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AcrAB-TolC Directs Efflux-Mediated Multidrug Resistance in *Salmonella enterica* Serovar Typhimurium DT104

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Multidrug-resistant *Salmonella enterica* serovar Typhimurium definitive phage type 104 (DT104) strains harbor a genomic island, called *Salmonella* genomic island 1 (SGI1), which contains an antibiotic resistance gene cluster conferring resistance to ampicillin, chloramphenicol, florfenicol, streptomycin, sulfonamides, and tetracyclines. They may be additionally resistant to quinolones. Among the antibiotic resistance genes there are two, i.e., *floR* and *tet*(G), which code for efflux pumps of the major facilitator superfamily with 12 transmembrane segments that confer resistance to chloramphenicol-florfenicol and the tetracyclines, respectively. In the present study we determined, by constructing *acrB* and *tolC* mutants, the role of the AcrAB-TolC multidrug efflux system in the multidrug resistance of several DT104 strains displaying additional quinolone resistance or not displaying quinolone resistance. This study shows that the quinolone resistance and the decreased fluoroquinolone susceptibilities of the strains are highly dependent on the AcrAB-TolC efflux system and that single mutations in the quinolone resistance-determining region of *gyrA* are of little relevance in mediating this resistance. Overproduction of the AcrAB efflux pump, as determined by Western blotting with an anti-AcrA polyclonal antibody, appeared to be the major mechanism of resistance to quinolones. Moreover, chloramphenicol-florfenicol and tetracycline resistance also appeared to be highly dependent on the presence of AcrAB-TolC, since the introduction of mutations in the respective *acrB* and *tolC* genes resulted in a susceptible or intermediate resistance phenotype, according to clinical MIC breakpoints, despite the presence of the FloR and Tet(G) efflux pumps. Resistance to other antibiotics, ampicillin, streptomycin, and sulfonamides, was not affected in the *acrB* and *tolC* mutants of DT104 strains harboring SGI1. Therefore, AcrAB-TolC appears to direct efflux-mediated resistance to quinolones, chloramphenicol-florfenicol, and tetracyclines in multidrug-resistant *S. enterica* serovar Typhimurium DT104 strains.

Multidrug-resistant *Salmonella enterica* serovar Typhimurium definitive phage type 104 (DT104) emerged during the 1980s as a global health problem because of its involvement in diseases in animals and humans (6, 13, 15, 23, 24, 40, 42, 43). Multidrug-resistant strains of this phage type were first identified from exotic birds in the United Kingdom in the early 1980s and in cattle and humans in the late 1980s but have since become common in other animal species such as poultry, pigs, and sheep. The serovar Typhimurium DT104 epidemic clone has now spread worldwide, with several outbreaks since 1996 in the United States and Canada (6, 15, 23, 40).

Multidrug-resistant *S. enterica* serovar Typhimurium DT104 strains are commonly resistant to ampicillin (AMP), chloramphenicol (CHL)-florfenicol (FLF), streptomycin (STR)-spectinomycin (SPT), sulfonamides (SULs), and tetracyclines (TETs). The antibiotic resistance genes are clustered in part of a 43-kb genomic island, called *Salmonella* genomic island I (SGI1), between the *thdF* and *int2* genes of the chromosome compared to the genome sequence of *S. enterica* serovar Typhimurium strain LT2 (8, 9). The antibiotic resistance gene cluster represents approximately one-third of SGI1 and is located at the 3′ end of the structure (8, 9). All resistance genes are clustered and are bracketed by two integron structures (4, 8–10, 13). The first integron carries the *aadA2* gene, which confers resistance to STR and SPT, and a truncated *sul1* (*sul1delta*) gene. The second contains the β-lactamase gene *pse-1*, which confers resistance to AMP, and a complete *sul1* gene, which confers resistance to the SULs. Flanked by these two integron structures are the *floR* gene (4), also called *floSt* (7) or the *cmlA*-like gene (10), which confers cross-resistance to CHL-FLF, and the TET resistance gene *tet*(G). The *floR* and *tet*(G) genes code for efflux pumps of the major facilitator superfamily (MFS) with 12 transmembrane segments.

The emergence of decreased susceptibility to fluoroquinolones in multidrug-resistant *S. enterica* serovar Typhimurium DT104 strains is causing particular concern (11, 35, 43, 45). The emergence and spread of such serovar Typhimurium DT104 strains followed the licensing of enrofloxacin (ENR) for veterinary use in 1993 (43). This fluoroquinolone antibiotic has subsequently been used to treat cattle and poultry, resulting in the selection of quinolone-resistant strains (43). It has been reported that the mechanisms of decreased susceptibility to fluoroquinolones in serovar Typhimurium DT104 isolates involve point mutations in the quinolone resistance-determining region (QRDR) of the target gene, *gyrA* (12, 13, 43). Another important mechanism may be active efflux due to overproduction of the AcrAB efflux pump (22). We have previously shown that the AcrB multidrug transporter plays a major role in the high-level fluoroquinolone resistance of *S.*

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enterica serovar Typhimurium DT204 strains carrying multiple target mutations in *gyrA*, *gyrB*, and *parC* (5).

In the present study we analyzed the role of the AcrAB-TolC efflux system in the multidrug resistance of *S. enterica* serovar Typhimurium DT104 by constructing mutants with mutations in the AcrB transporter and the TolC outer membrane component of the efflux system. We investigated in particular the role of the efflux system in quinolone, CHL-FLF, and TET resistance. For resistance to CHL-FLF and TETs, it was our hypothesis that the AcrAB-TolC efflux system might interplay with the secondary specific transporters FloR and Tet(G), respectively.

MATERIALS AND METHODS

Bacterial strains. Multidrug-resistant *S. enterica* serovar Typhimurium DT104 strains were isolated from cattle in Belgium (strain 543SA98) and France (strains BN10055, BN9945, and BN9181) (Table 1). Susceptible serovar Typhimurium DT104 control strain S/921495 was isolated from cattle in Scotland. Quinolone-susceptible serovar Typhimurium strain BN18 was isolated from a pigeon in France; and quinolone-resistant clones of this strain selected in vitro, strains BN18/21, BN18/41, and BN18/71, were used as controls for Western blot analysis for AcrA production. The MICs of ENR (Bayer AG, Leverkusen, Germany) for BN18 and the three clones were 0.125, 1, 4, and 16 μg/ml, respectively, and correlated well with the level of AcrA production (5, 22).

MIC determinations. MICs were determined by the standard agar doubling dilution method on Mueller-Hinton medium with inocula of 10^4 CFU per spot. The MIC was defined as the lowest concentration of the drug that completely inhibited visible growth after incubation for 18 h at 37°C. The following antibiotics were used: nalidixic acid (NAL; Sigma, Steinheim, Germany), flumequine (FLU; Sigma), ENR (Bayer AG), ciprofloxacin (CIP; Bayer AG), marbofloxacin (MAR; Vétoquinol, Lure, France), CHL (Boehringer, Mannheim, Germany), FLF (Schering-Plough Animal Health, Kenilworth, N.J.), and TET (Boehringer). The following MIC breakpoints (*c* and *C*), defined by the Comité de l'Antibiogramme de la Société Française de Microbiologie (34) or the manufacturers, were used to classify strains as susceptible (MIC $\leq c$), intermediate ($c <$ MIC $\leq C$), or resistant (MIC $> C$): NAL, 8 and 16 μg/ml; FLU, 4 and 8 μg/ml; ENR, CIP, and MAR, 1 and 2 μg/ml; CHL and FLF, 8 and 16 μg/ml; and TET, 4 and 8 μg/ml. The MICs of these antibiotics were also determined with 80 μg of the efflux pump inhibitor Phe-Arg-β-naphthylamide (PAβN; Sigma) per ml (30, 31).

PCR amplification and sequencing of QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* genes. The sequences of the primers used in the PCR amplifications of the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* are given in Table 2. Genomic DNA was extracted from overnight cultures in Luria-Bertani (LB) medium at 37°C by using the QIAamp DNA Mini extraction kit (Qiagen S.A., Courtaboeuf, France). PCR was performed with extracted genomic DNA in volumes of 100 μl containing 200 ng of template DNA, 100 pmol of each primer, 200 μM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, and 2 U of *Taq* polymerase (Promega, Madison, Wis.). After an initial denaturation step of 3 min at 94°C, amplification was performed over 30 cycles, with each cycle consisting of 1 min at 94°C, 1 min at the hybridization temperature (55°C for *gyrA*, 58°C for *gyrB*, and 52°C for *parC* and *parE*), and 1 min at 72°C, with a final extension step of 10 min at 72°C. Primers and free nucleotides were removed with a Qiaquick spin PCR purification kit (Qiagen S.A.). Nucleotide sequencing was performed with a Génome Express (Grenoble, France) sequencer. Sequence analysis was done by using the following programs: BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), FASTA (http://www2.ebi.ac.uk/fasta3/), and CLUSTALW (http://www2.ebi.ac.uk/clustalw/).

Construction of *acrB* mutants. *S. enterica* serovar Typhimurium *acrB* mutant strain LXA3, constructed by disrupting the *acrB* gene with the kanamycin resistance aminoglycoside 3′-phosphotransferase gene from plasmid pUC4K (Amersham Pharmacia Biotech, Orsay, France), was characterized previously (27). This strain was used to introduce the *acrB*::*kan* mutation in the strains used in this study by transduction with phage P22 (5, 41). The resulting *acrB*::*kan* mutants were selected on LB agar plates containing 50 μg of kanamycin per ml, and insertion of the kanamycin resistance gene into *acrB* was confirmed by PCR with primers Pr1 and Pr2 (Table 2).

Construction of *tolC* mutants. Deletion of the *tolC* gene was performed by the method described by Datsenko and Wanner (14). Briefly, the kanamycin resistance gene *kan* flanked by FLP recognition target sites was amplified by a standard PCR with the template plasmid pKD4 and hybrid primers. These primers, P1TolC and P2TolC (Table 2), were synthesized with 20 nucleotides (nt) of priming sites 1 and 2 of pKD4 and with 50 nt of the 5′ and 3′ ends of the *tolC* gene. The 1.6-kb PCR fragment was purified and electroporated into antibiotic-susceptible strain *S. enterica* serovar Typhimurium DT104 S/921495, into which Red recombinase expression plasmid pKD46 was introduced. Transformants were selected at 37°C on LB agar medium containing kanamycin at 50 μg/ml. Homologous recombination between the genomic DNA and the PCR product resulted in the deletion of the *tolC* sequence from nt 70 to 1426 (1,356-bp deletion) and its replacement with the *kan* gene. This was confirmed by three different PCRs. Deletion of *tolC* in the transformants was first shown by a negative result by PCR with primers TolC3 and TolC4 (Table 2), whose sequences correspond to internal sequences of the deleted *tolC* gene. To confirm the deletion, a second PCR was carried out with primers TolC5 and TolC6 (Table 2), whose sequences correspond to sequences flanking the *tolC* deletion and which resulted in a 2,400-bp fragment for the parental strains and a 2,644-bp fragment when *tolC* was deleted and replaced by the *kan* gene flanked by FLP recognition target sites. The third control PCR, with primers k2 and kt (6) (Table 2), was used to detect the 471-bp *kan* fragment.

The *tolC* gene deletion was further transducted into other strains by using phage P22, as described above.

SGI1 isogenic strain construction. Analysis of the *S. enterica* serovar Typhimurium LT2 genome revealed two AscI sites which flanked the *thdF* gene (also called the *trmE* gene), which has been shown to be the insertion site of SGI1 (8). No AscI sites were identified in the complete SGI1 sequence; therefore, the complete SGI1 sequence could be isolated on a 51-kb AscI fragment. Serovar Typhimurium DT104 strain 1948SA96 genomic DNA was cut with AscI and ligated into the MluI site of the suicide vector pMAKSACB (19). This ligation mixture was electrotransformed into *Escherichia coli* DH10B (Invitrogen), and colonies were selected on AMP-CHL at the permissive temperature. The plasmids from several colonies were isolated, and the presence of SGI1 was confirmed by restriction mapping with EcoRI and BamHI, targeting the ends of SGI1 by PCR, and sequencing across the cloning site. The plasmid, labeled pST-SGI1, was used to electrotransform antimicrobial-susceptible strain *S. enterica* serovar Typhimurium DT104 S/921495 with selection on AMP-CHL at the permissive temperature. No transformants could be obtained after several attempts, even though the pMAKSACB vector alone could be successfully maintained in serovar Typhimurium DT104 S/921495 at the permissive temperature. However, when a portion of the ligation mixture described above was electrotransformed into serovar Typhimurium DT104 S/921495, two colonies were isolated on AMP-CHL. PCR confirmed the presence of SGI1 but the absence of the *sacB* gene of the vector. Southern hybridization and further PCR analysis showed that the 51-kb AscI fragment was likely circularized and inserted into the genome by a single crossover event via the *thdF* end in one strain (strain ST-1) and the *yidY* end in the other strain (strain ST-2).

AcrA expression analysis by Western blotting. *acrA* is cotranscribed with *acrB*, and AcrA expression is indicative of expression of the AcrAB efflux pump. Overnight cultures grown in LB medium at 37°C were harvested by centrifugation and resuspended at an A_{600} of 10.0. Cells were diluted 1/2 in the sample buffer of Laemmli (28) and were heated for 10 min at 100°C. Whole-cell proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred at 0.8 mA/cm^2 to a nitrocellulose membrane. The membrane was washed three times with Tris-buffered saline (TBS; 0.15% NaCl, 10 mM Tris-HCl [pH 7.5]), saturated for 30 min at room temperature with TBS containing 1% skim milk, and incubated overnight at room temperature with an anti-AcrA polyclonal antibody (33) diluted 1/2,000 in TBS containing 0.05% Tween 20 (TBS-T) and 0.33% skim milk. After three washes in TBS-T, the membrane was incubated for 1 h with peroxidase conjugated to protein A (Sigma) diluted 1/1,000 in TBS-T. Finally, after three washes in TBS-T, the blot was developed by incubation at room temperature in a solution of TBS containing 0.06% 4-chloro-1-naphthol and 5 mM H_2O_2. The reaction was stopped by washes in distilled water.

RESULTS

Role of AcrAB-TolC in resistance to quinolones. Representative strains of *S. enterica* serovar Typhimurium DT104 (strains BN10055, 543SA98, and BN9945) showing increasing levels of resistance to NAL and decreased susceptibilities to fluoroquinolones were studied for their mechanisms of resistance to these antibiotics (Table 1). Strain BN9945 exhibited

TABLE 1. *S. enterica* serovar Typhimurium DT104 strains studied

Strain	Geographic origin[a]	SG11	Quinolone MIC (μg/ml)[b]					MIC of other drugs (μg/ml)			Substitution in *gyrA* QRDR	WB[c] AcrA
			NAL	FLU	ENR	MAR	CIP	CHL	FLF	TET		
BN10055	F	+	>1,024 (16)	64 (2)	4 (0.125)	2 (0.25)	2 (0.125)	256 (64)	128 (4)	128 (64)	Ser83Tyr	+++
BN10055*acrB*::*kan*		+	64	2	0.25	0.25	0.060	16	4	16	Ser83Tyr	ND[e]
BN10055Δ*tolC*::*kan*		+	32	1	0.060	0.125	0.060	16	4	8	Ser83Tyr	ND
543SA98	B	+	1,024 (16)	32 (1)	2 (0.125)	2 (0.25)	1 (0.125)	256 (64)	128 (16)	128 (64)	Ser83Phe	+++
543SA98*acrB*::*kan*		+	32	1	0.25	0.25	0.060	16	8	16	Ser83Phe	ND
543SA98Δ*tolC*::*kan*		+	16	1	0.125	0.125	0.060	16	8	8	Ser83Phe	ND
BN9945	F	+	512 (16)	32 (1)	1 (0.125)	1 (0.25)	0.5 (0.125)	256 (64)	128 (16)	128 (64)	—[f]	++
BN9945*acrB*::*kan*		+	32	1	0.25	0.125	0.060	16	8	8	—	ND
BN9945Δ*tolC*::*kan*		+	16	1	0.060	0.125	0.060	16	8	8	—	ND
BN9181	F	+	4 (0.5)	0.5 (0.03)	0.125 (0.008)	0.060 (0.030)	0.030 (0.015)	256 (64)	32 (2)	64 (32)	—	+
BN9181*acrB*::*kan*		+	0.5	0.060	0.008	0.030	0.004	16	2	16	—	ND
BN9181Δ*tolC*::*kan*		+	0.5	0.060	0.008	0.008	0.002	16	2	8	—	ND
Control strains												
S/921495	S	−	4 (0.5)	0.5 (0.03)	0.125 (0.008)	0.060 (0.008)	0.030 (0.008)	8 (0.5)	8 (1)	2 (0.5)	—	+
S/921495*acrB*::*kan*		−	0.5	0.060	0.008	0.008	0.004	0.5	1	0.5	—	ND
S/921495Δ*tolC*::*kan*		−	0.5	0.060	0.008	0.008	0.004	1	1	0.5	—	ND
ST-1[d]	C	+	4 (0.5)	0.5 (0.03)	0.125 (0.008)	0.060 (0.008)	0.030 (0.004)	256 (64)	64 (4)	128 (32)	—	+
ST-1*acrB*::*kan*		+	0.5	0.060	0.008	0.008	0.004	16	4	16	—	ND
ST1Δ*tolC*::*kan*		+	0.5	0.060	0.008	0.008	0.004	16	4	8	—	ND

[a] F, France; B, Belgium; S, Scotland; C, Canada.
[b] Values in parentheses are MICs in the presence of the efflux pump inhibitor PAβN at 80 μg/ml.
[c] WB, Western blotting with an anti-AcrA polyclonal antibody.
[d] ST-1 corresponds to strain S/921495 complemented with SGI1.
[e] ND, not determined.
[f] —, no substitution.

TABLE 2. Primers used for PCRs

Primer	Gene	Nucleotide sequence (5′–3′)
STGYRA1	*gyrA*	TGTCCGAGATGGCCTGAAGC
STGYRA12	*gyrA*	CGTTGATGACTTCCGTCAG
STGYRB5	*gyrB*	AACGGTCTGCTCATCAGAAAGG
STGYRB7	*gyrB*	GAAATGACCCGCCGTAAAGG
STPARC1	*parC*	ATGAGCGATATGGCAGAGCG
STPARC2	*parC*	TGACCGAGTTCGCTTAACAG
STPARE1	*parE*	GACCGAGCTGTTCCTTGTGG
STPARE2	*parE*	GCGTAACTGCATCGGGTTCA
Pr1	*acrB*	ACGGTATCGATAACCTGATG
Pr2	*acrB*	TTCTTCGCCGACAACCCAAT
P1TolC	*tolC-kan*	TCCCCATCCTTATCGGCCTGAGC CTGTCGGGGTTCAGCACACTA AGCCAGGTGTAGGCTGGAGC TGCTTC
P2TolC	*tolC-kan*	TCAATGCCGGAATGGATTGCCG TTATTGCTGTTGGCGCGAGCG GCGGTCGCATATGAATATCCT CCTTAG
k2	*kan*	CGGTGCCCTGAATGAACTGC
kt	*kan*	CGGCCACAGTCGATGAATCC
TolC3	*tolC*	AAGCACGCCTGAGCAACCCG
TolC4	*tolC*	AATGCCGGAATGGATTGCCG
TolC5	*tolC*	CTCTCTAGACGAACATCTCCAG CAGCCAC
TolC6	*tolC*	CTCGAGCTCGCACTGACCTTCG CCAAACT

high-level resistance to NAL (MIC, 512 μg/ml) and decreased susceptibilities to the fluoroquinolones ENR, MAR, and CIP (MICs, 0.5 to 1 μg/ml) without carrying any mutation in the QRDRs of target gene *gyrA*, *gyrB*, *parC*, or *parE*. This strongly suggests other quinolone resistance mechanisms such as active efflux. The levels of resistance to NAL, FLU, ENR, MAR, and CIP were slightly increased (two- to fourfold) in two other *S. enterica* serovar Typhimurium DT104 strains (strains 543SA98 and BN10055) carrying a single mutation at codon 83 of *gyrA*, leading to the amino acid changes Ser83Phe and Ser83Tyr, respectively. According to the clinical breakpoints, the latter strains were resistant or intermediate resistant to the fluoroquinolones ENR, MAR, and CIP (Table 1).

Evidence for active efflux was provided by Western blot analysis with an anti-AcrA polyclonal antibody, which showed overproduction of the AcrA periplasmic protein in the quinolone-resistant strains relative to the level of production by the susceptible control strains (Fig. 1). The level of production of AcrA correlated well with the level of resistance to NAL and fluoroquinolones; i.e., strains BN10055 and 543SA98 showed a higher level of production of AcrA than strain BN9945. These results support an active efflux mechanism due to overproduction of the AcrAB-TolC efflux system, which appears to be a primary mechanism of resistance in quinolone-resistant field strains of *S. enterica* serovar Typhimurium DT104.

The role of AcrAB-TolC was further confirmed by inactivating the *acrB* and *tolC* genes, located at different chromosomal loci, in the strains studied. This resulted in 32- to 64-fold decreases in the levels of resistance to NAL and the fluoroquinolones (Table 1). The decreases in the levels of resistance were approximately the same for the *acrB* and *tolC* mutants, suggesting that AcrB is the major quinolone transporter interacting with TolC. Interestingly, the amount of decrease in resistance was the same whether the strains carried a mutation in *gyrA* or not. Nevertheless, inactivation of *acrB* or *tolC* did not result in a complete reversal of susceptibility to the level observed in the NAL-susceptible control strains (strains BN9181 and S/921495), suggesting that other not yet identified mechanisms may participate in quinolone resistance (Table 1).

FIG. 1. Western blot analysis of AcrA protein production in *S. enterica* serovar Typhimurium control strains BN18 (lane 1), BN18/21 (lane 2), BN18/41 (lane 3), and BN18/71 (lane 4) and serovar Typhimurium DT104 strains S/921495 (lane 5), ST-1 (lane 6), BN9181 (lane 7), BN9945 (lane 8), 543SA98 (lane 9), and BN10055 (lane 10).

Active efflux was also finally confirmed by use of the efflux pump inhibitor PAβN, which was effective in decreasing the MICs of NAL and fluoroquinolones for the *S. enterica* serovar Typhimurium DT104 field strains studied to the same extent obtained by inactivating their respective *acrB* and *tolC* genes (Table 1).

Role of AcrAB-TolC in resistance to other antibiotics. Multidrug-resistant *S. enterica* serovar Typhimurium DT104 strains harbor a chromosomal antibiotic resistance gene cluster within SGI1 conferring resistance to AMP, CHL-FLF, STR-SPT, SULs, and TETs. Since some of these antibiotics, such as AMP, CHL, and TETs, are well-known substrates of the AcrB multidrug transporter, it was interesting to determine whether inactivation of *acrB* or *tolC* would affect the levels of resistance to these antibiotics in these strains. Antibiograms performed for the *S. enterica* serovar Typhimurium DT104 parental and mutant strains showed that only resistance to CHL-FLF and TET was affected in both the *acrB* and the *tolC* mutant strains (data not shown). As shown in Table 1, inactivation of *acrB* or *tolC* resulted in a 16- to 32-fold reduction in the level of resistance to CHL-FLF and in a 4- to 16-fold reduction in the level of resistance to TET, depending on the strain. As was the case for the decreases in the levels of resistance to NAL and fluoroquinolones, the decreases in the levels of resistance to CHL-FLF and TET were approximately the same for the *acrB* and *tolC* mutants. Interestingly, inactivation of *acrB* and *tolC* in *S. enterica* serovar Typhimurium DT104 strains harboring SGI1 resulted in an intermediate level of resistance to CHL (MIC, 16 μg/ml) and susceptibility to FLF (MICs, ≤8 μg/ml), according to the clinical breakpoints. In the case of the parental strains, the levels of resistance to FLF were two- to fourfold higher (MIC, 128 μg/ml) for the quinolone-resistant strains than for quinolone-susceptible strains BN9181 and ST-1 harboring SGI1 (MICs, 32 and 64 μg/ml, respectively) (Table 1). If this difference is significant, it could be due to the overproduction of AcrAB, as shown by Western blotting (Fig. 1). However, it was not evident for the CHL and TET resistance

levels; i.e., the MICs were the same for the quinolone-resistant and quinolone-susceptible strains. The serovar Typhimurium DT104 *acrB* and *tolC* mutant strains harboring SGI1 showed intermediate resistance to TET (MIC of 8 μg/ml) or resistance to TET close to the clinical breakpoint (MIC, 16 μg/ml). Together, these results indicate that resistance to CHL-FLF and TET in multidrug-resistant serovar Typhimurium DT104 strains harboring SGI1 is highly dependent on the presence of the AcrAB-TolC efflux system.

This was further confirmed by studying a set of isogenic *S. enterica* serovar Typhimurium DT104 strains, a susceptible strain (strain S/921495), a strain in which SGI1 had been inserted in vitro (strain ST-1), and ST-1 with inactivated *acrB* and *tolC* genes. Serovar Typhimurium ST-1 exhibited 32-fold (MIC, 256 μg/ml), 8-fold (MIC, 64 μg/ml), and 64-fold (MIC, 128 μg/ml) increases in the levels of resistance to CHL, FLF, and TET, respectively, compared to those for its parental strain, S/921495 (Table 1). These resistance levels are comparable to those seen for multidrug-resistant serovar Typhimurium DT104 field strains harboring SGI1. Inactivation of the *acrB* and *tolC* genes in strain ST-1 resulted in the same decrease in the levels of resistance to CHL-FLF and TET observed for the multidrug-resistant serovar Typhimurium DT104 field strains harboring SGI1, thereby confirming the major role of the AcrAB-TolC efflux system in drug resistance.

To further show the effect of the simultaneous expression of the FloR or the Tet(G) transporter with the multicomponent efflux system AcrAB-TolC, we compared the MICs of FLF, CHL, and TET for control strains S/921495 and ST-1 to those for their *acrB*-derived mutants. The MICs of FLF, CHL, and TET for strain S/921495 [which lacks SGI1 and the *floR* and *tet*(G) genes] were 8, 8, and 2 μg/ml, respectively, whereas in the absence of the AcrB transporter, the MICs of FLF, CHL, and TET decreased to 0.5, 1, and 0.5 μg/ml, respectively. Complementation of this strain with SGI1 (strain ST-1) resulted in increases in the FLF, CHL, and TET MICs to 256, 64, and 128 μg/ml, respectively. Inactivation of the *acrB* gene in the latter strain decreased the FLF, CHL, and TET MICs to 16, 4, and 16 μg/ml, respectively (Table 1). By calculating the ratio of these MICs, i.e., the MIC in the presence of the transporter considered divided by the MIC in its absence, we obtained the results shown in Table 3. For each antibiotic, the ratio in the presence of the two types of transporters [FloR and AcrB or Tet(G) and AcrB] corresponded approximately to the multiplication of the ratio obtained for each transporter alone [FloR, Tet(G), or AcrB]. This indicates that the simultaneous expression of these transporters and a multicomponent efflux pump results in levels of drug resistance higher than those provided by each of them alone. Thus, the antibiotics FLF, CHL, and TET can be directly exported by the AcrAB-TolC efflux system, as well as efficiently transported to the periplasm by FloR or Tet(G).

The efflux pump inhibitor PAβN was not as effective in decreasing the MICs of CHL, FLF, and TET to the same extent obtained by inactivating the *acrB* and *tolC* genes for most multidrug-resistant *S. enterica* serovar Typhimurium DT104 field strains studied (Table 1). By use of this efflux pump inhibitor, only the FLF resistance levels were decreased to the same extent obtained by inactivating *acrB* or *tolC* in strains BN10055, BN9181, and ST-1.

TABLE 3. Effect of simultaneous expression of single-component efflux pump FloR or Tet(G) and the multicomponent efflux system AcrAB-TolC

Transporter	Ratio of MICs of antibiotics for control strains		
	CHL	FLF	TET
AcrB[a]	16	8	4
FloR and Tet(G)[b]	32	8	64
FloR, Tet(G), and AcrB[c]	512	64	256

[a] MIC for strain S/921495/MIC for strain S/921495 *acrB*::*kan*.
[b] MIC for strain ST-1*acrB*::*kan*/MIC for strain S/921495*acrB*::*kan*.
[c] MIC for strain ST-1/MIC for strain S/921495::*kan*.

DISCUSSION

The mechanisms of decreased susceptibility to fluoroquinolones in *S. enterica* serovar Typhimurium DT104 isolates have principally been attributed to point mutations in the QRDR of the target gene, *gyrA* (12, 13, 43). Thus, it was thought that the mutation in *gyrA* constituted the major mechanism of resistance. Amino acid changes at Ser83 (to Phe, Tyr, or Ala) or Asp87 (to Gly, Asn, or Tyr) are the most frequently observed in NAL-resistant strains (12, 13, 21, 43). A previous study (22) showed the participation of active efflux in quinolone-resistant mutants of serovar Typhimurium selected in vitro. In such mutants quinolone selection resulted in a multiple-antibiotic-resistance phenotype with associated resistance to unrelated antibiotics (22). The efflux mechanism appeared prior to acquisition of target gene mutations in these in vitro-selected quinolone-resistant *Salmonella* mutants (22). In the first-step mutants lacking *gyrA* mutations, overproduction of the AcrAB efflux pump already resulted in an eightfold increase in the levels of resistance to most antibiotics studied, i.e., NAL, FLU, ENR, MAR, CHL, and FLF; and this accounted for clinical resistance to NAL and unrelated drugs, CHL and FLF. It was concluded that the primary mechanism of resistance to quinolones was active efflux (22). It was thus of interest to reassess more precisely the mechanisms of quinolone resistance in epidemic serovar Typhimurium DT104 strains.

The results of the present study are in accordance with previous observations for in vitro-selected quinolone-resistant *Salmonella* mutants, i.e., that active efflux by the AcrAB-TolC efflux system is also the main mechanism of resistance to quinolones in *S.* Typhimurium DT104 strains. First, quinolone-resistant *S. enterica* serovar Typhimurium DT104 strains, such as strain BN9945, exist without carrying any mutations in the QRDRs of the target genes. According to clinical breakpoints, strain BN9945 indeed displayed high-level resistance to NAL (MIC, 512 μg/ml) and reduced susceptibilities to the fluoroquinolones ENR, MAR, and CIP. This strain overproduced AcrAB, according to Western blot analysis with an anti-AcrA polyclonal antibody. Inactivation of the *acrB* and *tolC* genes in this strain further confirmed the importance of the AcrAB-TolC efflux system in quinolone resistance. Inactivation of the *acrB* and *tolC* genes in strains BN10055 and 543SA98 carrying a single point mutation in the QRDR of *gyrA* resulted in the same levels of susceptibility to quinolones observed for strain BN9945, which lacked any *gyrA* mutation. Thus, it appears from these data that the GyrA substitutions Ser83Tyr or

Ser83Phe are of little relevance in mediating quinolone resistance and that efflux by the AcrAB-TolC efflux system is the main mechanism that mediates quinolone resistance in serovar Typhimurium DT104 strains. Our results are also in accordance with those of Oethinger et al. (38), who reported that in *E. coli* deletion of the *acrAB* locus made strains, including those with double *gyrA* mutations, hypersusceptible to fluoroquinolones and certain other unrelated drugs. To obtain high-level fluoroquinolone resistance, as shown previously for serovar Typhimurium DT204 strains (5), both active efflux by AcrAB-TolC and multiple mutations in the target genes *gyrA*, *gyrB*, and *parC* are probably required.

In this study, we showed that resistance to CHL and FLF and to TET is due to efflux mechanisms both by specific transporters encoded by the *floR* and *tet*(G) genes located on SGI1 and by the multidrug efflux system AcrAB-TolC. In addition, there were no significant differences between the MICs of CHL, FLF, and TET for either the *acrB* or the *tolC* mutants, thereby suggesting that AcrB is the main multidrug transporter that interacts with TolC and that is involved in resistance to these drugs. Moreover, the resistance levels observed for *acrB* and *tolC* mutants and for a susceptible strain complemented with SGI1 indicated strong functional interactions between the MFS transporters FloR and Tet(G) and the multicomponent AcrAB-TolC efflux system. Data from the present study indicate that the simultaneous expression of the AcrAB-TolC efflux system and the FloR or Tet(G) transporter results in multiplicative increases in the levels of resistance to CHL, FLF, and TET. This observation is in accordance with what was previously observed for *E. coli* (29, 39), i.e., a synergistic effect resulting from the simultaneous expression of the multicomponent efflux system AcrAB-TolC, where AcrB is an RND transporter that interacts directly with TolC, and transporters of the MFS family, such as FloR and Tet(G), with the energies of both systems depending on the proton motive force (12, 13, 21, 29, 39, 43, 48). Thus, the global mechanism of CHL, FLF, and TET export would be for the MFS transporter FloR or Tet(G) to transport the antibiotics from the cytoplasmic membrane to the periplasm, which would then be recovered by the multidrug transporter AcrB to be extruded out of the cell by TolC. This point of view is consistent with the recent determination of the three-dimensional structure of AcrB, which is organized as a trimer, in which the three-dimensional structure revealed the presence of vestibules that permit direct access of the substrates from the periplasm or the outer leaflet of the cytoplasmic membrane to its central cavity, with the large periplasmic domains of AcrB being involved in substrate recognition (16, 17, 20, 32, 36, 44, 46, 47). Then, the TolC channel, also organized as a trimer (26), would be recruited to form with AcrB a direct transit structure to the extracellular medium (2, 16, 25, 36, 37).

The results of our study have some practical implications. As shown in the present study, clinical levels of resistance to CHL and FLF and to TET are highly dependent on the synergy between AcrAB-TolC and FloR and Tet(G), respectively. Thus, targeting of AcrB (5, 30–32) or TolC (1, 3, 18, 25), rather than the other transporters, to develop efflux pump inhibitors would probably be sufficient to develop combination therapies with such inhibitors and CHL, FLF, or TET as well as fluoroquinolones.

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