CHARACTERIZATION OF CLASS 1 INTEGRONS WITH UNUSUAL 3′ CONSERVED REGION FROM *SALMONELLA ENTERICA* ISOLATES

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Abstract. The unusual 3′ conserved sequence region of class 1 integrons was characterized in seven *Salmonella* isolates from swine and poultry. Three types of gene cassette arrays, *aadA2-cmlA1-aadA1*, *sat-psp-aadA2-cmlA1-aadA1* and *drfA12-orf-aadA2-cmlA1-aadA1*, were found to be linked to a genetic organization *qacH*-IS*440-sul3.* All class 1 integrons were located on a conjugative plasmid that could be transferred to *Escherichia coli*. The results support the notion that the use of an antibiotic can select for resistance not only to that specific agent, but also to other unrelated antimicrobials including those that are no longer approved for use in food animal production.

INTRODUCTION

Class 1 integrons play an important role in the dissemination of antimicrobial resistance in many multidrug resistance (MDR) gram-negative bacteria, including different zoonotic serovars of *Salmonella enterica* (Guerra *et al*, 2000; Goldstein *et al*, 2001; Randall *et al*, 2004; Fluit, 2005). Most of class 1 integrons comprise two conserved segments, 5′ CS and 3′ CS, separated by a variable region that contains one or more gene cassettes. The 5′ CS region contains integrase gene (*intI1*), integration site (*attI1*) and promoter region. The typical 3′ CS region usually consists of *qacEΔ1* (encoding resistance to quaternary ammonium compounds), *sul1* (encoding resistance to sulphonamide), ORF5 of unknown function and/or *tni* (transposition functions) (Fluit and Schmitz, 2004). The 3′ CS fragment of class 1 integrons is not always conserved and integrons that lack this region have been previously observed (Kazama *et al*, 1998; Naas *et al*, 1998). Recently, an unusual 3′ conserved sequence regions with *qacH* linked to a *sul3* domain was found in plasmid-borne class 1 integrons in different *Salmonella* serovars (Antunes *et al*, 2007). These atypical integrons carry different resistant gene arrays or hybrid genes suggesting genetic evolution by different recombination events (Antunes *et al,* 2007).

We previously reported that class 1 integrons are widely widespread in MDR *Salmonella* isolates from swine and poultry (Chuanchuen *et al*, 2007) and all of the cassette arrays were followed by a 3′ CS typical of *sul1*-associated integrons. In this study, we investigated the structure of the unusual integrons from a number of selected *Salmonella* strains. The genetic location and mobility of resistance genes were also examined. This will help to explore role of integrons in the dissemination of antibiotic resistance and explain mechanisms underlying multidrug resistance in *Salmonella*.

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MATERIALS AND METHODS

Bacterial strains, media and growth conditions

Seven *S. enterica* isolates that carried *intI1* but not the typical 3′ CS were identified

as previously described (Chuanchuen *et al,* 2007) and are shown in Table 1. All the bacterial strains were stored as 20% glycerol stocks at -80ºC. Bacteria were grown on Luria-Bertani (LB) media (Difco, BD Diagnostic Systems, MD, USA) or Muller Hinton Agar (MHA; Difco) at 37ºC.

Determination of antimicrobial susceptibility

Antimicrobial agents were purchased from Sigma-Aldrich (St Louis, MO, USA). Minimum inhibitory concentrations (MICs) were determined by two-fold agar dilution and/or two-fold microdilution technique according to Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (NCCLS, 2002). *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as control organisms.

PCR amplification and DNA sequencing

All of the *Salmonella* isolates were tested for the presence of *sul1*, *sul2* and *sul3* using PCR. Organizational structure of class 1 integrons with atypical 3′ CS was determined using several specific primer combinations. Primers used were as follows: qacHF (5′-CTC GCACTCAAGTCCATCC-3′), qacHR (5′-CTAA CGATAAGTC CCATGCC-3′), sul3F (5′-GGGA GCCGCTTCCAGTAAT-3′), aadA1F (5′-CTCC GCAGTGGATGGCGG-3′), aadA2F (5′-CATTGA GCGCCATCTGGAAT-3′), aadA2R (5′-ACATTT CGCTCATCGCCGGC-3′), dfrA12F (5′-TTCG CAGACTCACTGAGGG-3′), cmlAF (5′-TGG ACCGCTATCGGACCG-3′), cmlAR (5′-CGCAA GACACTTGGGCTGC-3′), 5′ CS (5′-GGCATCC AAGCAGCAAG-3′) (Levesque *et al*, 1995; Chuanchuen *et al,* In press). Template DNA was prepared by the boiled lysis procedure as described (Leverstein-van Hall *et al*, 2002). PCR was performed using Eppendrof® MasterMix (Eppendrof Hamburg, Germany) according to the manufacturer's instructions. PCR thermocycling conditions were as follows: denaturation at 94ºC for 5 minutes, following by 30 cycles of denaturation for 45 seconds at 94ºC, primer annealing for 3 minutes at 57ºC, and extension for 5 minutes at 72ºC, and a final extension for 10 minutes. PCR products were purified using QIAQuick Gel Extraction kit (Qiagen, CA, USA) and submitted for sequencing at Macrogen (Seoul, South Korea). Sequence comparisons were made using the BLAST program available at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

Conjugal transfer of plasmid-borne integrons

All *Salmonella* isolates were used as donors and the rifampicin-resistant derivative of *E. coli* K12 strain MG1655 (MG1655Rif^r, MIC = 256 μg/ml) was recipient. Transconjugants were selected on LB agar supplemented with 32 μg/ml of rifampicin and one of the following antibiotics: chloramphenicol (16 μg/ml), streptomycin (50 μg/ml). Transconjugants were confirmed to be *E. coli* by growth on MacConkey agar (Difco) and examined for susceptibility to antibiotics. Plasmid DNA were extracted from each transconjugant clone using QIAprep® Mini-spin kit (Qiagen) and examined for the presence of *intI1* and the resistance genes by PCR as described above. Plasmid DNA was also digested with the restriction enzyme *Bam*H1 and the restriction patterns were compared with those from the corresponding donors.

RESULTS

Structure of integrons

Class 1 integrons in all the *Salmonella* isolates were not of the *sul1*-type, and *sul3* was the only sulfonamide resistance gene detected in these isolates. PCR amplifications revealed that the gene cassettes in all strains were followed by an unusual 3′ CS liked to *qacH* and *sul3* and genetic organization of the integrons is shown in Fig 1. Three *S.* Kedougou isolates harbored the *sul3*-integron containing the following gene cassette organization: 5′ CS-

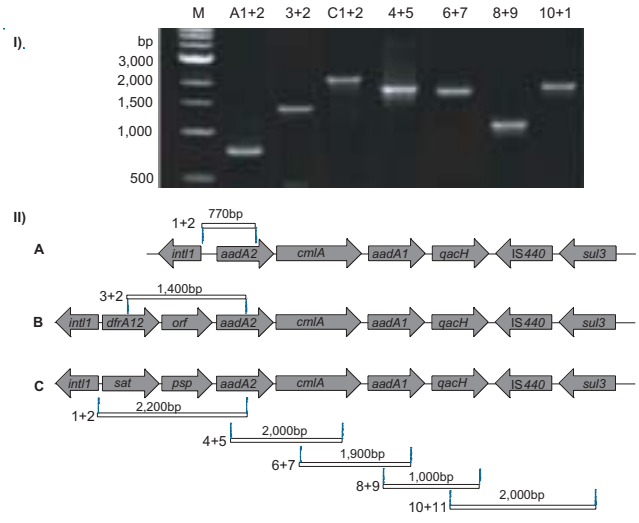

Fig 1–Organization of class 1 integrons in *Salmonella.* I) PCR products resulting from PCR amplifications using the combination of specific primers indicated in numbers above each lane. II) Organization of class 1 integrons in *Salmonella* deduced from the PCR amplicons (not to scale). Gene orientations are indicated by arrows. The solid bars and dashed lines indicate the PCR amplification regions with the size indicated above each bar. The specific primers used are shown in numbers as follows: 1, 5′CS; 2, aadA2R; 3, dfrA12; 4, aadA2F; 5, cmlAR; 6, cmlAF; 7, aadA1R; 8, aadA1F; 9, qacHR; 10, qacHF; 11, sul3. M, molecular weight marker.

sat-psp-aadA2-cmlA1-aadA1-qacH-IS*440-sul3* (Table 1). Class 1 integrons with the gene cassette 5′CS-*drfA12-orf-aadA2-cmlA1-aadA1-qacH*-IS*440-sul3* was identified in only one *S. enterica* serotype Stanley and those with the genetic structure 5′ CS-*aadA2-cmlA1-aadA1-qacH*-IS*440-sul3* were demonstrated in the other three isolates. The common genes *aadA1* and *aadA2, cmlA1* and *qacH* encode resistance to streptomycin, chloramphenicol and quaternary ammonium compounds, respectively. The *dfrA12* gene confers resistance to trimetroprim. For the *S.* Stanley class 1 integrons, *sat* and *psp* encodes streptothricin resistance and putative phosphoserine phosphatase respectively.

Localization and transfer of integrons

Four *Salmonella* isolates, SA077, SA076, SA075, and SA201, were able to transfer streptomycin and/or chloramphenicol resistance to *E. coli* MG1655Rif^r (Table 2). Cotransference between resistance to these two antibiotics was also observed and the transconjugants derived from SA077 were additionally resistant to trimethoprim. PCR analysis indicated that all transconjugant strains possessed class 1 integrons and *Bam*H1 digestion of plasmid from transconjugants and donors yielded the same restriction pattern (data not shown). The other 3 *Salmonella* isolates, SA043, SA044 and

Table 1
Characteristics of *Salmonella* isolates used in this study.

Strain	Serovar	Source	Gene cassette	Resistance phenotype
SA043	Kedougou	Swine	*aadA2-cmlA1-aadA1*	AMP-CHP-GEN-SPC-STR-SUL-TET
SA045	Kedougou	Poultry	*aadA2-cmlA1-aadA1*	AMP-CHP-GEN-SPC-STR-SUL-TET
SA076	Kedougou	Swine	*sat-psp-aadA2-cmlA1-aadA1*	AMP-CHP- GEN-STR-SUL-TET
SA077	Stanley	Swine	*drfA12-orf-aadA2-cmlA1-aadA1*	AMP-CHP-SPC-STR-SUL-TET-TRI
SA0161	Kedougou	Swine	*aadA2-cmlA1-aadA1*	CHP-SPC-SUL-STR-TRI
SA075	Kedougou	Poultry	*sat-psp-aadA2-cmlA1-aadA1*	AMP-CHP-GEN-SPC-STR-SUL-TET
SA201	Kedougou	Swine	*sat-psp-aadA2-cmlA1-aadA1*	AMP-CHP-GEN-SPC-STR-SUL-TET

AMP=ampicillin; CHP=chloramphenicol; GEN=gentamicin; SPC=spectinomycin; STR=streptomycin; SUL=sulfamethoxazole; TET=tetracycline; TRI=trimethoprim

Table 2
Antibiotic susceptibility of transconjugants.

Donor x recipient	MICs[a] (μg/ml)			
	STR	CHP	SUL	TRI
MG1655Rif^R	8	16	1,024	0.5
SA076 x MG1655Rif^R	256	64	1,024	ND
SA077 x MG1655Rif^R	512	128	1,024	>1,024
SA075 x MG1655Rif^R	1,024	64	1,024	ND
SA201 x MG1655Rif^R	>1,024	256	1,024	ND

[a]Minimum inhibitory concentration value of transconjugant from the corresponding donor (*Salmonella*) and recipient (*E. coli* MG1655Rif^R)
ND=Not detected

SA0161, could not transfer streptomycin and chloramphenicol resistance.

DISCUSSION

The *sul3* gene was originally identified on a conjugative plasmid in *E. coli* from a pig population (Perreten and Boerlin, 2003). It was later found to be associated with class 1 integrons in *Salmonella* from livestock (Antunes *et al* 2007) and in *E. coli* from humans (Bischoff *et al*, 2005). As this gene is part of the unusual integrons, it could facilitate the dissemination of class 1 integrons similar to that of *sul1*. As sulfonamides have been commonly used in the production of food animals, this will place an important pressure for selection of sulfonamide resistance and resistance to other antibiotics encoded by resistance genes in the integrons.

Chloramphenicol has been withdrawn from use in food animals in many countries including Thailand but the high prevalence of chloramphenicol-resistant *Salmonella* is remarkable (Abouzeed *et al*, 2000; Bischoff *et al,* 2005; Gebreyes and Thakur, 2005). In this study, co-transfer of chloramphenicol and streptomycin resistance was observed. Aminoglycosides such as streptomycin are commonly used in poultry and swine and therefore, selection of streptomycin resistance via *aadA* can also co-select for chloramphenicol resistance encoded by *cmlA* located in the same integron structure. This could be the explanation of the persistence of chloramphenicol-resistant *Salmonella* even in the absence of a direct chloramphenicol pressure (Bischoff *et al,* 2005). In addition, it is an indication of the presence of a direct genetic link between *cmlA* and *aadA* genes in a unique integron structure. The observed transconjugant strains possessing class 1 integrons suggests that integrons play a role in the wide distribution of antibiotic resistance among the *Enterobactericeae* family. However, strains SA043, SA044 and SA0161 did not transfer their streptomycin/chloramphenicol resistance to *E. coli* recipients, suggesting that class 1 integrons in these strains are chromosomally located. This can provide stable antibiotic resistance even in the absence of antibiotic selection pressure (Levings *et al*, 2005).

Three types of the integron structure identified in this study are identical to those previously reported in *Salmonella* and *E. coli* (Bischoff *et al* 2005; Antunes *et al* 2007). The resistance gene arrays *aadA2* and *drfA12-orf-aadA2* located at the 5′-end are similar to those widely disseminated among *Salmonella* as previously reported (Hsu *et al*, 2006). The *drfA12-orf-aadA2-cmlA1-aadA1-qacH*-IS*440-sul3* organization found in *S.* Kedougou from poultry and swine indicates the widespread

distribution and transfer of resistance determinants among pathogens in food animals. Use of common antibiotics in the production of these domestic livestock could be the explanation. The gene cluster *sat-psp-aadA2* was previously identified on a plasmid in an enterotoxigenic *E. coli* (Bischoff *et al,* 2005) and was also identified in *Salmonella* in our collection. Since identical genetic structures of class 1 integrons could be identified in the same and different bacterial species, in different hosts and in different geographical area, it suggests that such gene cassettes or integrons have been exchanged between intra- and interspecies and play an important role in the horizontal dissemination of antimicrobial resistance among bacteria (Hsu *et al,* 2006).

In summary, we have identified class 1 integrons with the unusual 3′ conserved segment linked to the *qacH-sul3′* domain in *Salmonella* isolates from poultry and swine in Thailand. The different organizations of gene cassette suggest a genetic evolution by different recombination events. Class 1 integrons containing variety of resistance gene cassette could play an important role in the dissemination and maintenance of antibiotic resistance in *Salmonella* isolates both in the presence and absence of selective pressure.

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