Molecular differentiation of common promoters in *Salmonella* class 1 integrons

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Abstract

The integron is a mobile gene element which harbors antibiotic-resistance gene cassettes capable of site-specific integration. Among the four known types of integrons, the class 1 integron has been associated with multidrug-resistance in pathogenic bacteria. These gene cassettes have been the focus of a series of studies. The gene cassettes share a common promoter, and their expression levels are affected not only by their proximity to the promoter, but also by the strength (weak, hybrid and strong) of the common promoter, P₁, as well as the presence of the additional promoter, P₂. In this study, we developed molecular methods for the differentiation of promoter structures using PCR, restriction enzyme analysis, and polyacrylamide gel electrophoresis, and have applied them to the characterization of class 1 integrons in 33 non-typhoidal *Salmonella* serotypes in Korea. Class 1 integrons were detected in four serotypes: *S. Derby* (SD), *S. Istanbul* (SI), *S. Paratyphi B* (SPB), and *S. Livingstone* (SL), and the amplicon sizes were 1.0 Kb (SD, SI and SPB) and 2.0 Kb (SL). All of the 1.0 kb amplicons harbored gene cassettes (*aadA1* or *aadA2*), but the 2.0 kb amplicon harbored three (*dhfrXII-orfF-aadA2*) gene cassettes, which conferred streptomycin/spectinomycin (*aadA*) and trimethoprim (*dhfr*) resistances. Our promoter structure study revealed three types of promoters; strong P₁ (SD), weak P₁ (SPB and SL), and weak P₁ + P₂ (SI). In conclusion, the class 1 integrons were detected in Korean NTS, and their promoter structures were found to be variable. Therefore, our methods may prove helpful in terms of our understanding of molecular diversity, as well as the transmission of class 1 integrons and phenotype-genotype relationships in antibiotic-resistance.

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1. Introduction

In the mid-1980s, the DNA sequencing of several seemingly unrelated antibiotic-resistance-associated genes revealed common upstream and downstream regions (Cameron et al., 1986; Hall and Vocker, 1987). The upstream harbored the *attI* site, a

common promoter sequence, P_{ant} (P₁), and the opposite strand, the integrase gene (*int*) (Hall and Collis, 1995; Recchia and Hall, 1995), whereas the 3'-conserved segment harbored an antiseptic resistance gene (*qacE1*), a sulphonamide resistance gene (*sulI*), and an open reading frame (*orf5*) of undetermined function (Hall and Stokes, 1993). The central variable region included varying combinations of inserted gene cassettes. Thus far, more than 60 different cassettes associated with resistance genes have been characterized, which consequently renders their bacterial hosts resistant to a broad spectrum of antimicrobial agents (Recchia and Hall, 1995). This genetic element lacks direct or indirect

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Table 1
Salmonella serotypes used in this study and their antibiotic-resistances

Serotype(serogroup)	Antibiotic resistance ^a	Serotype	Antibiotic resistance
<i>S. Agama</i> (B)	S	<i>S. Montevideo</i> (C1)	
<i>S. Bardo</i> (C3)		<i>S. Muenchen</i> (C2)	AM, S, Te, SAM (I) K, TIC, NA
<i>S. Bareilly</i> (C1)		<i>S. Nchanga</i> (E1)	AM, S, CF, FOX, CRO
<i>S. Blockley</i> (C2)	Te, K, TIC, NA	<i>S. Newport</i> (C2)	
<i>S. Bradford</i> (B)		<i>S. Nigeria</i> (C1)	S
<i>S. Braenderup</i> (C1)		<i>S. Othmarchen</i> (C1)	Te
<i>S. Brezany</i> (B)	S	<i>S. Paratyphi A</i> (A)	NA
<i>S. Derby</i> (B)	S, Te	<i>S. Paratyphi B</i> (B)	AM, C, S, Te, TIC, NA
<i>S. Give</i> (E1)	S	<i>S. Planckendael</i> (C1)	S
<i>S. Hadar</i> (C2)	S, Te	<i>S. Rissen</i> (C1)	Te
<i>S. Infantis</i> (C1)		<i>S. Scenftenberg</i> (E4)	
<i>S. Istanbul</i> (C3)	S, Te, GM	<i>S. Thompson</i> (C1)	S
<i>S. Kedougou</i> (G)	S	<i>S. Tibati</i> (E1)	
<i>S. Kentucky</i> (C3)	S	<i>S. Travis</i> (B)	Te
<i>S. Lindenberg</i> (C2)	NA	<i>S. Tumodi</i> (B)	S
<i>S. Livingstone</i> (C1)	AM, C, S, SXT, Te, CP, K, TIC, GM	<i>S. Uppsala</i> (B)	S
<i>S. Mbandaka</i> (C1)			

^a streptomycin (S), ampicillin (AM), chloramphenicol (C), gentamicin (GM), sulfonamids (Su), tetracyclin (Te), nalidixic acid(NA), cefoxitine (FX), ciprofloxacin (CIP), ampicillin/sulbactam (SAM), cephalothin (CF), sulfamethoxazol/trimetoprim (SXT), amikacin(AN), ticarcillin (TIC), kanamycin (K), sulfamethoxazol/trimetoprim (SXT), cefoxitim (FOX), ceftriaxone (CRO).

repeat sequences at its ends and many gene products are associated with transposition. For this reasons, the new element was termed integron in contrast to transposon. Thus far, four types of integrons, with different *int* genes, have been identified, and the majority of the integrons obtained from clinical isolates are members of class 1 (Recchia and Hall, 1995; Martinez-Freijo et al., 1999; Naas et al., 2001). Class 1 integrase catalyzes site-specific recombination between the *attI* of the integron and a 59-base element of the gene cassette, which is mobile and harbors a variety of antibiotic-resistance genes, as well as unknown open reading frames (Recchia and Hall, 1995). Most of the gene cassettes lack their own promoters, and integrons function as natural expression vectors by supplying a common promoter, P_{ant} (P1), which is located in the conserved sequences upstream of the inserted genes (Hall and Stokes, 1993; Hall and Collis, 1995). Mutations in the –35 and –10 regions of P_{ant} modulate levels of gene expression and the varying promoter types were classified into weak, hybrid, and strong promoters (Lévesque et al., 1994). Such single nucleotide polymorphisms (SNPs) provide selective advantages to bacteria during the course of a single infection, epidemic spread, or the long-term evolution of virulence especially if antibiotic treatment is involved (Sokurenko et al., 1999). In a few integrons a second promoter, P2, has been formed via the insertion of three G residues, which extends the length of DNA between the –35 and –10 boxes to 17 base pairs (Tenover et al., 1988; Schmidt et al., 1988). The expression level of the gene cassette is influenced not only by its proximity to P1 and the copy number of gene cassettes, but also by the potency of P1 and the presence or absence of P2 (Collis and Hall, 1995; Lévesque et al., 1994). Thus far, studies concerning the class 1 integrons have focused solely on the characterization of gene cassettes, and have generally ignored the structures of the promoters. In this study, we developed simple promoter characterization methods via restriction enzyme analysis (REA) and polyacrylamide gel electrophoresis (PAGE), and

applied them to the characterization of the class 1 integrons in non-typhoidal *Salmonella* cases in Korea.

2. Materials and methods

2.1. Bacteria

33 *Salmonella enterica* serotypes were isolated from the stools of patients hospitalized for acute gastroenteritis in 2000, and were characterized by the Laboratory of Enteric Infections, Department of Microbiology, Korea Center for Disease Control and Prevention, located in Seoul, Korea. *Salmonella* was serotyped via slide agglutination tests using O and H group antisera (Difco Laboratories, Detroit, MI, USA). The serotypes employed in this study are summarized in Table 1.

2.2. Antimicrobial susceptibility testing

Salmonella serotypes were evaluated for their susceptibility to 18 antimicrobial agents (Table 1) via disk diffusion assay following CLSI (formerly NCCLS) guidelines (NCCLS, 2003).

Fig. 1. Molecular typing of P_{ant} (P1) via restriction enzyme analysis. Lanes: M, 100 bp molecular weight marker (iNtRON Biotechnology, Seoul, Korea); 1, *S. Derby* (*AluI*); 2, *S. Derby* (*HincII*); 3, *S. Istanbul* (*AluI*); 4, *S. Istanbul* (*HincII*); 5, *S. Paratyphi B* (*AluI*); 6, *S. Paratyphi B* (*HincII*); 7, *S. Livingstone* (*AluI*); 8, *S. Livingstone* (*HincII*).

Table 2
Genetic structures of the variable regions of class 1 integrons and antibiotics resistances

Serotypes	Amplicon size (Kb)	Gene cassette	Antibiotic resistance
<i>S. Derby</i>	1.0	<i>aadA2</i>	S, Te
<i>S. Istanbul</i>	1.0	<i>aadA1</i>	S, Te, GM
<i>S. Paratyphi B</i>	1.0	<i>aadA2</i>	AM, C, S, Te, TIC
<i>S. Livingstone</i>	2.0	<i>dhfrXII-orfF-aadA2</i>	AM, C, S, SXT, Te, GM, K, TIC

2.3. Detection of class 1 integrons by PCR

All serotypes were evaluated for the presence of class 1 integrons using primers, 5'CS and 3'CS (Lévesque et al., 1995). The total *Salmonella* DNA was extracted using G-spin for Bacteria kit (iNtRON Biotechnology Co., Seoul, Korea) in accordance with the manufacturer's protocol. The PCR was conducted as before (Kwon et al., 2002).

2.4. Characterization of P_{ant} by REA and polyacrylamide gel electrophoresis (PAGE)

Proximity to the common promoter P_{ant} affects the expression of gene cassettes of the class 1 integrons, as does the strength of the promoter. According to the nucleotide sequence of the –35 and –10 regions of P_{ant} (P1) it can be divided into strong, hybrid and weak (Fig. 1). The –35 region of strong P1 and the –10 region of weak P1 are digested by *HincII* and *AluI*, respectively, but the –35 and –10 regions of hybrid P1 are not digested by the enzymes. In addition, three nucleotide (GGG) insertions have been shown to generate a new promoter, P2 downstream of P_{ant} . For the rapid and simple characterization of the promoter structure of class 1 integrons, we have also designed PCR primer sets for REA and sequencing (IntProF: 5'-ATG CCT CGA CTT CGC TGC T-3', IntProR: 5'-ACT TTG TTT TAG GGC GAC TGC-3', amplicon size 327 bp) and for PAGE (Int PT2F: 5'-TGG TAA CGG CGC AGT GGC-3', 5'-TTG CTG CTT GGA TGC CCG A-3', amplicon size 82 bp or 85 bp in the case of the three-nucleotide-insertion). The amplicons generated by the primer set (IntProF/ IntProR) were then treated with *HincII* and *AluI* in separate tubes as before (Kwon et al., 2000), and

electrophoresed in 2% agarose. In order to identify P2 (gel shift by 3 bp insertion), we conducted PAGE with 12% (19:1) polyacrylamide gel at 90 V for 4 h, and the gel was stained with ethidium bromide.

2.5. Sequencing of PCR products

PCR products, representing the different amplicons generated for the 33 *Salmonella* serotypes, were purified via simple ethanol precipitation and sequenced as described before (Kwon et al., 2000). The obtained DNA sequences were then compared to entries in the GenBank database of the National Center for Biotechnology Information BLAST network (Altschul et al., 1990).

2.6. Nucleotide sequence accession numbers

Nucleotide sequences were submitted to the GenBank database and the following accession numbers were assigned: *aadA1* in *S. Istanbul* (AY125351), *aadA2* in *S. Derby* (AY127872), in *S. Paratyphi B* (AY125352) and in *S. Livingstone* (AY128094), and *dhfrXII* in *S. Livingstone* (AY127874).

3. Results and discussion

3.1. Antimicrobial susceptibility

Ten serotypes—Bardo, Bareilly, Bradford, Braenderup, Infantis, Mbandaka, Montevideo, Newport, Senftenberg and Tibati—were found to be susceptible to all tested antibiotics (Table 1). Streptomycin- and tetracycline-resistance were most frequently detected, at 50% (16/33) and 32% (10/33), respectively. Multi-drug-resistant (MDR) *Salmonella* serotypes evidencing resistance to three or more different antibiotics were reported in serotypes Typhimurium, Ohio, Panama, Brandenburg, Virchow, and Hadar (Guerra et al., 2000). In Korea, MDR *S. Typhi*, *S. Typhimurium*, and *S. Enteritidis* were reported (Yang et al., 2001; Pai et al., 2003; Kim et al., 2004), but MDR had not been previously detected in serotypes Muenchen, Paratyphi B, and Livingstone. Therefore, the appearance of MDR in these minor NTS in Korea (Table 1) makes necessary further studies into the mechanisms underlying the evolution and spread of resistance, as well as effective control methods.

Table 3
Single nucleotide polymorphisms (SNPs) of *aadA2* in Korean pathogenic bacteria

Bacteria	Accession no.	SNP (amino acid changes and codon number)	Gene cassettes
<i>E. coli</i>	AY878717	— ^a	<i>dhfrXVI-aadA2</i>
	AY878718	GAG>GAA (151 ^b)	<i>aacA4-bla_{oxa-4}-aadA2</i>
<i>K. pneumoniae</i>	AF180731	GTA>GAA (V3E) ATT>GTT (I9V) GAC>GAT (69 ^b) TGG>TGT (W211C)	<i>dhfrXII-orfF-aadA2</i>
	AF227505	—	<i>aacA4-oxa-1-aadA2</i>
<i>P. aeruginosa</i>	AF418284	CTG>CAA (L29Q) GAG>GGG (E123G)	<i>blaVIM-2-catB8-aacA4-aadA2</i>
<i>S. Derby</i>	AY127872	GCG>ACG (A150T)	<i>aadA2</i>
<i>S. Paratyphi B</i>	AY125352	—	<i>aadA2</i>
<i>S. Livingstone</i>	AY128094	GAG>GGG (E123G)	<i>dhfrXII-orfF-aadA2</i>

^a No SNP.

^b Silent mutation.

Table 4
Molecular characterization of class 1 integron common promoter

Promoter type	–35 Region		Spacing		–10 Region		Serotypes
	Seq	<i>Hinc II</i> (GTY/RAC)	No. of nt	3-nt-insertion	Seq	<i>Alu I</i> (AG/CT)	
strong P1	gTTGACA	+	17		TAAACT	–	<i>S. Derby</i>
	cTTGTTA	–	14	–	TACAGT	–	
weak P1 + P2	gTGGACA	–	17		TAAGCT	+	<i>S. Istanbul</i>
	cTTGTTA	–	17	+	TACAGT	–	
weak P1	gTGGACA	–	17		TAAGCT	+	<i>S. Paratyphi B</i>
	cTTGTTA	–	14	–	TACAGT	–	<i>S. Livingstone</i>

3.2. Molecular characterization of gene cassettes

Class 1 integrons were detected in four (serotypes Derby, Istanbul, Paratyphi B and Livingstone) of 33 NTS serotypes. The amplicons generated by the 5'CS/3'CS primer set were approximately 1.0 and 2.0 Kb in size (Table 2). Nucleotide sequencing revealed *aadA2* (*S. Derby* and *S. Paratyphi B*) and *aadA1* (*S. Istanbul*) in the 1.0 kb amplicons, and *dhfrXII-orfF-aadA2* (*S. Livingstone*) in the 2.0 Kb amplicon (Table 2). Such gene cassettes had already been reported in Korea (Kwon et al., 2002; Kang et al., 2005). Not all of the streptomycin-resistant NTS serotypes harbored class 1 integrons, but the streptomycin resistance of the integron-positive serotypes could be attributed to *aadA* (Tables 1 and 2). The coding regions of the *aadA2* gene sequence identified in *S. Derby* (AY127872), *S. Paratyphi B* (AY125352) and *S. Livingstone* (AY128094) were compared with other *aadA2* genes detected in pathogenic bacteria in Korea (AF180731, AF257505, AF418284, AY878717, and AY878718). We detected six amino-acid-changing SNPs at codons 3, 9, 29, 123, 150, and 211, and two silent SNPs at codons 69 and 151 (Table 3). Each isolate harbored zero to four SNPs, and only the SNP at codon 123 was found to exist in common in *Pseudomonas aeruginosa* and *S. Livingstone*. The *AadA2* genes in *Escherichia coli* (AY878717, Jeong et al., 2005), *Klebsiella pneumoniae* (AF227505) and *S. Paratyphi B* harbored no SNPs, but the contents of their gene cassettes were completely different. In addition, *K. pneumoniae* (AF180730) and *S. Livingstone* evidenced identical contents, but their *AadA2* genes differed at codons 3, 9, 69, 123, and 211 (Table 3). SNPs in the *fimH* of *E. coli* and *S. Typhimurium* were associated with pathogenic adaptation (Sokurenko et al., 1998; Boddicker et al., 2002). Some of the SNPs in *spvR* were conserved in certain serotypes of *Salmonella*, and they could be used in the rough differentiation of *Salmonella* serotypes (Kwon et al., 2004). Therefore, SNPs identified in *aadA2* may prove useful as molecular markers for the study of the transmission and evolution of *aadA2*-bearing class 1 integrons.

Gene cassettes can be recombined into an integron, but conversely can be looped-out from an integron (Hall et al., 1991). The array of *dhfrXII-orfF-aadA2* gene cassettes can be formed by the additional recombination of *dhfrXII* into the integron bearing the *aadA2* cassette. The gene cassette most proximal to the common promoter (P1 and/or P2) is expressed more abundantly than others, and the localization of *dhfrXII* proximally to P1 in *S. Livingstone* represents selection pressure by trimethoprim (Hall and Collis, 1995). Resistances to

tetracycline, chloramphenicol, kanamycin, gentamicin, ampicillin, and nalidixic acid can be transferred by integrons; however, in the case of the NTS serotypes studied in this work, resistance was not associated with the Class 1 integron (Recchia and Hall, 1995; Martínez-Martínez et al., 1998).

3.3. Molecular characterization of common promoters of gene cassettes

According to the results of a promoter study involving CAT assays, the relative strengths of the strong P1 and the weak P1 + P2 to weak P1 were approximately 32-fold and 16-fold higher, respectively (Lévesque, 1994). The amplicons generated by the primer set (IntProF/ IntProR) were digested by *HincII* (*S. Derby*) or *AluI* (*S. Istanbul*, *S. Livingstone* and *S. Paratyphi B*) (Fig. 1), and gel shift via 3-nt-insertion was detected in *S. Istanbul*. Therefore, the class 1 integrons of *S. Derby*, *S. Livingstone* and *S. Paratyphi B* harbored strong and weak P1, respectively. *S. Istanbul* was found to harbor P2, as well as weak P1 (weak P1 + P2) (Table 4). All of the amplicons employed in this promoter study were sequenced, and all of the nucleotide variations were confirmed. *S. Derby* and *S. Paratyphi B* were similar with regard to the contents of the gene cassette, but were distinct in terms of the structures of the promoters. Therefore, our promoter structure assays may provide us with further insight into class 1 integron transmission and evolution, as well as the relationships between antibiotic-resistances and certain genotypes.

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