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NOTE

Assessment of Conjugal Transfer of Antibiotic Resistance Genes in *Salmonella* Typhimurium Exposed to Bile Salts

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This study was designed to evaluate the transfer potential of antibiotic resistance genes in antibiotic-resistant *Salmonella* Typhimurium (*S.* Typhimurium^{R}) in the presence of bile salts. The resistance of *S.* Typhimurium^{R} to ampicillin, kanamycin, and tetracycline was increased by 64-, 64-, and 512-fold, respectively. The highest transfer frequency from *S.* Typhimurium^{R} to *Escherichia coli* was observed at the bile salt concentration of 160 μg/ml (3.8×10^{-3} transferrants/cells). The expression of *traJ* and *traY* was suppressed in *S.* Typhimurium^{R} by bile salt. This study provides useful information for understanding the conjugative transfer of antibiotic resistance genes in *S.* Typhimurium under intestinal conditions.

***Keywords*:** *Salmonella* Typhimurium, antibiotic resistance, virulence, gene transfer, bile salt

The misuse and overuse of antibiotics has been a major cause of the emergency of antibiotic resistant bacteria in food products. Over the last few decades, numerous reports of outbreaks of foodborne diseases in association with antibiotic-resistant *Salmonella* strains have been a growing health concern worldwide (Jones *et al.*, 2002). The general symptoms of salmonellosis include abdominal cramps, diarrhea, nausea, vomiting, headache, chills, and fever. In addition, the antibiotic-resistant pathogenic *Salmonella* strains can encounter probiotic strains dwelling in the gastrointestinal tract and possibly transfer antibiotic-resistant genes in plasmids, transposons, and integrons (Fluit and Schmitz, 1999). Many virulence factors such as invasion genes (*invA*), pathogenicity islands, and plasmid-associated genes can contribute to the pathogenicity of *Salmonella* strains (Rotger and Casadesus, 1999). The plasmid-containing virulence genes (*spv* and *pef*) encode fimbriae and transfer genes (Fluit, 2005). The rapid dissemination of multidrug-resistant pathogens is attributed to the integron-mediated antibiotic resistance in *Salmonella* strains (Molla *et al.*, 2007). The acquisition and dissemination of antibiotic resistance determinants can occur through bacterial conjugation, regulated by *tra* operons (Grohmann *et al.*, 2003; de la Cruz *et al.*, 2009). The conjugal transfer of antibiotic resistance genes in bacteria can be triggered by small molecules or peptides originating from the host chromosome, regulated by plasmid- and host-encoded factors (Frost and Koraimann, 2010). However, relatively few studies have been conducted on the transfer potential of antibiotic resistance genes in the presence of bile salts. Therefore, the objective of this study was to investigate the possibility of conjugative transfer of antibiotic resistance and virulence determinants from antibiotic-resistant *S.* Typhimurium exposed to bile salts.

Strains of *S. enterica* serovar Typhimurium KCCM 40253, *S.* Typhimurium CCARM 8009, and *Escherichia coli* KACC 10115 were obtained from the Korean Culture Center of Microorganisms (KCCM; Korea), the Culture Collection of Antibiotic Resistant Microbes (CCARM, Korea), and the Korean Agricultural Culture Collection (KACC; Korea), respectively, and cultured in trypticase soy broth (TSB; Difco, BD Diagnostic Systems, USA) at 37°C for 24 h. Strains of *Lactobacillus rhamnosus* GG and *Lactobacillus paracasei* were kindly provided by Dr. Azlin Mustapha of the Department of Food Science at the University of Missouri-Columbia. Strains of *Lactobacillus brevis* KACC 10553 and *Lactobacillus casei* KACC 12413 were obtained from the Korean Agricultural Culture Collection (KACC). Probiotic strains were anaerobically cultivated in de Man, Rogosa, and Sharpe (MRS; BD Diagnostic Systems) broth supplemented with 0.05% cysteine hydrochloride at 37°C for 24 h in a GasPak anaerobic system (BBL, USA) with AnaeroGen (Oxoid Ltd., UK).

To evaluate the susceptibility of selected bacterial strains to bile salts (sodium salt mixture of cholic acid and deoxycholic acid) and antibiotics (ampicillin, AP; aztreonam, AT; cefotaxime, CX; ceftazidime, CZ; kanamycin, KM; erythromycin, EM; nalidixic acid, NA; norfloxacin, NF; streptomycin, SM; sulfamethoxazole, SZ; tetracycline, TC), the stock solutions for bile salts and antibiotics were prepared by dissolving them in sterile distilled water at concentrations of 51.2 mg/ml and 256 μg/ml, respectively, which were serially diluted two-fold in TSA and MRS agar to 256 and 0.25 μg/ml. The pathogenic and probiotic strains (1×10^{4} CFU/plate each) were plated on the TSA and MRS agar and incubated anaerobically at 37°C for 18 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of each bile salt or anti-

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Table 1. Minimum inhibitory concentrations (MICs, μg/ml) of antibiotics and bile salts against selected strains

Strain[a]	Antibiotic[b]											
	AP	AT	CX	CZ	EM	KM	NA	NF	SM	SZ	TC	BS
S. Typhimurium KCCM 40253	4	<0.25	<0.25	0.25	128	4	2	0.25	32	256	0.5	>51,200
S. Typhimurium CCARM 8009	>256	<0.25	<0.25	0.25	128	>256	2	0.25	>256	256	128	>51,200
E. coli KACC 10115	4	<0.25	<0.25	0.25	128	4	32	0.25	>256	256	0.25	25,600
E. coli^{TC}	>256	<0.25	<0.25	0.25	128	>256	32	0.25	>256	>256	0.25	25,600
L. brevis KACC 10553	2	>256	16	16	1	128	256	256	64	>256	8	1,280
L. casei KACC 12413	0.25	>256	4	4	0.25	>256	256	16	128	128	0.5	1,280
L. paracasei ATCC 25598	0.25	>256	16	16	0.25	>256	>256	32	256	256	0.5	1,280
L. rhamnosus GG	0.25	>256	4	16	0.5	256	128	16	128	128	1	2,560

[a] *E. coli*^{TC}, multiple antibiotic-resistant *E. coli* KACC 10115 transconjugant.
[b] AP, ampicillin; AT, aztreonam; CX, cefotaxime; CZ, cefatazidime; EM, erythromycin; KM, kanamycin; NA, nalidixic acid; NF, norfloxacin; SM, streptomycin; SZ, sulfamethoxazole; TC, tetracycline; BS, bile salts.

biotic at which no visible growth was observed.

The plasmid DNA was extracted using a Wizard® Plus Minipreps DNA Purification System (Promega Co., USA) according to the instruction manual. The isolates were tested for the presence of virulence genes, and class 1 integrons, which were determined by PCR amplification. The PCR mixture (50 μl) containing 2.5 U of Taq polymerase (Fermentas Inc., Canada), 1× Taq buffer (Fermentas Inc.), 25 pmol of each primer, 0.2 mM of dNTP, and 1.75 mM of $MgCl_2$ was amplified using a thermal cycler (XP Cycler; Bioer Technology Co. Ltd., China). The PCR products (10 μl) were separated by electrophoresis on a 1% (w/v) agarose gel stained with ethidium bromide (10 mg/ml) and then visualized using an ultraviolet transilluminator (Bio-Rad, UK).

The transferability of antibiotic-resistant genes was evaluated by broth and filter mating techniques (Garcia-Quintanilla *et al.*, 2008). Multiple antibiotic-resistant *S.* Typhimurium CCARM 8009 was used as donor strain, and *L. brevis*, *L. casei*, *L. paracasei*, *L. rhamnosus*, and *E. coli* were used as recipient strains. The donor and recipient cultures (10^9 CFU/ml each) were mixed in the same volume (500 μl each), centrifuged at 8,000 × g for 5 min, and then resuspended in 50 μl of TSB containing 0, 160, 320, and 640 μg/ml of bile salts. For broth mating, the mating mixtures were incubated anaerobically at 37°C for 4 h. For filter mating, the mating mixtures were filtered through a 0.45 μm membrane filter (Millipore, Corp., USA). The filters were aseptically placed on TSA plates containing 0, 160, 320, and 640 μg/ml of bile salts and incubated anaerobically at 37°C for 4 h. After incubation, the numbers of donors, recipients, and transconjugants were determined by the plate count method using selective agars: MRS agar for probiotic strains and MacConkey-sorbitol agar (Diagnostic Systems) for *E. coli*. Transconjugants were selected on MRS and MacConkey-sorbitol agar plates containing 4-fold MICs of erythromycin, kanamycin, tetracycline, and streptomycin against each recipient strain. The donor cells were eliminated on MRS agar plates containing 4-fold MICs of aztreonam and nalidixic acid against *S.* Typhimurium^{R}. The frequency of conjugal transfer was expressed as the numbers of transconjugants cells per donor cells.

For RNA extraction, each culture was mixed with 1 ml of RNAprotect Bacteria Reagent (Qiagen, Germany) and centrifuged at 5,000 × g for 10 min. The harvested cells were lysed in a buffer containing guanidine isothiocyanate and lysozyme. After lysis, binding conditions were adjusted by adding ethanol. The mixture was then loaded into a RNeasy mini spin column for RNA extraction. For cDNA synthesis, the RNA extract was mixed with a master mixture containing Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix and RNase-free water at 42°C for 15 min, immediately incubated at 95°C for 3 min to inactivate the enzyme, amplified with the PCR mixture (20 μl) containing 2X QuantiTect SYBR Green PCR Master (10 μl), 60 pmol primer (0.6 μl), cDNA (2 μl), and RNase-free water (6.8 μl) using an iCycler iQ™ System (Bio-Rad Laboratories, UK), and then denatured sequentially for 15 min at 95°C, 45 cycles at 94°C for 15 sec, 59°C for 20 sec, and 72°C for 15 sec. The primer sequences used for the analysis of conjugation regulation were as follows: *finP* forward 5′-TTCTCACGATGC GTCGGACACAT-3′, reverse 5′-TAAATCGCCGATACAGG GAG-3′; *sdhA* forward 5′-TGGCTACAGGTAGATTCACC-3′, reverse 5′-CACTTCTATTGCCTGATGGC-3′; *traJ* forward 5′-TCAGCCTCTTTCGGGAGATAGT-3′, reverse 5′-A GCGACTGACATTCAAGTTCCA-3′; *traY* forward 5′-GA GGGATCATCTGAAACGATATCC-3′, reverse 5′-AATGT GGACTCTGTTTCTTCAATTACCT-3′ (Serna *et al.*, 2010). The relative expression of targeted genes was estimated according to the comparative C_t method (Livak and Schmittgen, 2001).

All experiments were carried out in three replicates. The Statistical Analysis System software (SAS) was used to analyze the significant difference using a Fisher's least significant difference (LSD) procedure at $P<0.05$.

The MICs of antibiotics and bile salts against pathogenic and probiotic strains were determined as shown in Table 1. The *S.* Typhimurium CCARM 8099 donor strain exhibited higher resistance to AP, KM, SM, and TC than *S.* Typhimurium KCCM 40253. The resistance of *E. coli* KACC 10115 to AP and KM was noticeably enhanced after transconjugation, showing the MICs of more than 256 μg/ml (Table 1). Based on the results above, *S.* Typhimurium KCCM 40253, *S.* Typhimurium CCARM 8009, and transconjugant from *E. coli* KACC 10115 were assigned to antibiotic-susceptible *S.* Typhimurium (*S.* Typhimurium^{S}), multiple antibiotic-resistant *S.* Typhimurium (*S.* Typhimurium^{R}), and *E. coli* transconjugant (*E. coli*^{TC}), respectively. The resistance of *E. coli* KACC 10115 to AP and KM was increased to more than 64-fold after transconjugation. Compared to *S.* Typhimu-

rium, the NA resistance of *E. coli* was increased to 16-fold. The probiotic strains were intrinsically more sensitive to AP, EM, and TC and resistant to AT, CX, CZ, KM, NA, NF, SM, and SZ than pathogenic bacteria (Table 1). The observation that *S.* Typhimurium and *E. coli* were highly resistant to BS when compared to probiotic strains is in a good agreement with the previous report that Gram-negative strains had relatively higher resistance to BS than Gram-positive bacteria (Prouty *et al.*, 2004). In this study, the effect of BS on the horizontal gene transfer (HGT) was conducted at the sub-MICs (0 to 640 μg/ml) to avoid BS inhibitory activity against pathogenic and probiotic strains. Antibiotics, AT (1 μg/ml) and NA (8 μg/ml), were used as count selection markers to eliminate the donor cells.

The plasmid-mediated antibiotic resistance genes, virulence genes, and class 1 integrons were determined in *S.* Typhimurium^{R}, *E. coli* KACC 10115, and *E. coli*^{TC}. The presence of targeted genes was determined by PCR amplification. The antibiotic resistance genes (*aadA*, *aphA1-lab*, bla_{TEM}, and *tetA*) were detected in *S.* Typhimurium^{R} and *E. coli*^{TC} with the exception of the *tetA* gene which was absent in *E. coli*^{TC}. The *aadA*, *aphA1-lab*, bla_{TEM}, and *tetA* genes conferred resistance to SM, KM, AP, and TC, respectively. No antibiotic resistance genes, virulence genes, or class 1 integron were observed in *E. coli* KACC 10115. The plasmid-mediated virulence genes (*spvA* and *spvC*) were detected only in *S.* Typhimurium^{R}. The class 1 integron was detected in *S.* Typhimurium^{R} and *E. coli*^{TC}, while it was not detected in *E. coli* KACC 10115. The genes, bla_{TEM}, *aphA1-lab*, *aadA*, and *tetA*, responsible for AP, KM, SM, and TC resistance, respectively, were detected in the plasmids of *S.* Typhimurium^{R}, and bla_{TEM}, *aphA1-lab*, and *aadA* were also carried by *E. coli*^{TC}, corresponding to the antibiotic resistance profiles. Multiple antibiotic-resistant *Salmonella* strains commonly carry genes, including bla_{TEM}, *aphA1-lab*, *aadA*, *tetA*, *strAB*, and *grm* (Guerra *et al.*, 2002). Antibiotic resistance gene cassettes, class 1 integrons, are mobile genetic elements and the predominant type of integron identified in multiple antibiotic-resistant *Salmonella* spp. (Hsu *et al.*, 2006; Molla *et al.*, 2007), which are distributed with plasmid-mediated virulence factors (Villa and Carattoli, 2005). The class 1 integrons in *S.* Typhimurium^{R} were transferred to *E. coli* KACC 10115, whiles the virulence-associated genes (*spvA* and *spvC*) and antibiotic resistance gen (*tetA*) were not carried by *E. coli*^{TC}. This implies that the *spvA*, *spvC*, and *tetA* genes might be located in other chromosomal and plasmid regions.

The transfer frequency of conjugative plasmids was determined during broth and filter mating. The highest conjugative transfer frequency was 3.8×10^{-3} and 2.73×10^{-4} transferrants/recipients during the filter mating and broth mating in the presence of bile salts (160 μg/ml), respectively, followed by 6.37×10^{-4} at the filter mating in the absence of bile salts. The transfer rates were decreased with increasing bile salt concentration. Relatively low conjugative transfer frequencies ($<10^{-7}$) were observed in recipient strains of *L. brevis*, *L. casei*, *L. paracasei*, and *L. rhamnosus* GG. The conjugative transfer of antibiotic resistance genes has been more extensively studied between and within pathogenic bacteria rather than between pathogenic bacteria and probiotic strains (Grohmann *et al.*, 2003). In this study, the

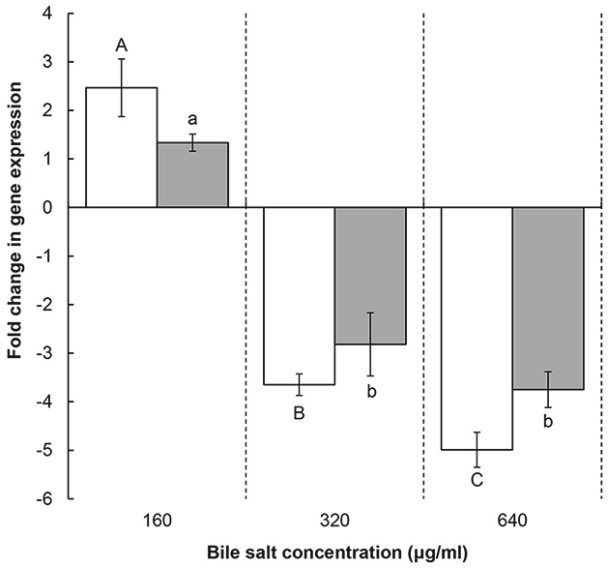

Fig. 1. Relative expression of *traJ* (□) and *traY* (■) in *S.* Typhimurium^{R} exposed to different bile salt concentrations. Different letters on the bars within *traJ* (A–C) and *traY* (a-b) are significantly different at $P<0.05$.

conjugative transfer of antibiotic resistance genes successfully occurred from *S.* Typhimurium^{R} to *E. coli* KACC 10115, while comparatively low conjugation frequencies were observed between *S.* Typhimurium^{R} and probiotic strains. This observation confirms that the conjugal gene transfer did not frequently occur between phylogenetically distant bacteria, specifically between Gram-positive bacteria and Gram-negative bacteria (Mazodier and Davies, 1991). The result that the conjugative transfer frequencies were higher in the filter mating method than the broth mating method are in a good agreement with the previous report that the conjugal interaction between donor and recipient cells was efficient in the solid phase (Romero and Mckay, 1985; West and Warner, 1985). In accordance with the previous reports (Garcia-Quintanilla *et al.*, 2008), the inhibitory effect of bile salts on HGT was increased with increasing bile salt concentration.

The expression patterns of *traJ* and *traY* in *S.* Typhimurium^{R} were investigated in the presence of bile salts (Fig. 1). The *traJ* and *traY* genes were overexpressed in *S.* Typhimurium^{R} exposed to a bile salt concentration of 160 μg/ml, showing a 2.5- and 1.3-fold increase in the relative expression levels of *traJ* and *traY* genes, respectively. The *traJ* and *traY* genes were suppressed in *S.* Typhimurium^{R} exposed to bile salt concentrations of 320 and 640 μg/ml. The relative expression levels of *traJ* and *traY* genes were 3.7- and 2.8-fold at 320 μg/ml bile salts, respectively, and 5.0- and 3.8-fold at 640 μg/ml. TraJ and TraY act as regulators of bacterial conjugation, which can positively activate the transcription of the *tra* operon (Frost and Koraimann, 2010). The expression of *traJ* and *traY* genes was activated at low concentration of bile salts (160 μg/ml) and then significantly decreased with increasing bile salt concentration (≥320 μg/ml). The expression patterns of *traJ* and *traY* genes were highly corresponded with the conjugative transfer frequencies between *S.* Typhimurium^{R} and *E. coli* KACC 10115,

suggesting that TraJ and TraY might be essential for the activation of bacterial conjugation. SdhCDAB and FinOP are known as negative regulators of bacterial conjugation (Koraimann *et al.*, 1996; Serna *et al.*, 2010). The expression of *finP* and *sdhA* genes was not detected in *S.* Typhimurium^{R} exposed to bile salts. This observation further confirms that bile salts may play an important role in activating the expression of *traJ* and *traY* genes (Bernstein *et al.*, 1999; Kristoffersen *et al.*, 2007). However, it needs to be done to further verify that bacterial conjugation is associated with bile salts.

In conclusion, the conjugative transfer of antibiotic resistance genes in *S.* Typhimurium varied with recipient strain and bile salt concentration. The multiple antibiotic resistance genes were transferred through plasmids and class 1 integrons to *E. coli* in the presence of bile salt. The most important finding was that the conjugative transfer frequency could depend on bile salt, leading to the down-regulation of *traJ* and *traY* genes at high concentrations of bile salts. Although the conjugative transfer of antibiotic resistance genes from *S.* Typhimurium to probiotic strains (*L. brevis*, *L. casei*, *L. paracasei*, and *L. rhamnosus*) was not observed in this study, the HGT cannot be underestimated in the human digestive tract. Therefore, this study could shed light on the acquired antibiotic resistance mechanism between pathogenic bacteria and probiotic strains encountered in the gastrointestinal tract.

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