Food Sci Biotechnol (2018) 27(3):921–928
https://doi.org/10.1007/s10068-018-0317-1

Characterization of β-lactamase- and efflux pump-mediated multiple antibiotic resistance in *Salmonella* Typhimurium

Md. Jalal Uddin[1] · Juhee Ahn[1]

Received: 21 November 2017 / Revised: 4 January 2018 / Accepted: 9 January 2018 / Published online: 13 January 2018

Abstract This study aimed to assess the β-lactamase- and efflux pump-mediated antibiotic resistance in *Salmonella* Typhimurium (WT-ST), ciprofloxacin-induced antibiotic-resistant *S*. Typhimurium (CI-ST), and clinically-acquired antibiotic-resistant *S*. Typhimurium (CA-ST). The β-lactamase activities were significantly increased up to 63 μmol/min/mL in CA-ST and 24 μmol/min/mL in CI-ST when compared to WT-ST (13 μmol/min/mL). The highest efflux pump activity was observed in CI-ST and CA-ST, showing more than 45%. The antibiotic susceptibilities of WT-ST, CI-ST, and CA-ST were increased in the presence of β-lactamase and efflux pump inhibitors. CA-ST showed the highest activity in AcrD, MdtABC, EmrAB, MdtK, and MacAB efflux pumps. The repressed *ompF* were responsible for the decreased susceptibility of CA-ST to ampicillin (MIC > 512 μg/mL). This study would provide useful information for better understating of the development of multidrug resistance in association with β-lactamase and efflux pump activities and designing new antibiotic chemotherapy in combination with inhibitors.

Keywords β-Lactamase · Efflux pump · Multidrug resistance · *Salmonella* · Porin · Virulence

✉ Juhee Ahn
juheeahn@kangwon.ac.kr

[1] Department of Medical Biomaterials Engineering and Institute of Bioscience and Biotechnology, Kangwon National University, Chuncheon, Gangwon 24341, Republic of Korea

Introduction

In recent year, the increasing emergence of multidrug resistant (MDR) bacteria has become a serious public health problem due to the leading cause of morbidity and mortality [1]. The misuse and overuse of antibiotics has accelerated the spread of MDR bacteria, resulting in treatment failure of these infectious diseases [2]. *Salmonella* strains can cause acute gastrointestinal and diarrheal disease, which are highly resistant to different classes of antibiotics such as β-lactam, chloramphenicol, aminoglycoside, fluoroquinolone, and tetracycline [3]. The zoonotic and nosocomial outbreaks of MDR *Salmonella* strains occurred through contaminated foods and among hospitalized humans [4]. The mechanisms of antibiotic resistance evolved in bacteria mainly include enzymatic degradation, target-site modification, permeability alteration, and metabolic bypass [5]. The MDR *Salmonella* strains are induced by several mechanisms, the important one of which is the production of β-lactamases and the other is efflux pump activity [5].

The production of β-lactamases plays a major role in bacterial resistance by hydrolyzing β-lactam antibiotics [6]. The β-lactamases are classified into four classes based on the amino acid sequence; class A (TEM, SHV, adn CTX-M), class B (IMP, VIM, and SPM), class C (AmpC, MIR, ACT, FOX, and MOX), and class D (OXA) [7]. The production of extended-spectrum β-lactamases (ESBL) is also involved in the induction of MDR bacteria [8]. The efflux pumps, including nodulation-division (RND) family, the major facilitator superfamily (MFS), the ATP-binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family, are responsible for substrate recognition and transportation in Gram-negative bacteria

[9]. The MDR in *Salmonella* strains are mainly attributed to the AcrABDF, MdsABC, and MdtABC efflux pumps beloning to the RND family [9]. Since more than one mechanism is involved in the emergence of MDR *Salmonella* strains, understanding the interplay of antibiotic resistance mechanisms is essential to optimize therapeutic approaches against MDR bacterial infections. However, there still remains unclear about the relationship among the mechanisms of MDR bacterial resistance. Therefore, this study was designed to understand the antibiotic resistance mechanisms underlying β-lactamase production and efflux pump activity in *S. enterica* serovar Typhimurium in comparison with ciprofloxacin-induced antibiotic-resistant and clinically-acquired antibiotic-resistant *S.* Typhimurium strains.

Materials and methods

Bacterial strains and culture conditions

Strain of *S.* Typhimurium ATCC 19585 and CCARM 8009 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and Culture Collection of Antibiotic Resistant Microbes (CCARM, Seoul, Korea), respectively. Strain of ciprofloxacin-induced *S.* Typhimurium ATCC 19585 was obtained by a serial passage method [10], serially cultured in trypticase soy broth (TSB; Difco, Becton, Dickinson and Co., Sparks, MD, USA) with increasing concentrations of ciprofloxacin (0–2 μg/mL). All strains were cultured in TSB at 37 °C for 20 h and collected by a centrifugation at 3000×*g* for 20 min at 4 °C. *S.* Typhimurium ATCC 19585, ciprofloxacin-induced antibiotic-resistant *S.* Typhimurium ATCC 19585, and clinically-acquired antibiotic-resistant *S.* Typhimurium CCARM 8009 were assigned to WT-ST, CI-ST, and CA-ST, respectively.

β-Lactamase activity assay

The β-lactamase activity was evaluated by using a nitrocefin hydrolyzing assay with minor modifications [11]. WT-ST, CI-ST, and CA-ST cells cultured with and without 1/2 MIC ceftriaxone were centrifugated at 3000×*g* for 20 min at 4 °C. The cell-free supernatants were mixed with 10 μL of 1.5 mM nitrocefin and incubated at 37 °C for 30 min. The absorbance was recorded every 5 min at 515 nm using a microplate reader (BioTek Instruments, Inc., Norwood, MA, USA). According to the protocol of β-lactamase activity assay kit (ancam, Seoul, Korea), the standard curve was generated and then the β-lactamase activity was expressed as μmol/min/mL.

Efflux pump activity assay

The efflux pump activity of WT-ST, CI-ST, and CA-ST was determined by using a ethidium bromide (EtBr) accumulation method. All strains (0.5 McFarland standard each) were incubated in phosphate-buffered saline (PBS; pH 7.2) containing EtBr (2 μg/mL) for 1 h at 25 °C. The EtBr-loaded cells were suspended with 0.4% glucose in PBS. The fluorescence was monitored for 1 h using a RF-5301PC spectrofluoro photometer (Shimadzu, Kyoto, Japan) at emission and excitation wavelengths of 580 and 500 nm, respectively. The efflux of EtBr was expressed as relative residual fluorescence intensities.

Antimicrobial susceptibility assay

The antibiotic susceptibilities of WT-ST, CI-ST, and CA-ST were determined according to a broth dilution assay [12]. Antibiotic stock solutions were prepared to obtain a final concentration of 2048 μg/mL. The stock solutions (100 μL each) were serially (1:1) diluted in 96-well microtiter plates and inoculated with WT-ST, CI-ST, and CA-ST cells (10^5 cfu/mL in 100 mL) with and without β-lactamase inhibitors [BLI-489 (BL), clavulanate (CA), sulbactam (SB) or tazobactam (TB)], purchased from Sigma Chemicals (St. Louis, MO, USA), at 4 μg/mL that cell viability was not affected or efflux pump inhibitors [carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP, 0.5 μg/mL) or phenylalanine-arginine-β-naphthylamide (PAβN, 8 μg/mL)]. The prepared microtiter plates were incubated for 18 h at 37 °C. After incubation, the MICs were determined at the lowest antibiotic concentration at which no visible growths were observed.

Quantitative RT-PCR assay

RNAs were extracted from WT-ST, CI-ST, and CA-ST according to the protocol of RNeasy Protect Bacteria Mini kit (Qiagen, Hilden, Germany). In brief, the cells (0.5 mL) were mixed with 1 mL of RNAprotect Bacteria Reagent and centrifuged at 5000×*g* for 10 min. The collected cells were lysed with a buffer containing lysozyme and then mixed with ethanol to extract RNA using an RNeasy mini column. The mixtures were rinsed with a Wipe buffer to remove the genomic DNA. The extracted RNA was mixed with a master mixture of reverse transcriptase, RT buffer, and RT primer mix and then incubated at 42 °C for 15 min followed by 95 °C for 3 min. The synthesized cDNA was used as a template for quantitative RT-PCR assay. The reaction mixture (20 μL) containing 10 μL of 2 × QuantiTect SYBR Green PCR Master, 2 μL of each primer, and 2 μL of cDNA, and 4 μL of RNase-free water was amplified using an iCycler iQ™ system (Bio-Rad

Laboratories, Hemel Hempstead, UK). The custom-synthesized oligonucleotides using IDT (Integrated DNA Technologies Inc., Coralville, IA, USA) as primers of *S*. Typhimurium are listed in Table 2. The PCR mixture was denatured at 95 °C for 30 s, followed by 45 cycles of 95 °C for 5 s, 55 °C for 20 s, and 72 °C for 15 s using an iCycler iQ^{TM} system (Bio-Rad Laboratories, Hemel Hempstead, UK). The relative gene expression levels were estimated using the comparative method [13]. The C_T values of target genes in CI-ST, and CA-ST were compared to the C_T values obtained from the WT-ST.

Results and discussion

Mechanism-mediated antibiotic resistance phenotypes in WT-ST, CI-ST, and CA-ST

The β-lactamase and efflux pump activities were evaluated in *S*. Typhimurium ATCC 19585 (WT-ST), ciprofloxacin-induced antibiotic-resistant *S*. Typhimurium ATCC 19585 (CI-ST), and clinically-acquired antibiotic-resistant *S*. Typhimurium CCARM 8009 (CA-ST) exposed to 1/2 MIC ceftriaxone and loaded with EtBr, respectively (Fig. 1). The constitutive β-lactamase activity was observed in WT-ST, while the inducible β-lactamase activity was observed in CI-ST and CA-ST. The β-lactamase activities were 11, 13, and 26 μmol/min/mL for WT-ST, CI-ST, and CA-ST in the absence of ceftriaxone (data not shown). The highest β-lactamase activity was observed

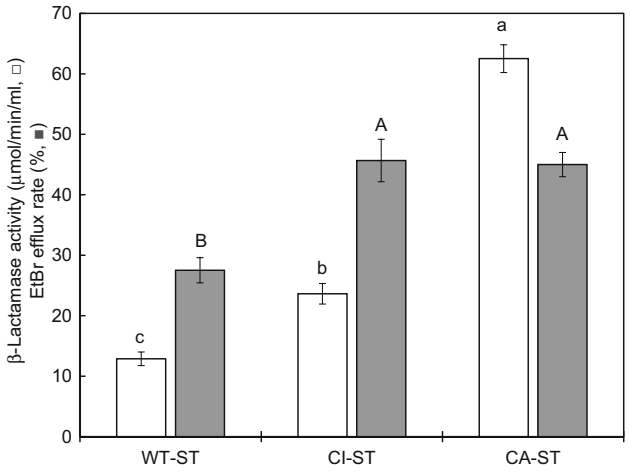

Fig. 1 Hydrolyzing activity of β-lactamases (open square) and efflux pump activity (filled square) produced by *S*. Typhimurium ATCC 19585 (WT-ST), ciprofloxacin-induced antibiotic-resistant *S*. Typhimurium ATCC 19585 (CI-ST), and clinically-acquired antibiotic-resistant *S*. Typhimurium CCARM 8009 (CA-ST). The different letters (a–c, A, B) on the bars indicate significant differences within β-lactamase production (open square) and efflux rate (filled square) at $p < 0.05$, respectively

in CA-ST (63 μmol/min/mL), followed by CI-ST (24 μmol/min/mL) when exposed to ceftriaxone. The ceftriaxone exposure can induce the production of β-lactamases in CI-ST and CA-ST strains. Furthermore, the production of β-lactamases results in the emergence of MDR by causing selective pressure on bacteria [14]. The noticeable increase in efflux pump activity was observed in CI-ST and CA-ST, showing EtBr efflux rates of 46 and 45%, respectively (Fig. 1). The MDR are attributed to the multidrug efflux pumps in bacteria by decreasing the intracellular accumulation of antibiotics [15].

The MICs of different classes of antibiotics against WT-ST, CI-ST, and CA-ST were determined in the absence and presence of β-lactamase inhibitors [BLI-489 (BL), clavulanate (CA), sulbactam (SB) or tazobactam (TB)] and efflux pump inhibitors [carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) or phenylalanine-arginine-β-naphthylamide (PaβN)] as shown in Table 1. According to the MIC breakpoints [12], the antibiotic susceptibilities of WT-ST, CI-ST, and CA-ST were assigned as antibiotic-sensitive (S), intermediate (I), and resistant (R). WT-ST was relatively more susceptible to all antibiotics than CI-ST and CA-ST (Table 1). After serial exposure to ciprofloxacin (CI-ST), WT-ST exhibited more than 2-fold decrease in susceptibility to all antibiotic used in this study (Table 1). The susceptibility of CI-ST to cefoxitin, ceftriaxone, chloramphenicol, meropenem, ciprofloxacin, and levofloxacin was decreased to 4-, 8-, 4-, 8-, 128-, and 32-fold when compared to WT-ST. CI-ST was resistant to cefoxitin (8 μg/mL), ciprofloxacin (2 μg/mL), and tobramycin (8 μg/mL), and CA-ST was highly resistant to ampicillin (> 512 μg/mL). The results imply that the ciprofloxacin selective pressure induced MDR CI-ST in association with efflux pumps [16].

The susceptibilities of WT-ST, CI-ST, and CA-ST varied in the presence of β-lactamase inhibitors (BL, CA, SB, and TB) and efflux pump inhibitors (CCCP and PAβN) (Table 1). All β-lactamase inhibitors were ineffective for chloramphenicol, showing no changes in susceptibility of WT-ST, CI-ST, and CA-ST, while those were effective for tobramycin in WT-ST. BL and SB were most effective for ampicillin and ceftriaxone in WT-ST, whereas CA was not effective for ampicillin and ceftriaxone, respectively, in CI-ST and CA-ST. The hydrolysis of ampicillin was not inhibited by CA (Table 1). Thus, the resistance of CI-ST to ampicillin may not be attributed to the production of TEM-1 and AmpC β-lactamases because these β-lactamases are resistant to CA [14]. TB was not effective for cefoxitin, ceftriaxone, chloramphenicol, meropenem, tetracycline, and tobramycin in CA-ST but effective for β-lactams in WT-ST and CI-ST. This indicates that WT-ST and CI-ST can produce SHV and CTX-M type β-lactamases, which are inactivated by TB [17].

Table 1 Minimum inhibitory concentrations (MICs) of *S.* Typhimurium ATCC 19585 (WT-ST), ciprofloxacin-induced antibiotic-resistant *S.* Typhimurium ATCC 19585 (CI-ST), and clinically-acquired antibiotic-resistant *S.* Typhimurium CCARM 8009 (CA-ST) in absence and presence of β-lactamase inhibitors [BLI-489 (BL), clavulanate (CA), sulbactam (SB) or tazobactam (TB)] and efflux pump inhibitors [carbonyl cyanide-m-chlorophenyl hydrazine (CCCP) and phenylalanine-arginine-β-naphthylamide (PaβN)]

Strain	Antibiotic	Control	β-Lactamase inhibitor				Efflux pump inhibitor	
			BL	CA	SB	TB	CCCP	PAβN
WT-ST	Ampicillin	4(S)	1(S)	2(S)	1(S)	2(S)	4(S)	4(S)
	Cefoxitin	2(S)	1(S)	1(S)	0.5(S)	1(S)	2(S)	2(S)
	Ceftriaxone	0.063(S)	0.016(S)	0.031(S)	0.016(S)	0.016(S)	0.063(S)	0.063(S)
	Chloramphenicol	2(S)	2(S)	2(S)	2(S)	2(S)	0.5(S)	0.5(S)
	Ciprofloxacin	0.016(S)	0.008(S)	0.008(S)	0.008(S)	0.008(S)	0.008(S)	0.008(S)
	Levofloxacin	0.016(S)	0.008(S)	0.004(S)	0.008(S)	0.008(S)	0.004(S)	0.008(S)
	Meropenem	0.031(S)	0.016(S)	0.031(S)	0.008(S)	0.008(S)	0.016(S)	0.016(S)
	Tetracyline	1(S)	1(S)	0.5(S)	0.5(S)	0.5(S)	0.25(S)	0.5(S)
	Tobramycin	4(I)	0.5(S)	0.5(S)	0.5(S)	0.5(S)	2(S)	4(I)
CI-ST	Ampicillin	8(S)	4(S)	8(S)	2(S)	4(S)	8(S)	8(S)
	Cefoxitin	8(R)	4(I)	4(I)	4(I)	4(I)	4(I)	4(I)
	Ceftriaxone	0.5(S)	0.125(S)	0.063(S)	0.25(S)	0.125(S)	0.5(S)	0.5(S)
	Chloramphenicol	8(I)	8(I)	8(I)	8(I)	8(I)	4(S)	4(S)
	Ciprofloxacin	2(R)	0.25(S)	0.5(S)	0.25(S)	0.5(S)	0.5(S)	0.5(S)
	Levofloxacin	0.5(S)	0.125(S)	0.25(S)	0.25(S)	0.25(S)	0.125(S)	0.25(S)
	Meropenem	0.25(S)	0.125(S)	0.063(S)	0.25(S)	0.125(S)	0.125(S)	0.125(S)
	Tetracyline	2(I)	1(S)	2(I)	1(S)	1(S)	1(S)	2(I)
	Tobramycin	8(R)	4(I)	8(R)	8(R)	4(I)	4(I)	8(R)
CA-ST	Ampicillin	> 512(R)	128(R)	128(R)	64(R)	128(R)	> 512(R)	> 512(R)
	Cefoxitin	8(R)	2(S)	4(I)	4(I)	8(R)	4(I)	4(I)
	Ceftriaxone	0.125(S)	0.063(S)	0.125(S)	0.125(S)	0.125(S)	0.125(S)	0.125(S)
	Chloramphenicol	2(S)	2(S)	2(S)	2(S)	2(S)	2(S)	2(S)
	Ciprofloxacin	0.031(S)	0.008(S)	0.008(S)	0.016(S)	0.008(S)	0.016(S)	0.016(S)
	Levofloxacin	0.063(S)	0.008(S)	0.031(S)	0.016(S)	0.016(S)	0.016(S)	0.016(S)
	Meropenem	0.031(S)	0.016(S)	0.031(S)	0.016(S)	0.031(S)	0.016(S)	0.016(S)
	Tetracyline	2(I)	1(S)	2(I)	1(S)	2(I)	1(S)	2(I)
	Tobramycin	4(I)	2(S)	2(S)	2(S)	4(I)	2(S)	4(I)

S, I, and R indicate the antibiotic-sensitive, intermediate, and resistant strains, respectively

The susceptibilities of WT-ST to β-lactams (ampicillin, cefoxitin, and ceftriaxone) were not changed in the presence of efflux pump inhibitors (Table 1). CCCP and PAβN were effective for ciprofloxacin, levofloxacin, and meropenem in all WT-ST, CI-ST, and CA-ST strains. CCCP was effective for tetracycline and tobramycin in CI-ST and CA-ST, while PAβN was not effective. The fluoroquinolone susceptibilities of WT-ST, CI-ST, and CA-ST were increased in the presence of β-lactamase inhibitors and efflux pump inhibitors. The hydrolysis of β-lactams was inhibited in WT-ST by β-lactamase inhibitors (BL, CA, SB, and TB), while that was not completely inhibited in CI-ST and CA-ST. The efflux pump systems in WT-ST, CI-ST, and CA-ST were not effectively inhibited by efflux pump inhibitors (CCCP and PAβN). The efflux-mediated MDR is induced through the proton motive force-driven and substrate competitive extrusions, which can be disrupted by CCCP and PAβN, respectively [18, 19].

The substrate profiles varied with *Salmonella* efflux pumps. The overlapped substrate profiles were observed in this study. As the results of Table 2, WT-ST exhibited the highest activity in AcrAB, AcrEF, and MdfA efflux pumps, CI-ST was active in AcrAB, AcrEF, EmrAB, MdfA, and MdtK, and CA-ST has AcrD, MdtABC, EmrAB, and MdtK efflux pumps. The efflux pump inhibitors used in this study did not completely inactivate the different families of efflux pumps. This explains the reason why the susceptibilities of all strains to antibiotics were not increased in the presence of CCCP and PAβN [20]. Furthermore, the intrinsic resistance of WT-ST, CI-ST, and CA-ST to

Table 2 Primer sequences used in this study and relative gene expression in ciprofloxacin-induced antibiotic-resistant *S*. Typhimurium ATCC 19585 (CI-ST) and clinically-acquired antibiotic-resistant *S*. Typhimurium CCARM 8009 (CA-ST)

Type	Gene	Annotation	Primer	Fold change	
				CI-ST	CA-ST
RND (AcrAB)	*acrA*	Periplasmic adaptor protein	F: AAAACGGCAAAGCGAAGGT	2.39	2.19
			R: GTACCGGACTGCGGGAATT		
	acrB	Multidrug transporter	F: TGAAAAAAATGGAACCGTTCTTC	1.97*	1.19
			R: CGAACGGCGTGGTGTCA		
	marA	Transcriptional activator	F: CGCAACACTGACGCTATTAC	1.44*	0.45
			R: TTCAGCGGCAGCATATAC		
	ramA	Transcriptional activator	F: CCAGAAGGTGTATGATATTTGTCTCAAG	2.25	1.14
			R: GGTTGAACGTGCGGGTAAA		
	soxS	Transcriptional activator	F: CATATCGACCAACCGCTA	0.92	2.23
			R: CGGAATACACGCGAGAAG		
	tolC	Outer membrane channel	F: GCCCGTGCGCAATATGAT	3.39	2.83
			R: CCGCGTTATCCAGGTTGTTG		
RND (AcrD)	*acrD*	Aminoglycoside	F: CCTTTGTCAAAGCCTCGATTATC	1.49	2.20
			R: CAGGAACAGATACATCACCAG		
RND (AcrEF)	*acrF*	Aminoglycoside	F: GTTCCTGTCGTGTTGCTA	2.55	3.66
			R: CAAACATCGTCAGGGTATTG		
RND (MdsABC)	*mdsA*	Multidrug transporter	F: TGCTAAAGCCCTTAGCCGTACA	3.87	5.28
			R: GCGCGGCCAGAAAACC		
	mdsB	Multidrug transporter	F: CGATATGTTGATGGTGGTT	2.71	3.50
			R: GATGGCGAAGTTAGACAG		
	mdsC	Multidrug transporter	F: TCGTAACGCGCTGGAATTG	3.70	4.67
			R: TTTAGTCGACGCGACAGTTCA		
RND (MdtABC)	*mdtB*	Multidrug transporter	F: TATCGGCTATCGCTTCCT	1.99	2.90
			R: TAGAGCGTAACAACCTGAAT		
	mdtC	Multidrug transporter	F: GAGGTAGAAGAGACACTGGCTATCTCT	3.68	4.59
			R: CGGAGCGCAGGAATAAAAAC		
MFS (EmrAB)	*emrA*	Multidrug transporter	F: GCGCAAAGCGACCTTAACC	0.79	0.81
			R: CTTCCCGGCCAATAAGATTG		
	emrB	Multidrug transporter	F: TCTGGTCAATGACCGTCATTG	1.33	2.31
			R: TGTAGCCCCCCAGAATCG		
MFS (MdfA)	*mdfA*	Multidrug transporter	F: TTTGAGGAGGCGGTGTGTATAA	0.71	1.17
			R: AGCGGCGCGATTAACG		
MATE (MdtK)	*mdtK*	Multidrug transporter	F: TTTGTTTGGTCACGGCTTACTG	1.05	2.62*
			R: GCCGGAGCCATTGAGTTG		
ABC (MacAB)	*macA*	Regulator	F: CGCGCCAGCAGCAGTTA	0.33	− 0.13
			R: CGCCGCGGTATCCAGAT		
	macB	Regulator	F: ACAGCAGCAGCGTGTCAGTATT	− 0.17	− 0.58
			R: TCGGCTCATCTGCCAGAATC		
Porin	*ompC*	Outer membrane protein	F: TCGCAGCCTGCTGAACCAGAAC	1.95	2.42*
			R: ACGGGTTGCGTTATAGGTCTGAG		
	ompD	Outer membrane protein	F: GCAACCGTACTGAAAGCCAGGG	4.29	5.06
			R: GCCAAAGAAGTCAGTGTTACGGT		
	ompF	Outer membrane protein	F: CGGAATTTATTGACGGCAGT	3.99*	− 1.17
			R: GAGATAAAAAAACAGGACCG		
Resistance	*cat*	Chloramphenicol acyltransferase	F: GCAAGATGTGGCGTGTTAC	3.58*	1.38
			R: GGGGGCGAAGAAGTTGTC		

Table 2 continued

Type	Gene	Annotation	Primer	Fold change	
				CI-ST	CA-ST
β-Lactamase	*blaTEM*	β-Lactamase	F: ATGAGTATTCAACATTTCCG R: CCAATGCTTAATCAGTGAGG	3.54	7.03*
Virulence	*invH*	Adherence/invasion	F: CCCTTCCTCCGTGAGCAAA R: TGGCCAGTTGCTCTTTCTGA	2.49	3.92*
	orf245	SPI-2/PhoP	F: CAGGGTAATATCGATGTGGACTACA R: GCGGTATGTGGAAAACGAGTTT	2.51	3.80
	pipB	Type III secreted effector	F: GCTCCTGTTAATGATTTCGCTAAAG R: GCTCAGACTTAACTGACACCAAACTAA	1.74	2.85
	sipA	SPI-1	F: CAGGGAACGGTGTGGAGGTA R: AGACGTTTTTGGGTGTGATACGT	2.29	4.36*
	ssaB	SPI-1/SPI-2	F: ATTCAGG ATATCAGGGCCGAAGGT R: GTGCTGCAAGCAGTAGTGTCACAT	5.80	5.41

*Indicates the significant difference at $p < 0.05$

antibiotics could cause the decrease in susceptibility regardless of efflux pump activity [21].

Differential expression of mechanism-related genes in WT-ST, CI-ST, and CA-ST

The relative expression of efflux pump-, porin-, and resistance-, β-lactamase-, and virulence-related genes were observed in CI-ST and CA-ST in comparison with WT-ST as control strain (Table 2). The relative expression levels of all genes were increased in both CI-ST and CA-ST with the exception of CI-ST (*macB*) and CA-ST (*macA*, *macB*, and *ompF*). The genes, *mdsA*, *mdsC*, *mdtC*, *ompD*, bla_{TEM}, and *ssaB*, were overexpressed by more than threefold in both CI-ST and CA-ST. The results of β-lactamase and efflux pump activities corresponded well with overall antibiotic susceptibility and relative gene expression in WT-ST, CI-ST, and CA-ST.

The transcriptional levels of *acrAB*, *tolC*, and *ompF* were regulated by *marA*, *soxS*, and *ramA* as transcriptional activators belonging to the AraC/XylS family [20, 22]. The overexpression of *marA* (0.45-fold) and *ramA* (1.14-fold) in CA-ST was in good agreement with the suppression of *ompF* (Table 2). The overexpression of *marA*, *soxS*, and *ramA* contributes to the increased efflux activity of AcrAB-TolC and reduction of OmpF, resulting in MDR [20]. The AcrB and TolC are required not only to induce MDR but also to produce virulence factors in *S.* Typhimurium [23]. The substrates of tripartite multidrug efflux system AcrAB-TolC include β-lactams, chloramphenicols, macrolides, novobiocin, tetracyclines, sulfonamides, and trimethoprim [22]. The substrates of AcrAD efflux pump are aminoglycosides (kanamycin and streptomycin) [24]. The *acrB*, *acrF*, and *acrD* have common transcriptional regulators, *marA* and *soxS* [15]. MdsAB acts as multidrug transporter of *Salmonella* with either MdsC or TolC [25]. MdtB functions together with MdtA and TolC, leading to MDR *S.* Typhimurium to β-lactam and novobiocin [26]. The substrate of EmrAB efflux pump is nalidixic acid, which functions with TolC [25]. The expression of *mdfA* contributes to the enhanced resistance to tetracycline, chloramphenicol, norfloxacin, and doxorubicin [25]. The *mdtK* gene confers to resistance to norfloxacin [25]. The transcription of *macAB* is suppressed by two-component regulatory system PhoP/PhoQ which regulates *Salmonella* virulence [25].

The altered porin channels function as a permeable barrier, which can influence on bacterial susceptibility to antibiotics [27]. The reduced permeability of porin channels plays an important role in MDR bacteria by decreasing the influx of different types of antibiotics such as β-lactams, chloramphenicol, fluoroquinolones, and tetracycline [27, 28]. CA-ST was highly resistant to ampicillin (> 512 μg/mL) which is blocked by the OmpF porin in its zwitterionic form [29]. OmpF and OmpC are mainly responsible for the porin-mediated antibiotic resistance by regulating the permeability to hydrophilic antibiotics, while the two-component regulatory systems (PhoP/PhoQ and PmrA/PmrB) are involved in the permeability to hydrophobic antibiotics, including aminoglycosides, macrolides, and cationic peptides, through lipid bilayers in *S.* Typhimurium [29]. The increased resistance of CI-ST and CA-ST to ciprofloxacin, levofloxacin, and tetracycline was due to the porin- and lipid-mediated permeability barriers [27]. The overexpression of *ompD* in CI-ST (> fourfold increase) and CA-ST (> fivefold increase) is

related to the increased efflux activity responsible for conferring MDR [30].

The chloramphenicol acetyltransferase (*cat*) encodes the resistance to chloramphenicol [31]. The *cat* gene was strongly overexpressed by 3.6-fold and 1.4-fold, respectively, in CI-ST and CA-ST, confirming more chloramphenicol resistance in CI-ST than CA-ST. CA were least effective for ampicillin in CI-ST and ceftriaxone in CA-ST (Table 1). The expression levels of bla_{TEM} in CI-ST and CA-ST were increased by 3.5-fold and sevenfold, respectively (Table 2). This is in good agreement with the report that TEM-type β-lactamases were resistant to CA [14]. The antibiotic activity of ampicillin was increased by adding β-lactamase inhibitors, which can inhibit plasmid-encoded TEM-1 and TEM-2 penicillinases produced by CA-ST. The *Salmonella* Type III secreted effector-related genes (*orf245*, *pipB*, *sipA*, and *ssaB*) and invasion-related gene (*invH*) were overexpressed in CI-ST and CA-ST, which are essential for bacterial virulence and survival [32]. The expression of virulence-related genes is directly associated with the expression of multidrug efflux pump-related genes. The RND family is specific to various antibiotics, showing diverse substrate profiles [33]. These results suggest that efflux pump-related genes are required for not only antibiotic resistance but also bacterial virulence [25]. The efflux pump-related genes are regulated by transcriptional regulators, local repressors, and global response regulators [9]. The various types of efflux pumps are interplayed to avoid redundant expression and wasteful metabolism. The antibiotics in combination with β-lactamase inhibitors and efflux pump inhibitors can be used to give synergistic effects [34, 35].

In conclusion, this study describes the MDR mechanisms underlying β-lactamase production and efflux pump activity in WT-ST, CI-ST, and CA-ST. The antibiotic susceptibilities of WT-ST, CI-ST, and CA-ST varied depending on the types of *β*-lactamase and efflux pump inhibitors. The SHV and CTX-M type β-lactamases could be produced by WT-ST and CI-ST, while the resistance of CI-ST might not be attributed to the TEM-1 and AmpC β-lactamases. It is worth noting that the multidrug efflux pumps conferred resistance to specific antibiotics as substrates. The various types of multidrug efflux pump systems were responsible for the MDR in CI-ST (AcrAB, AcrEF, EmrAB, MdfA, and MdtK) and CA-ST (AcrD, MdtABC, EmrAB, and MdtK). The altered porin channels resulted in the reduced permeability that can be used as barrier to antibiotics. The results obtained in this study can help us understand the β-lactamase production and efflux pump activity with regard to multiple antibiotic resistance and bacterial virulence. However, further study is needed to better understand the interplay between the phenotypic resistance and genotypic resistance in association with various antibiotic resistance mechanisms in *S.* Typhimurium.

Acknowledgement This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2016R1D1A3B01008304).

References

1. Eng S-K, Pusparajah P, Ab Mutalib N-S, Ser H-L, Chan K-G, Lee L-H. *Salmonella*: a review on pathogenesis, epidemiology and antibiotic resistance. Front. Life Sci. 8: 284–293 (2015)
2. Currie CJ, Berni E, Jenkins-Jones S, Poole CD, Ouwens M, Driessen S, de Voogd H, Butler CC, Morgan CL. Antibiotic treatment failure in four common infections in UK primary care 1991–2012: longitudinal analysis. Br. Med. J. 349: g5493 (2014)
3. Mather AE, Reid SW, Maskell DJ, Parkhill J, Fookes MC, Harris SR, Brown DJ, Coia JE, Mulvey MR, Gilmour MW, Petrovska L, de Pinna E, Kuroda M, Akiba M, Izumiya H, Connor TR, Suchard MA, Lemey P, Mellor DJ, Haydon DT, Thomson NR. Distinguishable epidemics of multidrug-resistant *Salmonella* Typhimurium DT104 in different hosts. Science 341: 1514–1517 (2013)
4. Smith AM, Mthanti MA, Haumann C, Tyalisi N, Boon GPG, Sooka A, Keddy KH. Nosocomial outbreak of *Salmonella enterica* serovar Typhimurium primarily affecting a pediatric ward in South Africa in 2012. J. Clin. Microbiol. 52: 627–631 (2014)
5. Miró E, Vergés C, García I, Mirelis B, Navarro F, Coll P, Prats G, Martínez-Martínez L. Resistance to quinolones and β-lactams in *Salmonella enterica* due to mutations in topoisomerase-encoding genes, altered cell permeability and expression of an active efflux system. Enferm. Infect. Micribiol. Clin. 33: 204–211 (2015)
6. Lakshmi R, Nusrin KS, Ann GS, Sreelakshmi KS. Role of beta-lactamases in antibiotic resistance. Int. Res. J. Pharm. 5: 37–40 (2014)
7. Barry GH, Barlow M. Revised Ambler classification of β-lactamases. J. Antimicrob. Chemother. 55: 1050–1051 (2005)
8. Shaikh S, Fatima J, Shakil S, Rizvi SMD, Kamal MA. Antibiotic resistance and extended spectrum beta-lactamases: types, epidemiology and treatment. Saudi J. Biol. Sci. 22: 90–101 (2015)
9. Sun J, Deng Z, Yan A. Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. Biochem. Biophys. Res. Commun. 453: 254–267 (2014)
10. Michéa-Hamzehpour M, Kahr A, Pechère JC. In vitro stepwise selection of resistance to quinolones, β-lactams and amikacin in nosocomial gram-negative *bacilli*. Infection 22: S105–S110 (1994)
11. Sharma S, Ramnani P, Virdi JS. Detection and assay of β-lactamases in clinical and non-clinical strains of *Yersinia enterocolitica* biovar 1A. J. Antimicrob. Chemother. 54: 401–405 (2004)
12. CLSI. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard-Tenth Edition. CLSI document M07-10. Wayne, PA. Clinical and Laboratory Standards Institute (2015)
13. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Method. 25: 402–408 (2001)
14. Féria C, Ferreira E, Correia JD, Gonçalves J, Caniça M. Patterns and mechanisms of resistance to β-lactams and β-lactamase

inhibitors in uropathogenic *Escherichia coli* isolated from dogs in Portugal. J. Antimicrob. Chemother. 49: 77–85 (2002)
15. Nikaido E, Yamaguchi A, Nishino K. AcrAB multidrug efflux pump regulation in *Salmonella enterica* serovar Typhimurium by RamA in response to environmental signals. J. Biol. Chem. 283: 24245–24253 (2008)
16. Kang H-W, Woo G-J. Increase of multidrug efflux pump expression in fluoroquinolone-resistant *Salmonella* mutants induced by ciprofloxacin selective pressure. Res. Vet. Sci. 97: 182–186 (2014)
17. Manoharan A, Premalatha K, Chatterjee S, Mathai D. Correlation of TEM, SHV and CTX-M extended-spectrum beta lactamases among enterobacteriaceae with their in vitro antimicrobial susceptibility. Indian J. Med. Microbiol. 29: 161–164 (2011)
18. Pagès J-M, Masi M, Barbe J. Inhibitors of efflux pumps in Gram-negative bacteria. Trends Mol. Med. 11: 382–389 (2005)
19. Matsumoto Y, Hayama K, Sakakihara S, Nishino K, Noji H, Iino R, Yamaguchi A. Evaluation of multidrug efflux pump inhibitors by a new method using microfluidic channels. PLoS ONE 6: e18547 (2011)
20. Ferrari RG, Galiana A, Cremades R, Rodríguez JC, Magnani M, Tognim MCB, Oliveira TCRM, Royo G. Expression of the *marA*, *soxS*, *acrB* and *ramA* genes related to the AcrAB/TolC efflux pump in *Salmonella* enterica strains with and without quinolone resistance-determining regions *gyrA* gene mutations. Braz. J. Infect. Dis. 17: 125–130 (2013)
21. Clothier KA, Kinyon JM, Frana TS. Comparison of *Salmonella* serovar isolation and antimicrobial resistance patterns from porcine samples between 2003 and 2008. J. Vet. Diag. Invest. 22: 578–582 (2010)
22. Zheng J, Tian F, Cui S, Song J, Zhao S, Brown EW, Meng J. Differential gene expression by RamA in ciprofloxacin-resistant *Salmonella* Typhimurium. PLOS ONE 6: e22161 (2011)
23. Wang-Kan X, Blair JMA, Chirullo B, Betts J, La Ragione RM, Ivens A, Ricci V, Opperman TJ, Piddock LJV. Lack of AcrB efflux function confers loss of virulence on *Salmonella enterica* serovar Typhimurium. mBio 8: e00968–00917 (2017)
24. Sulavik MC, Dazer M, Miller PF. The *Salmonella* typhimurium mar locus: molecular and genetic analyses and assessment of its role in virulence. J. Bacteriol. 179: 1857–1866 (1997)
25. Nishino K, Latifi T, Groisman EA. Virulence and drug resistance roles of multidrug efflux systems of *Salmonella enterica* serovar Typhimurium. Mol. Microbiol. 59: 126–141 (2006)
26. Nishino K, Nikaido E, Yamaguchi A. Regulation of multidrug efflux systems involved in multidrug and metal resistance of *Salmonella enterica* serovar Typhimurium. J. Bacteriol. 189: 9066–9075 (2007)
27. Delcour AH. Outer membrane permeability and antibiotic resistance. Biochem. Biophys. Acta 1794: 808–816 (2009)
28. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol. Mol. Biol. Rev. 67: 593–656 (2003)
29. Nestorovich EM, Danelon C, Winterhalter M, Bezrukov SM. Designed to penetrate: time-resolved interaction of single antibiotic molecules with bacterial pores. Proc. Natl. Acad. Sci. 99: 9789–9794 (2002)
30. Santiviago CA, Toro CS, Hidalgo AA, Youderian P, Mora GC. Global regulation of the *Salmonella enterica* serovar Typhimurium major porin, OmpD. J. Bacteriol. 185: 5901–5905 (2003)
31. Bolton LF, Kelley LC, Lee MD, Fedorka-Cray PJ, Maurer JJ. Detection of multidrug-resistant *Salmonella enterica* serotype typhimurium DT104 based on a gene which confers cross-resistance to florfenicol and chloramphenicol. J. Clin. Microbiol. 37: 1348–1351 (1999)
32. Li G, Yan C, Xu Y, Feng Y, Wu Q, Lv X, Yang B, Wang X, Xia X. Punicalagin inhibits *Salmonella* virulence factors and has anti-quorum-sensing potential. Appl. Environ. Microbiol. 80: 6204–6211 (2014)
33. Yu EW, Aires JR, Nikaido H. AcrB multidrug efflux pump of *Escherichia coli*: composite substrate-binding cavity of exceptional flexibility generates its extremely wide substrate specificity. J. Bacteriol. 185: 5657–5664 (2003)
34. Blair JM, Piddock LJ. How to measure export via bacterial multidrug resistance efflux pumps. MBio 7: e00816–e00840 (2016)
35. Michael GB, Schwarz S. Antimicrobial resistance in zoonotic nontyphoidal *Salmonella*; an alarming trend? Clin. Microbiol. Infect. 22: 968–974 (2016)