ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2011, p. 921–924 Vol. 55, No. 2
0066-4804/11/$12.00 doi:10.1128/AAC.00996-10

Expression of Multidrug Efflux Pump Genes *acrAB-tolC*, *mdfA*, and *norE* in *Escherichia coli* Clinical Isolates as a Function of Fluoroquinolone and Multidrug Resistance▽‡

Michelle C. Swick,[1,2] Sonia K. Morgan-Linnell,[2]† Kimberly M. Carlson,[2,3] and Lynn Zechiedrich[1,2,3]*

Interdepartmental Program in Cell and Molecular Biology,[1] Department of Molecular Virology and Microbiology,[2] and Verna and Marrs McLean Department of Biochemistry and Molecular Biology,[3] Baylor College of Medicine, Houston, Texas 77030

Received 20 July 2010/Returned for modification 25 August 2010/Accepted 11 November 2010

In a single quantitative study, we measured *acrA*, *acrB*, *tolC*, *mdfA*, and *norE* expression in *Escherichia coli* clinical isolates by using real-time PCR. *acrA* and *acrB* overexpression strongly correlated with fluoroquinolone and multidrug resistance; *tolC*, *mdfA*, and *norE* expression did not. The order of abundance of efflux pump transcripts in all fluoroquinolone-susceptible isolates was *tolC* (highest), then *acrA* and *acrB*, and then *mdfA* and *norE*. Our findings suggest *acrAB* overexpression is an indicator of multidrug resistance.

Multidrug resistance (MDR) is an increasing public health concern worldwide (7, 11). There is a growing epidemic of multidrug-resistant Gram-negative pathogens and a dwindling arsenal of antibiotic options. MDR is most commonly defined as resistance to ≥3 classes of antibiotics (4). Increased efflux pump expression has been documented in association with resistance to several antibiotic classes, including the fluoroquinolones (reviewed in reference 11). Of more than 40 putative transporters in *Escherichia coli*, *acrAB-tolC*, *mdfA*, and *norE* affect fluoroquinolone MICs when expressed with their own promoters under laboratory growth conditions (14, 17). Only AcrAB-TolC overproduction, however, has been shown to contribute to clinical fluoroquinolone resistance. Additionally, plasmid-borne efflux pump gene *qepA* was found in a small percentage of *E. coli* isolates (10, 16), and it confers resistance to fluoroquinolones and aminoglycosides (9). Despite these findings, the link between efflux pump expression and multidrug resistance in the clinical setting is unclear.

We quantified expression of the efflux pump genes known to affect fluoroquinolone resistance in a single quantitative study. From our earlier study of 214 fluoroquinolone-resistant isolates and 27 fluoroquinolone-susceptible isolates from Ben Taub General Hospital in Houston, TX (2), 24 susceptible isolates and 36 resistant isolates that represented a full range of fluoroquinolone MICs were analyzed. RNA was stabilized in RNAprotect bacterial reagent. RNA was isolated using RNeasy minicolumns (Qiagen, Valencia, CA). RNase-free DNase I was incubated on-column for digestion of genomic DNA. RNA concentrations were assessed by using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Reverse transcription was completed using the ABI high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA). Quantitative PCRs (qPCRs) were performed in triplicate on a 7500 Fast PCR system from Applied Biosystems using 2× *Power* SYBR green chemistry. PCR-grade water served as a negative control. Genomic DNA from the *E. coli* ATCC 25922 strain was the positive control, and its cDNA was the calibrator. The primer concentrations (Table 1) equaled 300 nM, and melt curve analysis ensured that only a single PCR product was amplified.

In our previous study, ~30% of fluoroquinolone-resistant isolates overproduced AcrA, but fluoroquinolone-susceptible isolates had normal AcrA levels (6). Relative to the housekeeping gene *rpsL*, the average levels of expression of *acrA* and *acrB* in the fluoroquinolone-susceptible strains were 1.8- ± 0.7-fold and 2.0- ± 0.6-fold, respectively, compared to those of the ATCC 25922 standard *E. coli* strain. In fluoroquinolone-resistant isolates, the expression level of *acrA* averaged 4.5- ± 2.0-fold (Fig. 1A) and the expression level of *acrB* averaged 4.6- ± 2.5-fold (Fig. 1B). For both genes, the difference between the two groups was significant by Wilcoxon rank sum ($P < 0.001$). Overall, of 37 fluoroquinolone-resistant isolates, 22 overexpressed *acrA* and 25 overexpressed *acrB* more than

TABLE 1. Primers used in this study

Primer	Sequence (5′–3′)	Amplicon length (bp)[a]	% primer efficiency (*E*)[a,b]
acrA-F	CTCTCAGGCAGCTTAGCCCTAA	107	95
acrA-R	TGCAGAGGTTCAGTTTTGACTGTT		
acrB-F	GGTCGATTCCGTTCTCCGTTA	107	95
acrB-R	CTACCTGGAAGTAAACGTCATTGGT		
rpsL-F	GCAAAAACGTGGCGTATGTACTC	104	97
rpsL-R	TTCGAAACCGTTAGTCAGACGAA		
mdfA-F	CATTGGCAGCGATCTCCTTT	103	97
mdfA-R	TTATAGTCACGACCGACTTCTTTCA		
norE-F	CTGGCGGCAGCGGTAA	108	94
norE-R	TGCCATACAGACACCCACCATA		
tolC-F	AAGCCGAAAAACGCAACCT	100	95
tolC-R	CAGAGTCGGTAAGTGACCATC		

[a] Amplicon lengths and primer efficiencies in the rows for forward (F) primers correspond to the respective primer pairs.

[b] As measured by the efficiency equation, $E = 10^{(-1/m)} - 1$, where m is $\Delta C_T/\Delta$[cDNA].

* Corresponding author. Mailing address: Department of Molecular Virology and Microbiology, Baylor College of Medicine, One Baylor Plaza, Mail Stop BCM-280, Houston, TX 77030-3411. Phone: (713) 798-5126. Fax: (713) 798-7375. E-mail: elz@bcm.edu.

‡ Supplemental material for this article may be found at http://aac.asm.org/.

† Present address: Department of Investigational Cancer Therapeutics, M. D. Anderson Cancer Center, Houston, TX 77030.

▽ Published ahead of print on 22 November 2010.

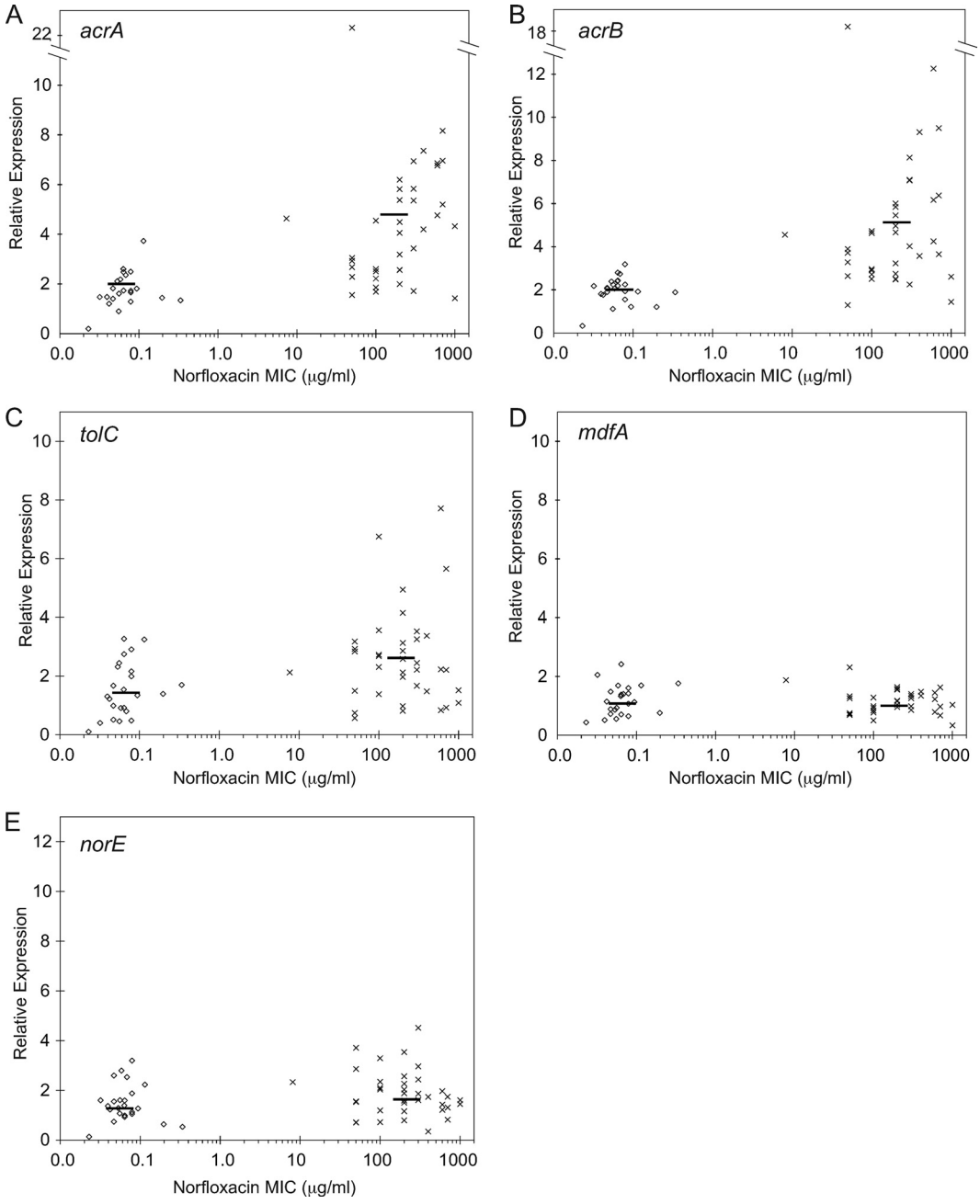

FIG. 1. Multidrug efflux pump expression in *E. coli* clinical isolates. Transcript levels of *acrA* (A), *acrB* (B), *tolC* (C), *mdfA* (D) and *norE* (E) were determined by qPCR and are shown normalized to their expression in the standard *E. coli* strain ATCC 25922, which had a norfloxacin MIC of 0.032 μg/ml. The housekeeping gene *rpsL* was used to calculate relative expression. Data are displayed relative to the MIC (μg/ml) of the historically relevant fluoroquinolone norfloxacin as measured in our laboratory. Isolates were classified as either susceptible (◇) or resistant (×) to fluoroquinolones as determined by the hospital. Each point is the average of three experiments. Lines represent the average relative expression values for all of the isolates in the norfloxacin-susceptible (◇) and -resistant (×) groups. Overexpression was defined as greater than two standard deviations above the mean for the 24 fluoroquinolone-susceptible isolates.

two standard deviations above the respective means for the fluoroquinolone-susceptible isolates. *acrA* and *acrB* coexpression was plotted ($r^2 = 0.75$; Fig. 2A). The best-fit line had a slope of 1.05, fitting the 1:1 ratio expected.

Two fluoroquinolone-resistant *Shigella* clinical isolates overexpressed *tolC* in response to ciprofloxacin (5). Otherwise, *tolC* expression has not been previously assessed. In three fluoroquinolone-resistant isolates, *tolC* expression was increased ~7-fold. Two of the isolates that overexpressed *tolC* also overexpressed *acrA* and *acrB* (Fig. 2B, arrows). Overall, the average *tolC* expression levels (Fig. 1C) of the fluoroquinolone-susceptible and fluoroquinolone-resistant clinical isolates did not differ statistically. *tolC* did not correlate with either *acrA* (Fig. 2B) or *acrB* (data not shown). It does not appear that *tolC* is

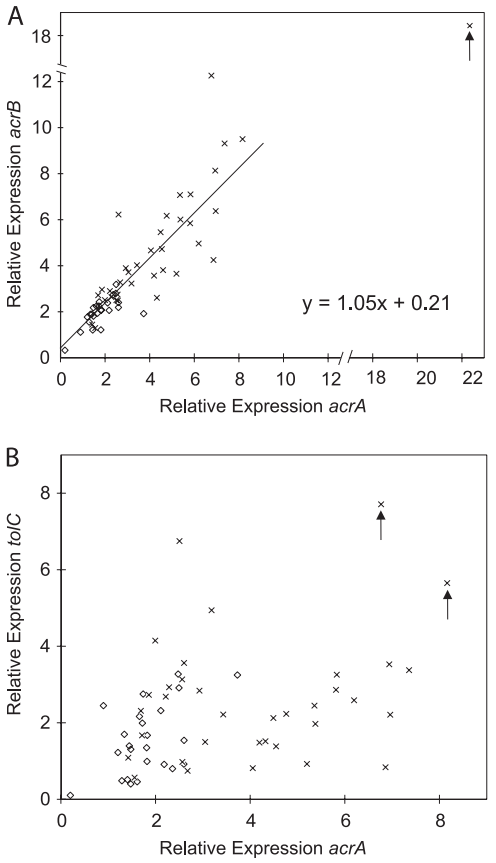

FIG. 2. Correlation of efflux pump expression levels. For each isolate, the transcript levels of *acrB* and *acrA* (A) and *tolC* and *acrA* (B) were plotted. Clinical isolates were either susceptible (◇) or resistant (×) to fluoroquinolones as determined by the hospital. In panel A, isolate ELZ4033 (arrow) was determined to be an outlier by the extreme studentized deviate test statistic and was removed from the best-fit regression. The arrows in panel B denote isolates ELZ4000 and ELZ4001, which significantly overexpressed *acrA* and *tolC* relative to the fluoroquinolone-susceptible isolates ($P < 0.05$).

overexpressed with *acrAB* for acquisition of fluoroquinolone resistance despite the ability of all three genes to respond to MarA regulation (1), which indicates complex regulation differences between these two promoters.

We previously found that known genotypic alterations could not explain the fluoroquinolone MICs in ~30% of the fluoroquinolone-resistant clinical isolates, suggesting that additional unknown mechanisms exist (2, 6). In laboratory strains, the overexpression of *mdfA* or *norE* causes 2- to 4-fold increased ciprofloxacin and norfloxacin MICs but has no effect on levofloxacin MICs (17). Overexpression of *acrAB* and either *mdfA* or *norE* synergistically increases fluoroquinolone MICs (17). In *Shigella*, transcript levels of *ydhE* (*norE*) and *mdfA* were increased in two fluoroquinolone-resistant isolates exposed to ciprofloxacin (5). Thus, increased expression of *mdfA* and *norE* could contribute to fluoroquinolone resistance, especially if combined with overproduction of AcrAB-TolC. Expression levels in fluoroquinolone-susceptible and fluoroquinolone-resistant clinical isolates, respectively, were 1.2- ± 0.5-fold and 1.1- ± 0.4-fold for *mdfA* and 1.5- ± 0.8-fold and 1.8- ± 0.9-fold for *norE* (Fig. 1D and E). Thus, *E. coli* isolates do not stably overexpress *mdfA* or *norE* in fluoroquinolone-resistant clinical isolates.

Although the genes encoding each of the three pumps, AcrAB-TolC, MdfA, and NorE, when overexpressed, increase fluoroquinolone MICs similarly, only the deletion of *acrAB* decreases MICs (8, 17). There are several possible explanations for these findings, but one simple explanation is that *acrAB* is normally expressed at higher levels than *mdfA* and *norE* and thus masks their contributions. Earlier exponential amplification of a gene, as shown by a lower threshold cycle (C_T) value, may be inferred as greater transcript abundance in the template (3); every 3.32 C_T values indicates a 10-fold difference in abundance. In the ATCC 25922 strain, the C_T value was 18.5 for *tolC*, 20.3 for *acrA* and *acrB* (each), and 22.6 and 22.7 for *mdfA* and *norE*, respectively. This order was observed for all fluoroquinolone-susceptible isolates (see Table S1 in the supplemental material).

In spite of the very low prevalence of the *qepA* gene (0.3% in Japan [15] and, to our knowledge, none yet in the United States), we screened 78 of our fluoroquinolone-resistant isolates for *qepA* by colony PCR using the primers 5′-CGAACC GATGACGAAGCACAG and 5′-CTCGCTTCCTGCCCGA GTAT. We found no isolate that harbored this gene.

AcrAB-TolC overproduction affects the MICs of several antimicrobial agents. To determine whether increased *acrAB* expression correlated with MDR, we analyzed drug resistance data generated at the hospital (described in references 2 and 6) for each *E. coli* isolate. We classified the antibiotics that were tested at the hospital into the following classes: aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, monobactams, nitrofurans, penicillins, combination penicillins, and sulfamethoxazole-trimethoprim. We then grouped the isolates into MDR classifications as follows: "non-MDR" if the isolate was resistant to fewer than three drug classes, "$MDR_{(\geq 3)}$" if the isolate was resistant to three or more drug classes, and "$MDR_{(\geq 5)}$" if the isolate was resistant to five or more drug classes. The non-MDR and $MDR_{(\geq 3)}$ classifications were chosen to model the most common definition of MDR (4). $MDR_{(\geq 5)}$ isolates are highly multidrug resistant, akin to extremely drug-resistant (XDR) *Mycobacterium tuberculosis*.

In general, the more severe the MDR phenotype, the higher the probability that the isolate also overexpressed *acrAB* (Table 2). Interestingly, no isolate categorized as $MDR_{(\geq 5)}$ was fluoroquinolone susceptible. While direct drug efflux has been demonstrated for some fluoroquinolones and a few additional agents (12), most of the drugs to which these clinical isolates were resistant have been shown to be unaffected by *acrAB*

TABLE 2. *acrAB* overexpression in MDR isolates

Resistance	No. (%) of isolates with *acrAB* expression	
	Normal	Increased
Non-MDR	19	3 (13.6)
$MDR_{(\geq 3)}$	17	17[a] (50.0)
$MDR_{(\geq 5)}$	5	8[b] (61.5)

[a] Significantly increased relative to the value for the non-MDR classification ($P < 0.05$).
[b] Significantly increased relative to the value for the non-MDR classification ($P < 0.01$).

overexpression or deletion in laboratory experiments (12). This introduces the possibility of an underlying correlation between fluoroquinolone resistance and MDR. Because fluoroquinolones are heavily prescribed (13), there is strong selective pressure for bacteria to become resistant to them. If exposure to other antibiotics occurs prior to exposure to fluoroquinolones, an isolate that subsequently overexpresses *acrAB* following fluoroquinolone treatment might simultaneously become both fluoroquinolone resistant and MDR. Thus, *acrAB* may not cause MDR but rather is indicative of an MDR phenotype in isolates that overexpress it. Regardless of the specific mechanisms through which bacteria become MDR, these data indicate that *acrAB* overexpression is a biomarker for MDR.

We thank Lauren Becnel Boyd, Rainer B. Lanz, and Richard Sucgang of Baylor College of Medicine (Houston, TX) for helpful discussions and critically reading the manuscript, Joseph F. Petrosino of Baylor College of Medicine (Houston, TX) for equipment, and Samuel Eguae of Paul Quinn College (Dallas, TX) for technical assistance during his visiting professorship funded by the American Society of Cell Biology.

M.C.S. is a fellow of the Pharmacoinformatics Training Program of the W. M. Keck Center for Computational and Structural Biology of the Gulf Coast Consortia (NIH grant T90 DK070109). S.K.M.-L. was supported by the Houston Area Molecular Biophysics training programs of the W. M. Keck Center of the Gulf Coast Consortia (NIH grant T32 GM008280). This work was supported by NIH grant R01 AI054830 (to L.Z.).

REFERENCES

1. **Barbosa, T. M., and S. B. Levy.** 2000. Differential expression of over 60 chromosomal genes in *Escherichia coli* by constitutive expression of MarA. J. Bacteriol. **182:**3467–3474.
2. **Becnel Boyd, L., et al.** 2009. Relationships among ciprofloxacin, gatifloxacin, levofloxacin, and norfloxacin MICs for fluoroquinolone-resistant *Escherichia coli* clinical isolates. Antimicrob. Agents Chemother. **53:**229–234.
3. **D'haene, B., J. Vandesompele, and J. Hellemans.** 2010. Accurate and objective copy number profiling using real-time quantitative PCR. Methods **50:** 262–270.
4. **Falagas, M. E., P. K. Koletsi, and I. A. Bliziotis.** 2006. The diversity of definitions of multidrug-resistant (MDR) and pandrug-resistant (PDR) *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. J. Med. Microbiol. **55:**1619–1629.
5. **Kim, J. Y., et al.** 2008. Resistance to fluoroquinolones by the combination of target site mutations and enhanced expression of genes for efflux pumps in *Shigella flexneri* and *Shigella sonnei* strains isolated in Korea. Clin. Microbiol. Infect. **14:**760–765.
6. **Morgan-Linnell, S. K., L. Becnel Boyd, D. Steffen, and L. Zechiedrich.** 2009. Mechanisms accounting for fluoroquinolone resistance in *Escherichia coli* clinical isolates. Antimicrob. Agents Chemother. **53:**235–241.
7. **Nikaido, H.** 2009. Multidrug resistance in bacteria. Annu. Rev. Biochem. **78:**119–146.
8. **Nishino, K., and A. Yamaguchi.** 2001. Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. J. Bacteriol. **183:**5803–5812.
9. **Périchon, B., et al.** 2008. Sequence of conjugative plasmid pIP1206 mediating resistance to aminoglycosides by 16S rRNA methylation and to hydrophilic fluoroquinolones by efflux. Antimicrob. Agents Chemother. **52:**2581–2592.
10. **Périchon, B., P. Courvalin, and M. Galimand.** 2007. Transferable resistance to aminoglycosides by methylation of G1405 in 16S rRNA and to hydrophilic fluoroquinolones by QepA-mediated efflux in *Escherichia coli*. Antimicrob. Agents Chemother. **51:**2464–2469.
11. **Poole, K.** 2005. Efflux-mediated antimicrobial resistance. J. Antimicrob. Chemother. **56:**20–51.
12. **Poole, K.** 2004. Efflux-mediated multiresistance in Gram-negative bacteria. Clin. Microbiol. Infect. **10:**12–26.
13. **SDI/Verispan.** 14 May 2009, posting date. 2008 top 100 branded drugs by retail dollars. http://formularyjournal.modernmedicine.com/formulary/ArticleStandard/Article/detail/598276.
14. **Sulavik, M. C., et al.** 2001. Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. Antimicrob. Agents Chemother. **45:**1126–1136.
15. **Yamane, K., J. Wachino, S. Suzuki, and Y. Arakawa.** 2008. Plasmid-mediated *qepA* gene among *Escherichia coli* clinical isolates from Japan. Antimicrob. Agents Chemother. **52:**1564–1566.
16. **Yamane, K., et al.** 2007. New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an *Escherichia coli* clinical isolate. Antimicrob. Agents Chemother. **51:**3354–3360.
17. **Yang, S., S. R. Clayton, and E. L. Zechiedrich.** 2003. Relative contributions of the AcrAB, MdfA and NorE efflux pumps to quinolone resistance in *Escherichia coli*. J. Antimicrob. Chemother. **51:**545–556.