas other two isolates without class 1 integron (one of serotype 6 and one of serotype y variant) were susceptible to both.

The above findings suggest that (i) class 2 integrons confer resistance to trimethoprim and streptomycin and (ii) class 1 integrons that contain cassettes of *dfv* or *dfv* + *aadA5* or *dfv* + *aadA1* confer resistance to trimethoprim, in *Shigella* spp. isolates. In addition, introduction of pUC-C202 into *E. coli* DH5α conferred host resistance to both trimethoprim and streptomycin, confirming that the class 2 integron is responsible for resistance to both trimethoprim and streptomycin.

All *Shigella* isolates carrying the SAI (found in 28 *S. flexneri* isolates and 1 *S. sonnei* isolate) were resistant to ampicillin, but some of the isolates that contain no SAI were also resistant to ampicillin (3 of 4 *S. flexneri* isolates tested and 9 of 30 *S. sonnei* isolates were resistant), suggesting that the SAI, as well as other determinants, are responsible for host resistance to ampicillin in *Shigella* isolates. Moreover, the SAI-mediated resistance to ampicillin was directly demonstrated by acquisition of resistance to ampicillin in susceptible host *E. coli* DH5α after transformation with pSU-C101 plasmid containing the SAI element.

Because all *Shigella* isolates carrying the SAI also contain class 2 integrons, the SAI-mediated streptomycin resistance could not be evaluated in these isolates. To our surprise, however, although the MIC for the transformant ( *E. coli* DH5α harbouring pUC-C101) of streptomycin, as determined by the agar dilution method, was increased from 0.25 mg/L in the control transformant ( *E. coli* DH5α harbouring pUC19) to 4.0 mg/L, introduction of pUC-C101 into *E. coli* DH5α did not confer resistance to streptomycin, as shown in a test using the disc diffusion method.

The gene cassettes of *aadA2*, *dfv* or *aadA5* or *dfv* + *aadA1* of class 1 integrons and *bla* or *aad* of the SAI element did not confer resistance to streptomycin in *Shigella* spp. in this study. Similar observations were made in several previous reports. Roe et al.\(^{34}\) reported that resistance to streptomycin was not observed in three *E. coli* isolates harbouring a class 1 integron that contains a single cassette of *aadA1*, but resistance to kanamycin in the same antibiotic class was observed. White et al.\(^{35}\) observed that, although an *E. coli* isolate harbouring a class 1 integron that contains cassettes of *dfv* + *aadA5* was resistant to both trimethoprim and streptomycin, *dfv* alone conferred a high level of resistance to trimethoprim, whereas *aadA5* conferred resistance to spectinomycin but not to streptomycin.

Conclusions

The genetic characteristics of class 1, SAI and class 2 integrons and their association with antibiotic resistance were examined in clinical *Shigella* isolates collected in Hangzhou, China. It was observed that the gene cassettes of class 1, SAI and class 2 integrons are responsible for mediating resistance to commonly used antibiotics, such as trimethoprim, streptomycin and ampicillin, in these *Shigella* spp. isolates. Our data indicate that multiple and complex mechanisms involving class 1 and class 2 integrons and antibiotic resistance have been developed in the evolution of *Shigella* strains.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References