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Expression of *acrB*, *acrF*, *acrD*, *marA*, and *soxS* in *Salmonella enterica* Serovar Typhimurium: Role in Multiple Antibiotic Resistance

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Comparative reverse transcription-PCR in combination with denaturing high-pressure liquid chromatography analysis was used to determine the levels of expression of *soxS*, *marA*, *acrF*, *acrB*, and *acrD* in multiple-antibiotic-resistant (MAR) *Salmonella enterica* serovar Typhimurium isolates and mutants of *S. enterica* serovar Typhimurium SL1344 with defined deletions. Posttherapy MAR clinical isolates had increased levels of expression of all genes except *soxS*. *S. enterica* serovar Typhimurium SL1344 Δ*acrB* expressed 7.9-fold more *acrF* than the parent strain. A strain with an *acrF* deletion expressed 4.6-fold more *acrB*. Deletion of *acrB* and/or *acrF* resulted in 2.7- to 4.3-fold more *marA* mRNA and 3.6- to 4.9-fold increases in the levels of expression of *acrD* but had a variable effect on the expression of *soxS*. All mutants were hypersusceptible to antibiotics, dyes, and detergents; but the MIC changes were more noticeable for SL1344 with the *acrB* deletion than for the mutant with the *acrF* disruption. These mutants had different but overlapping phenotypes, and the concentrations of ciprofloxacin accumulated by the mutants were different. These data suggest that *acrB*, *acrF*, and *acrD* are coordinately regulated and that their expression influences the expression of the transcriptional activators *marA* and *soxS*.

Decreased drug permeation and active efflux can give rise to multiple antibiotic resistance (MAR) in bacteria (40). Overproduction of the AcrAB efflux pump confers MAR to a variety of compounds in both *Escherichia coli* (35, 37) and *Salmonella enterica* serovar Typhimurium (12, 17, 18, 25, 27). In *E. coli*, *acrD* and the *acrEF* operon also encode efflux pumps (31, 40), and AcrD has been shown to efflux aminoglycosides (26, 31, 33). AcrE and AcrF are 65 and 77% identical to AcrA and AcrB, respectively (19). Genomic analysis reveals that *S. enterica* serovar Typhimurium LT2 AcrB and AcrF are 96 and 88% identical, respectively, to their *E. coli* homologues. Furthermore, AcrB is 80% identical to AcrF. *S. enterica* serovar Typhimurium LT2 AcrD is 64 and 65% identical to AcrB and AcrF, respectively (D. J. Eaves, unpublished data).

In *E. coli*, the *acrRAB* locus is a member of the *mar* and *sox* regulons, and both MarA and SoxS can activate *acrAB* expression (33). The *marRAB* operon was first described in *E. coli* (11) but has since been identified in several bacteria including *S. enterica* (34). MarR is a repressor, and MarA is a transcriptional activator. This operon confers MAR by production of MarA in response to environmental agents, including some antibiotics and disinfectants. In *E. coli* more than 60 chromosomal genes are part of the *mar* regulon (1, 3, 20). The *soxRS* operon is activated in response to oxidative stress, with SoxS being the activator. Both MarA and SoxS are members of the AraC family of transcriptional activators (21). MarA can also activate many of the stress response genes that are activated by SoxS (21, 22). Constitutive overexpression of *soxRS* can also contribute to antibiotic resistance in clinically relevant *S. enterica* isolates (16).

It was hypothesized that overexpression of *acrB*, *acrF*, and/or *acrD* could confer MAR in *S. enterica* serovar Typhimurium. It was further hypothesized that expression of one or more of these efflux pump genes in *S. enterica* serovar Typhimurium was regulated by homologues of *E. coli acrR*, *marA*, and *soxS* and that overexpression of *marA* and/or *soxS* conferred MAR. To measure the levels of expression of several genes in parallel from a single mRNA preparation, the technique of comparative reverse transcription (RT)-PCR (C-RT-PCR) was combined with the rapid and high-throughput technique of denaturing high-pressure liquid chromatography (DHPLC) analysis of amplimers.

While quantitative-competitive RT-PCR (QC-RT-PCR) offers the most accurate way to determine transcript concentrations, it requires several concentrations of a competitor template that is included in the reverse transcription reaction, making this procedure both time-consuming and expensive (9, 29, 34). C-RT-PCR measures transcript levels on the basis of the amount of amplification product relative to the amount of an internal control, and then the amount is compared to that for the parent strain. C-RT-PCR allows a comparison of relative mRNA levels between several strains, provided that each sample is processed in parallel (24).

In the present study C-RT-PCR was used to assess the levels of expression of *marA*, *soxS*, *acrB*, *acrF*, and *acrD* in defined Δ*acrB*, Δ*acrF*, and Δ*acrB* Δ*acrF* mutants of *S. enterica* serovar Typhimurium SL1344. The levels of expression of these genes were also determined for MAR clinical isolates originating from patients who failed ciprofloxacin therapy (28, 29).

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MATERIALS AND METHODS

Clinical isolates. The four *S. enterica* serovar Typhimurium clinical isolates have all been previously described in detail (28, 29) and originated from patients who failed ciprofloxacin therapy. Isolate L3 was isolated prior to treatment, and

TABLE 1. MICs of antibiotics, detergents, and dyes and concentrations of ciprofloxacin accumulated[a]

Strain	Genotype	MIC (μg/ml)								SSC[b] (ng/mg) of CIP	
		NAL	CIP	CHL	TET	EtBr	ACR	SDS	CTAB	Alone	With CCCP
L3	Pretherapy	8	0.015	4	4	32	64	256	128	98.6 ± 6.3	**117.1 ± 6.1**
L5[c]	*gyrA*	16	0.12	64	8	32	64	256	128	28.3 ± 6.2	**123.1 ± 3.5**
L10	Unknown	32	0.12	32	16	>256	256	256	>256	21.9 ± 1.7	**107.5 ± 9.4**
L12	Unknown	64	0.5	32	16	>256	128	256	>256	28.8 ± 5.6	**72.6 ± 2.6**
SL1344	Parent	4	0.06	2	2	>256	128	>256	>256	25.9 ± 5.7	27.7 ± 4.4
L561	Δ*acrF*	4	0.06	4	1	128	128	>256	256	11.3 ± 1.7	**38.1 ± 2.7**
L644	Δ*acrB*	1	0.015	2	0.5	64	32	256	64	18.4 ± 0.9	19.3 ± 2.6
L646	Δ*acrB* Δ*acrF*	1	0.015	2	0.5	16	16	128	64	17 ± 1.4	15.7 ± 2.1

[a] Abbreviations: NAL, nalidixic acid; CIP, ciprofloxacin; CHL, chloramphenicol; TET, tetracycline; EtBr, ethidium bromide; ACR, acriflavine; SDS, sodium dodecyl sulfate; CTAB, cetytrimethylammoniumbromide.
[b] SSC, steady-state concentration in dry cells. Boldface type indicates a statistically significant difference between ciprofloxacin alone and ciprofloxacin plus CCCP.
[c] L5 also contains a mutation in *gyrA* (28, 29).

three posttherapy isolates (isolates L5, L10, L12) were MAR and accumulated low concentrations of ciprofloxacin (Table 1).

Construction of mutants. The *acrB* and *acrF* genes were inactivated in *S. enterica* serovar Typhimurium SL1344 (38) by a one-step technique previously described for *E. coli* (5). Ampicillin- and kanamycin-resistant transformants were selected on Lennox agar containing 80 μg of ampicillin per ml or 25 μg of kanamycin per ml, respectively. A 1.7-kb PCR product was amplified from template pKD4 by using primers acrBp1pKD4 and acrBp2pKD4. The linear PCR product was recombined into the chromosome of SL1344 by the use of helper plasmid pKD46. A 1.8-kb PCR product was amplified from the resulting kanamycin-resistant intermediate strain by using primers usacrB and dsacrB. Primer pairs usacrB-k1 and dsacrB-k2 amplified products of 880 and 1.2 kb, respectively. A PCR product with a size (3.7 kb) identical to that amplified from SL1344 was amplified from the resulting Δ*acrB* mutant, mutant L644, with primer pair usacrF-dsacrF. The kanamycin resistance cassette was excised from the chromosome with helper plasmid CP20, which encodes the FLP recombinase under the control of a temperature-sensitive promoter. Afterwards, the PCR amplimers amplified from L644 with primers usacrB and dsacrB were reduced from 1.8 kb to 600 bp, as predicted for the loss of the 1.2-kb kanamycin resistance cassette from the deleted *acrB* site. No products were obtained with primer pairs containing either k1 or k2, also consistent with the loss of the kanamycin resistance gene from the chromosome.

Strain L561 was constructed by deleting *acrF* from strain SL1344. The Δ*acrF* construct (strain L561) was checked with primer sets analogous to those used for L644 (Table 2). Strain L646 was constructed by deleting the *acrB* gene from strain L561. The 1.7-kb PCR product amplified from template pKD4 by the use of primers acrBp1pKD4 and acrBp2pKD4 was recombined onto the chromosome of L561. Kanamycin-resistant recombinants were checked thoroughly by PCR with all possible combinations of primer pairs.

Determination of antibiotic susceptibility. The MIC of each agent was determined by the agar doubling dilution procedure on solid medium (2).

Accumulation of ciprofloxacin. The concentration of ciprofloxacin accumulated by each mutant and parent strain after 5 min of exposure to 10 μg of ciprofloxacin per ml was determined by a modified fluorescence method, as described previously (29). After 5 min of exposure, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 100 μM) was added to a parallel set of tubes. The accumulation data were expressed as nanograms of quinolone per milligram (dry weight) of cells. All experiments were performed in duplicate on at least three separate occasions, and the mean values and standard deviations (SDs) were determined. To determine whether any differences in the data were statistically significant, the values obtained after 5 min of exposure to each agent were compared by Student's two-tailed *t* test. A *P* value less than 0.05 was considered significant.

RNA isolation. Bacteria were cultured overnight in nutrient broth (Oxoid) at 37°C and were then diluted 100-fold in prewarmed medium and cultured with shaking until they mixture reached an $A_{600\ nm}$ of 0.4 to 0.5. Cultures (3 ml) were chilled on ice and harvested by centrifugation at 4°C. Total RNA was isolated by using RNAce Spin Cell Mini kits (Bioline) with the following optimizations: recombinant RNasin RNase inhibitor (Promega) was added during cell lysis, and the lysate was immediately applied to the DNA binding column. Additional RNasin was added after the final RNA elution step. RNA preparations were treated with DNA-free DNase (Ambion) and assessed for purity by gel electrophoresis and PCR. The RNA preparations were then quantified by using Gene Tools software (Syngene). At least two independent RNA preparations were isolated for each strain.

RT. cDNA was synthesized from RNA template by using Superscript II H^- reverse transcriptase (GibcoBRL) and random primer oligonucleotides (Invitrogen) according to the instructions of the manufacturers. RNA (150 ng) was retrotranscribed for 50 min at 42°C with 200 U of reverse transcriptase by using 100 ng of random primers in a 24-μl reaction mixture. The enzyme was inactivated by heating at 70°C for 15 min.

Multiplex PCR amplification of *soxS*, *marA*, *acrB*, *acrF*, and *acrD*. PCR amplification of cDNA was carried out with the primer sets described in Table 2. Regions within *soxS* (229 bp), *marA* (358 bp), *acrB* (397 bp), *acrF* (195 bp), *acrD* (341 bp), and the internal control 16S rRNA (247 bp) were amplified to assess the levels of mRNA for each gene. All six primer sets gave individual amplimers from the cDNA independently of each other and produced single amplicons of the predicted sizes. No minor amplification products were detected. The PCR conditions were optimized as described previously (10, 14) by titration of both cDNA template concentrations (1- to 2,000-fold dilutions) and primer concentrations (0 to 0.3 μM) to give quantities of product that were in the linear range of amplification (data not shown). *MarA* and *soxS* were amplified in a multiplex PCR with a neat cDNA preparation as the template. *AcrF* and *acrD* were amplified separately from neat cDNA. 16S rRNA and *acrB* required 1,000- and 5-fold dilutions of cDNA, respectively. The PCR mixtures (50 μl) contained PCR Master Mixture (1.5 mM $MgCl_2$; ABgene), cDNA (2 μl), and 0.3 μM (each) primer. DNA was amplified with a Techne thermal cycler with an initial denaturing step at 95°C (5 min); this was followed by 30 cycles of 95°C (30 s), 50°C (30 s), and 68°C (1 min and 20 s).

PCR product quantification by DHPLC analysis. The PCR products were quantified by DHPLC by using the Wave DNA fragment analysis system (Transgenomic Inc.). This system uses a combination of heat denaturation (50°C) and ion-pair chromatography to separate DNA fragments by size. The PCR product (40 μl) was loaded on the DNASepCartridge (Transgenomic Inc.) in 55% eluent A–45% eluent B. The gradient for separation was a 12.5-min linear gradient from 50% eluent B to 65% eluent B. The flow rate was 0.9 ml min^{-1}. Eluent A contained 0.1 M TEAA (triethylammonium acetate), and eluent B contained 0.1 M TEAA in 25% (vol/vol) acetonitrile. The column was subsequently washed in 100% eluent B for 30 s and then reequilibrated in 55% eluent A–45% eluent B. The DNA fragment elution profiles were captured and quantified according to their peak areas with NAVIGATOR (version 1.4) software (Transgenomic).

C-RT-PCR analysis. The 16S rRNA gene was assumed to be maximally transcribed under the growth conditions used throughout this study (29, 36). Differences in amplification efficiencies among cDNA preparations were normalized by referring the peak area of each amplicon resulting from the 16S rRNA amplification (used as the reference gene) to that of each amplicon resulting from strain NCTC74 16S rRNA amplification. The 16S rRNA was amplified from both strains on 10 separate occasions to establish an accurate mean ± SD value for this peak area. Significant variations in the levels of 16S rRNA amplification from samples indicated relative differences in mRNA levels within the total RNA preparations used. Peak areas for *soxS*, *marA*, *acrB*, *acrF*, and *acrD* were adjusted accordingly. To minimize these differences, samples from different bacterial strains were handled in parallel. The data are presented as the means ± SDs from at least three independent PCR amplifications. Comparisons between groups were done by a Student's *t* test. A *P* value less than 0.05 was considered significant.

TABLE 2. Primers used in this study

Primer purpose and gene	Primer	Primer sequence (5′→3′)	Reference or EMBL accession no.
Construction of deletion mutants			
acrB	acrBplpKD4 forward	GAAAATAGCCAGTACGGTTGCCGTTACCATCCCGCCGTGTAGGCTGGAG CTGCTTC	5
	acrBp2pKD4 reverse	GCGATCCTCAAATTGCCGGTAGCGCATATCCGACGACATATGAATATCC TCCTTCG	
acrF	acrFp1pKD4 forward	ACATGGCAAACTTTTTTATTAGACGTCCTATTTTCGGTGTAGGCTGGAG CTGCTTC	5
	acrFp2pKD4 reverse	GACGCCTCTGTTTACTGGTTAATCATGATGGCGATTCATATGAATATCC TCCTTCG	
acrB	usacrB forward	GGATCACACCTTATTGCCAG	AE008717
	dsacrB reverse	CGGCCTTATCAACAGTGAGC	
acrF	usacrF forward	CCGACGTTACCGTAGATGAA	AE008856
	dsacrF reverse	GTGGTAAGCAATGCAACTGG	
kan	k1 forward	CAGTCATAGCCGAATAGCCT	5
	k2 reverse	CGGTGCCCTGAATGAACTGC	
C-RT-PCR			
acrB	*acrB* forward	GACGTCCTATTTTCG	AE008717
	acrB reverse	CGAAGACGCCTCTGT	
acrF	*acrF* forward	TCGTGTTGCTAGGCACTT	AE008856
	acrF reverse	GGATCTGCGACATGGATT	
acrD	*acrD* forward	CTGCGCTGGATTCTGATT	AE008811
	acrD reverse	ATAATGGCGAACGAGGAG	
marA	*marA* forward	CGCAACACTGACGCTATTAC	U61147
	marA reverse	TTCAGCGGCAGCATATAC	
soxS	*soxS* forward	CATATCGACCAACCGCTA	U54468
	soxS reverse	CGGAATACACGCGAGAAG	
16S rRNA	16S forward	CCTCAGCACATTGACGTTAC	U90316
	16S reverse	TTCCTCCAGATCTCTACGCA	

RESULTS AND DISCUSSION

The growth rates for all clinical isolates and the strains with deletions were identical to those of the parent strain (SL1344) or the pretherapy isolate (isolate L3) in nutrient broth (data not shown), which ensured that total RNA was prepared from isolates in equivalent growth phases. Compared with the pretherapy isolate (isolate L3), the posttherapy MAR isolates (isolates L5, L10, and L12) had all been shown previously to have two- to eightfold decreased susceptibilities to nalidixic acid, ciprofloxacin, chloramphenicol, tetracycline, ethidium bromide, and acriflavine (Table 1) (28). The data obtained by the C-RT-PCR method agreed with those obtained by Northern blotting and QC-RT-PCR, or the method proved more sensitive than Northern blotting and QC-RT-PCR and was sufficiently sensitive to detect changes in levels of expression as low as 1.3-fold. Recently, microarray-determined gene expression has also found significant changes as low as 1.3-fold that correlated with physiological function (4, 32). This suggests that the significant differences determined by C-RT-PCR in the present study are important. This may be particularly important for regulatory genes, in which a small change in expression can alter expression of all members of a regulon.

Strains constructed to lack selected efflux pump genes demonstrated that there are some similarities between *E. coli* and *S. enterica* serovar Typhimurium; however, there are also distinct differences. Strain L644 (Δ*acrB*) was more susceptible to nalidixic acid, ciprofloxacin, tetracycline, ethidium bromide, and acriflavine (Table 1). A similar phenotype was seen for *E. coli* Δ*acrAB* (33), except that this mutant was also more susceptible to chloramphenicol and sodium dodecyl sulfate (SDS). L644 overexpressed *acrF* and *acrD* (Table 3), supporting the hypothesis made for *E. coli* that overproduction of AcrF and/or AcrD can partially compensate for a nonfunctional AcrB (J. Xu, M. L. Nilles and K. P. Bertrand, Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993, abstr. K-169, p. 290, 1993). L644 accumulated less ciprofloxacin than SL1344, but this decrease was unaffected by the addition of the proton motive force inhibitor CCCP (Table 1) and was therefore not

TABLE 3. Fold change in *soxS*, *marA*, *acrB*, *acrF*, and acrD expression measured by C-RT-PCR

Strain	Fold change in gene expression measured by C-RT-PCR[a]				
	soxS	*marA*	*acrB*	*acrF*	*acrD*
L3	1.0	1.0	1.0	1.0	1.0
L5	1.3 (<2)	**1.7** (<2)	1.4 (2.5)	1.1	**1.7**
L10	**0.8** (<2)	**1.9** (<2)	**6.0** (4)	**1.8**	**1.7**
L12	**0.8** (<2)	**1.4** (<2)	**2.3** (<2)	**1.6**	**1.3**
SL1344 (parent)	1.0	1.0	1.0	1.0	1.0
L561 (Δ*acrF*)	0.6	**2.7**	**4.6**		**4.7**
L644 (Δ*acrB*)	**3.1**	**3.3**		7.9	**3.6**
L646 (Δ*acrB* Δ*acrF*)	**3.6**	**4.3**			**4.9**

[a] Boldface type indicates statistically significant change compared to the result for parent strain L3 or SL1344, as determined by Student's *t* test ($P < 0.05$). Values in parentheses are those obtained previously by Northern blotting or QC-RT-PCR (29).

due to a proton antiport mechanism such as AcrF or AcrD. Deletion of the *acrB* gene (strain L644) resulted in increased levels of expression of *soxS* and *marA* (Table 3). In *E. coli* increased levels of expression of *micF* due to overexpression of *marA* gives rise to decreased levels of expression of *ompF* and, hence, decreased levels of accumulation of some antibiotics. It may be that the same is true for *S. enterica* serovar Typhimurium L644, explaining the lack of an effect of CCCP (6).

The susceptibility of strain L561 (Δ*acrF*) to antibiotics, dyes, and detergents was similar to the susceptibilities of SL1344 (Table 1) and *E. coli* Δ*acrEF* (33). The lack of changes in the substrate profile may be due to the apparent increase in the level of AcrB and/or AcrD expression (Table 3) that may compensate for the *acrF* deletion. This hypothesis is supported by the observed decrease in the level of ciprofloxacin accumulated by L561 that increased upon the addition of CCCP (Table 1). The level of *soxS* expression was reduced in strain L561 (Δ*acrF*), but the level of *marA* expression was increased (Table 3). These data suggest that overproduction of MarA in the absence of *acrF* is independent of a decrease in the level of *soxS* expression; this could be a secondary effect of the increase in the level of *acrB* expression. In L644 (Δ*acrB*) the levels of both *soxS* and *marA* expression were increased, which may account for the larger increase in the level of *acrF* expression.

With the exception of ethidium bromide, acriflavine, and SDS, the susceptibility of L646 (Δ*acrB* Δ*acrF*) to antibiotics, dyes, and detergents was identical to that of the strain with a single deletion, *acrB* (L644) (Table 1). The level of expression of *acrD* increased, but only to the level seen in L644, suggesting a maximal level of expression (Table 3). As for L644 (Δ*acrB*), the concentration of ciprofloxacin that accumulated was also reduced in a proton motive force-independent manner. The levels of expression of both *soxS* and *marA* were increased in L646 (Table 3). It is interesting that these levels of expression were greater than those by L644 or L561.

Compared with the pretherapy isolate (isolate L3), the levels of expression of one or more of the *acrB*, *acrF*, and *acrD* genes were increased in all three posttherapy clinical isolates (Table 3). These isolates also accumulated less ciprofloxacin; the level of accumulation could be increased by the addition of CCCP (Table 1). This is consistent with the overproduction of RND-type pumps. Ciprofloxacin has been shown to be a substrate of AcrB in *E. coli* (33); however, the 6.0-fold increase in the level of *acrB* expression in L10 was not mirrored by a large decrease in the level of ciprofloxacin accumulation; indeed, the level of accumulation was similar to that by L5, in which no significant change in the level of *acrB* expression was detected. Isolates L10 and L12 showed moderate increases in the levels of *acrF* expression. The reduction in level of accumulation was accompanied by an increase in the level of expression of *acrD*, known as an aminoglycoside efflux pump in *E. coli* (31, 33). No change in the level of *soxS* expression was detected, but the level of *marA* expression was increased in the posttherapy clinical isolates. The increase in the level of *marA* expression most likely explains the general increases in the levels of *acrB*, *acrF*, and *acrD* expression in these strains. The levels of *soxS* expression varied between the clinical isolates and the defined efflux pump mutants. The pretherapy isolate (isolate L3) contained double the amount of *soxS* transcript contained in laboratory strain SL1344. Variable *soxS* levels were observed in the efflux pump mutants, presumably as a result of different levels of oxidative stress.

It is hypothesized that deletion of either *acrB* or *acrF* in *S. enterica* triggers a stress response that in turn causes altered expression of other efflux pump genes. In *E. coli*, the level of AcrAB expression is inversely proportional to the growth rate and is greatest at late stationary phase (30). Deletion of an efflux pump may lead to increased levels of accumulation of the relevant environmental stress signals and switch on expression of the *mar* regulon genes, including *acrB*, independently of the nutrient supply. The periplasmic loops in AcrB (residues 29 to 339 and 558 to 872) have recently been shown to be involved in substrate specificity in *E. coli* (8). AcrB and AcrF are 80% identical in *S. enterica*, with 78% identity over the periplasmic loop regions. This may account for their overlapping substrate profiles. In *E. coli*, it has been shown that AcrA can interact with AcrF (15) or AcrD to form a functional efflux system (8). It is possible that similar interactions can occur between AcrB and AcrE (or AcrF and AcrA) in strains of *S. enterica* serovar Typhimurium from which *acrF* or *acrB* is deleted. In *S. enterica* AcrD shares 64% identity with both AcrF and AcrB. The level of expression of *acrD* was increased upon deletion of either *acrB* or *acrF*, but it did not increase further in the mutant with double gene deletions. It is hypothesized that expression of *acrD* can compensate for deficiencies in either of the other genes but is regulated by an independent mechanism or is simply limited by the amount of AcrA available to form a functional efflux pump module in the membrane. These data indicate that AcrD of *S. enterica* serovar Typhimurium may have a broader substrate range than its *E. coli* counterpart.

A rapid C-RT-PCR method was used to determine the levels of expression of several genes in MAR *S. enterica* serovar Typhimurium. This method had several advantages over others, demanded little method development, and did not require the synthesis of a competitor molecule. Inclusion of an internal 16S rRNA control abolished the need for several control reactions or absolute determination of cDNA concentrations. The multiplex PCR option cut down on cost and increased time efficiency, giving this technique the potential to screen for large numbers of bacterial strains. This C-RT-PCR method can be adapted to determine changes in the levels of expression of any gene and provides an alternative to LightCycler and

microarray techniques without the expense. The screening of fluoroquinolone-resistant bacteria for mutations in topoisomerase genes by using Wave DHPLC analyses is an established method (7, 13). The addition of a C-RT-PCR method now allows isolates to be screened for changes in MAR-linked gene expression by high-throughput analysis.

This is the first documentation indicating increased levels of expression of *acrF* and *acrD* (or *acrB* and *acrD*) in response to deletion of *acrB* (or *acrF*) in salmonella. The levels of expression of *acrF* and *acrD* appeared to be limited to a maximum in both clinical isolates and deletion mutants, regardless of the original parent strain. Maximal levels of expressions of efflux pumps have previously been observed in *E. coli* (39). The AcrAB-TolC efflux pump has recently been shown to be a trimer of AcrB subunits (23). Efflux capacity may be limited by the availability of AcrA and AcrE in the membrane to form functional efflux pumps. Data from the present study suggest that there is a significant coordination in the regulation of efflux systems with overlapping substrate specificities. AcrF and AcrD are both derepressed in response to the deletion of AcrB but are repressed in the presence of a functional AcrB. Interplay between different pump systems is likely to be of importance for the prevention of inappropriate expression of a redundant system that is metabolically wasteful and that may have an effect on membrane stability. A general increased, but limited, level of expression of *acrB*, *acrF*, and *acrD* may be a global response to stressful conditions. This response may be overlaid by a more specific induction of an efflux pump in response to an inducer such as an antibiotic.

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