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Medium Plays a Role in Determining Expression of *acrB*, *marA*, and *soxS* in *Escherichia coli*

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Analysis of expression of *acrB*, *marA*, and *soxS* in rich and minimal media, at early and late logarithmic growth phases, showed that *acrB* had increased expression in minimal medium compared to rich medium, but expression decreased dose dependently upon exposure to ciprofloxacin.

The AcrAB-TolC tripartite efflux pump confers resistance to a wide range of antibiotics, dyes, and biocides and is found in many members of the family *Enterobacteriaceae* (7, 11, 12). Regulation of the pump is provided locally by AcrR, which represses the expression of the cotranscribed *acrAB* (11). The system is also regulated globally by the transcriptional activators MarA and SoxS (1). The *marA* regulon contains a large number of genes including *acrB* (3). The mar operon can be induced by compounds such as salicylate (5). The *soxRS* regulon is induced by compounds such as hydrogen peroxide (1) and also includes *acrB* (8). The expression of genes within the *Escherichia coli* transcriptome has been widely studied, mainly in rich media such as Luria-Bertani or nutrient broth (13, 17). Data reported suggest that many genes are up-regulated in minimal medium ($n = 225$) compared to rich medium (18). Other factors such as cell density and growth rate have also been shown to affect gene expression (9, 15). Furthermore, a number of studies have demonstrated that fluoroquinolone antibiotics can induce a stress response within *E. coli* at high concentrations (14, 16, 19). Prior microarray work in our laboratory (S. J. Cariss and L. J. V. Piddock, unpublished data) surprisingly suggested that ciprofloxacin exposure did not induce expression of known effectors of multiple antibiotic resistance, such as *marA*, when tested in rich medium. We hypothesized that rich media, such as Luria-Bertani, may be inappropriate for the study of these genes. Regulation of efflux systems is complex, and it is possible that elements present in undefined rich medium can affect their expression (M. A. Webber and L. J. V. Piddock, unpublished data). To investigate this, expression of genes that confer ciprofloxacin resistance was determined after exposure to ciprofloxacin in minimal medium. Furthermore, it was hypothesized that previous microarray experiments may have been insensitive to small but statistically significant changes in expression of *acrB*, *marA*, and *soxS* in *E. coli*. Therefore, reverse transcription-PCR was used to measure changes in expression of these genes after ciprofloxacin exposure in this investigation, to see whether similar effects were still observed during growth in minimal medium.

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Escherichia coli MG1655 was used throughout this study (4). The MIC of ciprofloxacin was determined using the agar doubling dilution method (2) and found to be 0.015 μg/ml in both medium types. MG1655 was grown in 20 ml of broth (minimal or rich; Teknova, United States; based upon the work of Neidhardt et al. [10]) overnight at 37°C and subcultured into fresh corresponding medium using a 4% inoculum. Growth phases were defined as follows: early logarithmic phase, rich medium, 1 h, optical density at 660 nm (OD_{660}) = 0.3 (1×10^7 CFU/ml); minimal medium, 4 h, OD_{660} = 0.2 (1×10^7 CFU/ml); late logarithmic phase, rich medium, 2.5 h, OD_{660} = 1.2 (1×10^8 CFU/ml); minimal medium, 6 h, OD_{660} = 0.75 (1×10^8 CFU/ml). On six separate occasions, single cultures were divided into four equal aliquots. Ciprofloxacin was added at 0.5, 1, or 2× MIC; one aliquot served as the antibiotic-free control. The cultures were incubated at 37°C for 30 min at 100 rpm, followed by immediate RNA extraction using a Bioline RNAce Spin Cell minikit (Bioline, United Kingdom). The concentration of RNA in each RNA preparation was quantified using the GeneTools program (Syngene, United Kingdom) and an Agilent 2100 Bioanalyzer automated analysis system (Agilent Technologies, United States). RNA preparations were diluted to 5 mg/ml, and cDNA was synthesized with Superscript III and random hexamers (Invitrogen, United Kingdom). The reaction mixture was incubated at room temperature for 5 min and then in a T-Personal PCR machine (Biometra, United Kingdom) cycled at 50°C for 60 min followed by 70°C for 15 min.

PCRs and denaturing high-pressure liquid chromatography analysis (Transgenomic Ltd., United Kingdom) were performed at least three times from each RNA preparation and were as previously described (6). Primers were designed using Primer software (Table 1). Data were normalized to the expression of 16S rRNA to minimize cell density-dependent errors by calculating the mean 16S expression over all four ciprofloxacin concentrations per RNA preparation. Subsequently, area under the curve values for each test gene from the same biological repeat were normalized as described previously (6). Gene expression changes were expressed as *n*-fold in comparison to the antibiotic-free control. The mean and standard deviation of the six repeats for each data set were calculated, followed by two-tailed, two-sample, equal variance Student *t* tests to compare gene expression over all concentrations against the antibiotic-free control.

TABLE 1. PCR parameters[a]

Primer	Sequence (5′ to 3′)
16SF	GCTAATACCGCATAACGTCG
16SR	TAGCTGGTCTGAGAGGATGA
marAF	ATCGAGGACAACCTGGAATC
marAR	CATTAGGCCAATACATCCGC
soxSF	CGCATGGATTGACGAGCATA
soxSR	TGATATCGCAATGGACCTGG
acrBF	CATGACGCAGGAGGATATCT
acrBR	GGTGGTGAGAACTACGACAT

[a] The annealing temperature was 56°C, the extension time was 1 min, and the primer concentration was 25 μM.

The expression of 16S rRNA was used to normalize expression data, as it was shown to be invariant (data not shown). In minimal medium and with no ciprofloxacin present, during early logarithmic growth phase the expression of *marA* and *soxS* was 1.20-fold and 1.42-fold lower, respectively, than that in rich medium ($P = 0.011$ and 0.043, respectively) (Fig. 1A and B). Expression of *acrB* was 1.49-fold higher in minimal medium ($P = <0.001$) (Fig. 1E). During late logarithmic growth phase with no ciprofloxacin present, there was no significant difference in the expression of either *marA* or *soxS* in rich or minimal medium (Fig. 1B and D), whereas the expression of *acrB* was 1.23-fold higher in minimal medium than in rich medium ($P = <0.001$) (Fig. 1F).

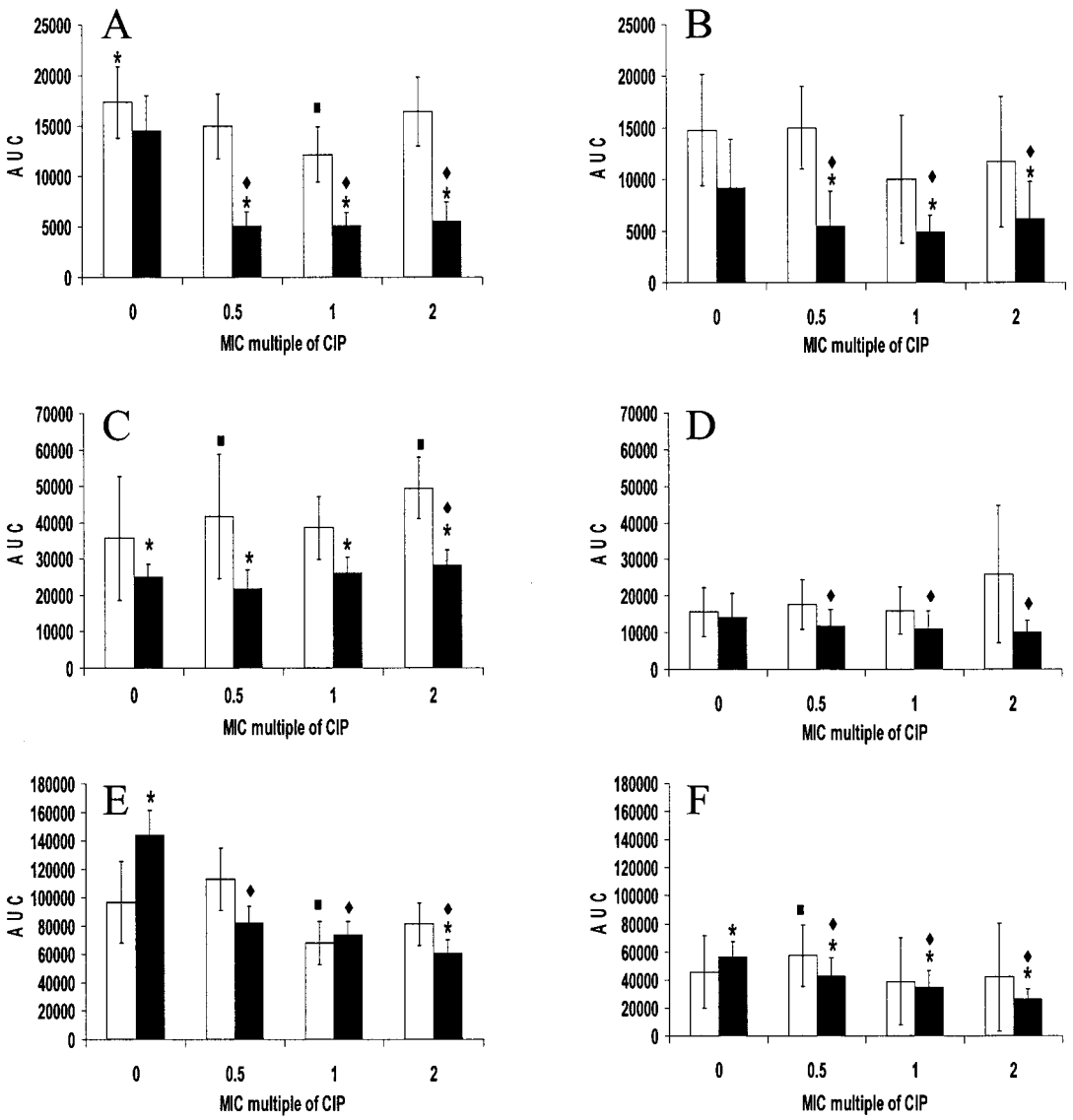

FIG. 1. Expression of (A) *marA* in early logarithmic phase, (B) *marA* in late logarithmic phase, (C) *soxS* in early logarithmic phase, (D) *soxS* in late logarithmic phase, (E) *acrB* in early logarithmic phase, and (F) *acrB* in late logarithmic phase. Unfilled bars, rich medium; filled bars, minimal medium; asterisk, significant difference between corresponding rich and minimal medium pairs at the same MIC and growth phase; square, significant difference between the antibiotic-free control and corresponding MIC multiple (rich medium); diamond, significant difference between the antibiotic-free control and corresponding MIC multiple (minimal medium); error bars, standard deviation of the mean of each value. CIP, ciprofloxacin.

During early logarithmic growth phase in minimal medium, the expression of *marA* significantly decreased upon the addition of ciprofloxacin (Fig. 1A). At 0.5× MIC of ciprofloxacin expression was 2.83-fold less than that in the antibiotic-free control ($P < 0.001$). At the MIC expression was 2.87-fold less ($P < 0.001$), and at 2× MIC expression was 2.60-fold less ($P < 0.001$). The expression of *soxS* remained constant upon the addition of ciprofloxacin, apart from at 2× MIC, when expression was 1.13-fold higher ($P = 0.004$) than that for the antibiotic-free control (Fig. 1C). Expression of *acrB* was 1.75-fold, 1.95-fold, and 2.38-fold lower in a dose-dependent manner at 0.5× MIC, 1× MIC, and 2× MIC of ciprofloxacin, respectively ($P < 0.001$ for all three values) (Fig. 1E).

Addition of ciprofloxacin during late logarithmic growth in minimal medium led to reduced expression of all three genes, compared with the antibiotic-free controls, with the reduction in expression of *soxS* and *acrB* being dose dependent. Expression of *marA* was 1.68-fold, 1.87-fold, and 1.49-fold lower, respectively, over the concentration range (all three, $P < 0.001$) (Fig. 1B). Expression of *soxS* was 1.22-fold, 1.31-fold, and 1.43-fold lower, respectively, in a dose-dependent manner over the concentration range ($P = 0.0110$, 0.0115, and <0.001, respectively) (Fig. 1D). Expression of *acrB* was 1.32-fold, 1.63-fold, and 2.17-fold lower, respectively, also in a dose-dependent manner over the concentration range (all three, $P < 0.001$) (Fig. 1F).

During early logarithmic growth in rich medium, addition of ciprofloxacin had less effect than in minimal medium. Compared with the antibiotic-free control, expression of *marA* was different only at 1× MIC, 1.43-fold lower ($P = 0.0042$) (Fig. 1A). At 0.5× MIC and 2× MIC of ciprofloxacin, expression of *soxS* was 1.15- and 1.13-fold higher, respectively ($P = 0.00336$ and 0.0373, respectively). At 1× MIC, *soxS* expression was not significantly different from that of the antibiotic-free control (Fig. 1C). At 1× MIC, expression of *acrB* was 1.95-fold lower ($P = <0.001$) than the control but constant at 0.5 and 1× MIC of ciprofloxacin (Fig. 1E). During late logarithmic growth phase in rich medium, expression of both *marA* and *soxS* was not significantly different at all concentrations of ciprofloxacin examined (Fig. 1B and D). Expression of *acrB* was similar at 1× MIC and 2× MIC; however, expression was 1.25-fold higher than that in the antibiotic-free control at 0.5× MIC ($P = 0.00284$) (Fig. 1F).

In this study, statistically relevant differential gene expression was observed, with all three conditions tested producing significant changes in gene expression. Expression of *marA*, *soxS*, and *acrB* was significantly higher during early logarithmic phase than during late logarithmic phase in antibiotic-free medium whereas prior work has reported *acrAB* to be expressed maximally during late logarithmic and stationary phases (15). However, under no condition did ciprofloxacin induce expression of *acrB*, *soxS*, or *marA*. The decreases in expression of *marA* and *soxS* in minimal medium compared to rich medium could be part of a global stress response to nutrient limitation, leading to decreased expression of *acrB*, as both *marA* and *soxS* encompass *acrB* in their respective regulons (1, 3). However, it is interesting that, without exposure to ciprofloxacin, *marA* and *soxS* are expressed in lower amounts in minimal medium than in rich medium, whereas expression of *acrB* is higher. The contrast seen between rich and minimal growth media after ciprofloxacin exposure was marked. The observed dose-dependent decreases in minimal medium of *acrB* and *soxS* expression compared to that in rich medium, upon exposure to ciprofloxacin, supports the hypothesis that rich growth medium ameliorates the effect(s) of antibiotic exposure, and possibly other test conditions, upon the *E. coli* transcriptome.

The general decrease of expression of *marA* and *acrB* seen in minimal medium upon antibiotic exposure in the present study is counterintuitive; it would be expected that up-regulation of these three genes would be advantageous, as they confer ciprofloxacin resistance. While the method is insensitive to small gene expression changes as reported in this study, further microarray analysis of the transcriptome under these conditions would allow the range of the effects to be defined at a global level, rather than the focused set of genes used in this study. These experiments would provide insight into the complex interactions between growth conditions and antibiotic exposure and define their end effect upon *E. coli* transcription in vivo. It is clear that the composition of growth medium can greatly affect expression of bacterial genes, and care should be taken to ensure that in vitro experiments include relevant growth conditions to enable changes in genes of interest to be identified. As many pathogens grow in a nutrient-limited environment in the host, it is suggested that in vitro experiments should be performed in minimal medium.

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REFERENCES

1. **Amabile-Cuevas, C. F., and B. Demple.** 1991. Molecular characterization of the *soxRS* genes of *Escherichia coli*: two genes control a superoxide stress regulon. Nucleic Acids Res. **19:**4479–4484.
2. **Andrews, J. M.** 2001. Determination of minimum inhibitory concentrations. J. Antimicrob. Chemother. **48**(Suppl. 1)**:**5–16.
3. **Barbosa, T. M., and S. B. Levy.** 2000. Differential expression of over 60 chromosomal genes in *Escherichia coli*. J. Bacteriol. **182:**3467–3474.
4. **Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277:**1453–1474.
5. **Cohen, S. P., S. B. Levy, J. Foulds, and J. L. Rosner.** 1993. Salicylate induction of antibiotic resistance in *Escherichia coli*: activation of the *mar* operon and a *mar*-independent pathway. J. Bacteriol. **175:**7856–7862.
6. **Eaves, D. J., V. Ricci, and L. J. Piddock.** 2004. Expression of *acrB*, *acrF*, *acrD*, *marA*, and *soxS* in *Salmonella enterica* serovar Typhimurium: role in multiple antibiotic resistance. Antimicrob. Agents Chemother. **48:**1145–1150.
7. **Fernandes, P., B. S. Ferreira, and J. M Cabral.** 2003. Solvent tolerance in bacteria: role of efflux pumps and cross-resistance with antibiotics. Int. J. Antimicrob. Agents **22:**211–216.
8. **Hidalgo, E., J. M. Bollinger, Jr., T. M. Bradley, C. T. Walsh, and B. Demple.** 1995. Binuclear [2Fe-2S] clusters in the *Escherichia coli* SoxR protein. J. Biol. Chem. **270:**20908–20914.
9. **Ihssen, J., and T. Egli.** 2004. Specific growth rate and not cell density controls the general stress response in *Escherichia coli*. Microbiology **150:** 1637–1648.
10. **Neidhardt, F., P. Bloch, and D. Smith.** 1974. Culture medium for enterobacteria. J. Bacteriol. **119:**736–747.
11. **Nikaido, H.** 1996. Multidrug efflux pumps of gram-negative bacteria. J. Bacteriol. **178:**5853–5859.
12. **Okusu, H., D. Ma, and H. Nikaido.** 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J. Bacteriol. **178:**306–308.
13. **Patten, C. L., M. G. Kirchhof, M. R. Schertzberg, R. A. Morton, and H. E. Schellhorn.** 2004. Microarray analysis of RpoS-mediated gene expression in *Escherichia coli*. Mol. Genet. Genomics **272:**580–591.
14. **Piddock, L. J. V., R. N. Walters, and J. M. Diver.** 1990. Correlation of quinolone MIC and inhibition of DNA, RNA, and protein synthesis and induction of the SOS response in *Escherichia coli*. Antimicrob. Agents Chemother. **34:**2331–2336.

15. **Rand, J. D., S. G. Danby, D. L. Greenway, and R. R. England.** 2002. Increased expression of the multidrug efflux genes *acrAB* occurs during slow growth of *Escherichia coli*. FEMS Microbiol. Lett. **207:**91–95.
16. **Shaw, K. J., N. Miller, X. Liu, D. Lerner, J. Wan, A. Bittner, and B. J. Morrow.** 2003. Comparison of the changes in global gene expression of *Escherichia coli*. J. Mol. Microbiol. Biotechnol. **5:**105–122.
17. **Snyder, J. A., B. J. Haugen, E. L. Buckles, C. V. Lockatell, D. E. Johnson, M. S. Donnenberg, R. A. Welch, and H. L. Mobley.** 2004. Transcriptome of uropathogenic *Escherichia coli* during urinary tract infection. Infect. Immun. **72:**6373–6381.
18. **Tao, H., C. Bausch, C. Richmond, F. R. Blattner, and T. Conway.** 1999. Functional genomics: expression analysis of *Escherichia coli* growing. J. Bacteriol. **181:**6425–6440.
19. **Walters, R. N., L. J. Piddock, and R. Wise.** 1989. The effect of mutations in the SOS response on the kinetics of quinolone killing. J. Antimicrob. Chemother. **24:**863–873.