Regulation of acrAB expression by cellular metabolites in Escherichia coli

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Objectives: Multidrug efflux pumps mediate resistance to antibiotics and other toxic compounds. We studied the role of AcrAB-TolC, the main efflux pump in Escherichia coli, in regulating gene expression.

Methods: Deletion mutants, an acrAB-lacZ fusion and reverse transcription–real-time quantitative PCR experiments were used to study the role of AcrAB-TolC and metabolism in regulating gene expression of the acrAB operon and its transcriptional regulators.

Results: Deletion of the acrB gene increased the expression of the acrAB operon. A similar induction of acrAB was found when acrA or tolC was deleted, and when the pump function was inhibited using phenylalanine-arginine-β-naphthylamide. The induction of acrAB in the ΔacrB strain was totally (AcrR or SoxS) or partially (SoxR or MarA) prevented when the genes for these acrAB regulators were also deleted. The expression of soxS and marA, but not of acrR, was increased in the ΔacrB strain, which also showed altered expression of many other genes related to different cellular processes, including motility. Deletion of the metabolic genes entA and entE (enterobactin biosynthesis), gpx (glutathione peroxidase), cysH (cysteine biosynthesis) and purA (purine biosynthesis) also prevented activation of the acrAB promoter in the ΔacrB strain. Addition of the enterobactin biosynthesis intermediate metabolite 2,3-dihydroxybenzoate induced the expression of acrAB.

Conclusions: These results together suggest a model in which the AcrAB-TolC pump effluxes cellular metabolites that are toxic and/or have a signalling role. If the pump is inactivated or inhibited, these metabolites would accumulate, inactivating AcrR and/or up-regulating soxS and marA expression, ultimately triggering the up-regulation of acrAB expression to restore homeostasis.

Keywords: AcrAB-TolC, multidrug efflux, gene regulation, acrR, soxS, marA

Introduction

AcrAB-TolC is the main multidrug efflux pump in Escherichia coli and other Enterobacteriaceae, and its overexpression is commonly found in multidrug-resistant clinical isolates. The AcrAB-TolC pump effluxes many different classes of antibiotics, including β-lactams, fluoroquinolones and tetracyclines, host factors such as bile salts and antimicrobial peptides, and many other toxic compounds such as acriflavine, triclosan, detergents, dyes and organic solvents.1–3

AcrAB-TolC is a tripartite transporter that captures substrates from the periplasm and effluxes them across the outer membrane and out of the cell. It is composed of the proteins AcrA, AcrB and TolC.1,2 AcrB is an inner membrane resistance–nodulation–cell division efflux protein that also extends into the periplasm, AcrA is a periplasmic adaptor protein and TolC is the outer membrane channel for this pump and at least eight other efflux pumps in E. coli.1,2 However, none of these other pumps seems to significantly contribute to resistance to most drugs in the presence of a functional AcrAB-TolC pump.3–5 The three proteins are encoded in two separate operons, acrAB and tolC-ygiAB, whose expression is activated by the transcriptional regulators MarA, SoxS and Rob.3 The regulator AcrR specifically represses acrAB and its own transcription.3,6

Besides its role in the efflux of exogenous toxic compounds, the AcrAB-TolC pump affects virulence in Salmonella.7 In E. coli, AcrB is also involved in contact-dependent growth inhibition.8 Furthermore, mutants lacking tolC have pleiotropic phenotypes, such as defects in cell division and growth when cultured in minimal glucose medium,9 altered intra- and extracellular concentrations...
of some metabolites like cAMP, porphyrins, cysteine and enterobactin (see Zgurskaya et al.10 for a review) and increased expression of marA, soxS and MarA/SoxS/Rob-regulated genes and increased Rob activity.11 However, the mechanisms and pump(s) involved in these tolC-associated phenotypes remain unknown because inactivation of acrAB or other known TolC-dependent efflux pumps singly did not reproduce the tolC phenotypes.9–11

We have found that the AcrAB-TolC pump regulates the expression of the acrAB operon in response to cellular metabolism. It does so by affecting the expression or activity of specific acrAB transcriptional regulators.

Materials and methods

Growth conditions

Cultures were grown in lysogeny broth (LB) medium (per L: 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl) at 37°C with agitation, except for those experiments described in Figure 1(a) to have been performed on M9 medium (per L: 6 g of Na2HPO4, 3 g of KH2PO4, 0.5 g of NaCl, 1 g of NH4Cl, 1 mM of MgSO4, and 0.2% glucose or glycerol). Antibiotics were used at 100 mg/L (ampicillin), 50 mg/L (kanamycin) and 25 mg/L (chloramphenicol).

Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype/relevant characteristics</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli strains</td>
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<tr>
<td>BW25113</td>
<td>(wild-type) F-, λ-, Δ(arad-araB)567, ΔlacZ4787::ramB-3, rph-1, Δ(rhaD-rhaB)568, hsdR514</td>
<td>CGSC (Keio)13</td>
</tr>
<tr>
<td>CR5000</td>
<td>BW25113 ΔacrB</td>
<td>this studya</td>
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<td>BW25113 ΔacrA</td>
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<tr>
<td>JW5503</td>
<td>BW25113 ΔtolC::kan</td>
<td>CGSC (Keio)13</td>
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<tr>
<td>CR7061</td>
<td>BW25113 ΔacrB ΔtolC::kan</td>
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<td>BW25113 ΔacrR</td>
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<td>BW25113 ΔacrB ΔmarR</td>
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<td>BW25113 Δrob::kan</td>
<td>CGSC (Keio)13</td>
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<td>BW25113 ΔacrB ΔacrR ΔsoxS::kan</td>
<td>P1 JW5503 x CR7021</td>
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<td>Other ΔacrB regulatory gene mutants (ΔmarA::kan, Δrob::kan, ΔsoxR::kan or ΔsoxS::kan derivatives of CR5000; Figure 2), KanR</td>
<td>P1 Keo mutant x CR5000</td>
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<td>Metabolic gene mutants (BW25113 derivatives; Figure 4), KanR</td>
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<td>ΔacrB metabolic gene mutants (CR5000 derivatives; Figure 4), KanR</td>
<td>P1 Keo mutant x CR5000</td>
<td></td>
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</tbody>
</table>

| Plasmids | | |
| pkD4 | template for amplifying the kan gene, KanR | 14 |
| pCP20 | plasmid for excision of kan markers by FLP-mediated site-specific recombination, AmpR, ChlR | 14 |
| pNN608 | single copy, acrABp-lacZ, ChlR | 6 |

9These strains were obtained by excising the kanamycin cassette from the corresponding Keio collection mutants13 obtained from the CGSC.
10Deletion of acrB in CR7021 or of marR in CR7035 were constructed using CR5000 as the parental strain and the λ Red recombinase method to create ΔacrB::kan or ΔmarR::kan derivatives whose kanamycin cassette was then removed using plasmid pCP20. Maintenance of the acrB deletion and the in-frame deletion of acrB or marR were confirmed by PCR amplification and sequencing using specific primers flanking the deleted genes.

Strains, plasmids and general genetic procedures

The bacterial strains and plasmids used in this study are listed in Table 1. PCR, phage P1 transduction to transfer KanR gene deletions between strains, and plasmid electroporation were performed according to standard procedures.12 KanR gene deletions were either obtained from the Keio collection13 via the E. coli Genetic Stock Center at Yale University (CGSC), or constructed using the λ Red recombinase method,14 plasmid pKD4 and the corresponding primers described in the Keio collection webpage (http://www.shigen.nig.ac.jp/ecoli/strain/top/top.jsp) for each gene to be deleted. Briefly, these primers were used to generate a PCR product of the kan gene of pKD4 with sequences flanking the desired gene at both ends. This product was used to replace the desired wild-type gene in the strain of interest, which was confirmed by PCR amplification and sequencing using specific primers flanking the deleted gene. When necessary, removal of the kanamycin cassette of constructed deletion mutants or Keio collection strains was performed using plasmid pCP20 as previously described,15 and confirmed by PCR amplification and sequencing using specific primers flanking the deleted genes. DNA sequencing was performed at the Tufts University Core Facility.
described. Briefly, cells were grown overnight, diluted 1:1000 in fresh LB and grown for 3 h to about 0.3 OD600. Then, the total RNA in each culture was stabilized using RNAProtect Bacteria Reagent (Qiagen), isolated by using an RNeasy Mini Kit (Qiagen) and two DNA removal steps, and its purity and concentration determined in a NanoDrop ND-1000 spectrophotometer. The RNA was reverse transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen). The obtained cDNA was then quantified in an Mx3000P detection system (Stratagene) using QuantiTect SYBR Green qPCR Master Mix from Qiagen and gene-specific standard plots. For each gene studied, the specific primers used for the RT and qPCR reactions are described elsewhere. \(^\text{16}\) gapA, which encodes the GAPDH enzyme, was used as a control gene according to Viveiros et al. \(^\text{16}\)

**β-Galactosidase assays**

The expression of the \(acrABp\)-lacZ transcriptional fusion in single-copy plasmid pNN608 was determined by measuring β-galactosidase activity from exponential phase or stationary (overnight) phase cultures as previously described. \(^\text{17}\) All experiments were performed using cells grown in LB medium except for those samples specified in Figure 1(a).

**Motility experiments**

Migration through (swimming) or across (swarming) semisolid agar was determined using LB plates supplemented with 0.3% or 0.6% (w/v) agar, respectively. Plates were inoculated with each strain by stabbing them with a sterile toothpick. The zones of migration through the agar were measured after 16, 24 and 48 h incubation at 30°C. Data were obtained in at least three separate experiments, each performed in duplicate.

**Statistical analysis**

Statistically significant differences in RT-qPCR, β-galactosidase and motility experiments were identified with the t-test (two independent samples with equal variance, two-tailed distribution) using Microsoft Excel 2010 software.

**Microarray experiments**

Cells were grown in LB medium and the total RNA in each culture was extracted as described in the ‘RNA experiments’ section. Three independent cultures of the wild-type (BW25113) and \(acrB\) (CR5000) strains were used. RNA quality and concentration were determined in an Agilent 2100 BioAnalyzer. RNA samples were then processed, hybridized to GeneChip E. coli Genome 2.0 arrays (Affymetrix) and analysed in the Keck Biotechnology Resource Laboratory at Yale School of Medicine. Determination of statistically differentially expressed genes was performed on the microarray data in the Computational Genomics Core at Tufts University. The full lists of genes up- or down-regulated in the \(acrB\) mutant compared with the wild-type are given in Table S1 (available as Supplementary data at JAC Online).

**Results**

**Inactivation or inhibition of AcrAB-ToIC activates the acrAB promoter**

Using an \(acrABp\)-lacZ fusion and measuring β-galactosidase activity from cells grown in LB medium, we found that the deletion of \(acrB\) increased the activity of the \(acrAB\) promoter. The increase was similar in cells in the exponential phase (2.2-fold, data not given in Table S1 (available as Supplementary data at JAC Online).}

**Figure 1.** Effect of AcrAB-ToIC inactivation or inhibition on \(acrAB\) expression. (a) \(acrABp\)-lacZ expression in the wild-type and \(acrB\) strains measured by β-galactosidase assay using cells grown in different culture media. Statistically significant differences between both strains in each medium are shown as **\(P<0.01\) or ***\(P<0.0001\). The exact \(acrB^+/acrB^+\) ratios—i.e. the induction of \(acrAB\) expression in the \(acrB\) mutant compared with the wild-type—are shown above each pair of bars. (b) Effect of deletion of different components of the AcrAB-ToIC pump on \(acrABp\)-lacZ expression measured using cells grown in LB. Significant differences between each mutant and the wild-type are shown as **\(P<0.01\) or ***\(P<0.0001\). (c) Cells were grown in LB medium in the presence of increasing concentrations of the efflux pump inhibitor PA\(\beta\)N to measure its effect on \(acrABp\)-lacZ expression. Statistically significant differences between the wild-type and the \(acrB\) mutant both treated with the same concentration of PA\(\beta\)N were found at concentrations <100 \(\mu\)M (**\(P<0.0001\) but not >100 \(\mu\)M (\(P>0.09\). (a–c) All results are presented as average ± SEM (\(n=3–4\) and are shown normalized to \(acrABp\)-lacZ expression in the wild-type strain grown in LB. WT, wild-type.
Figure 2. Effect of deletion of acrAB regulators on ΔacrB-mediated induction of acrABp-lacZ expression. Known regulators of acrAB were each deleted in both the wild-type (acrB⁺, light grey) and ΔacrB (acrB⁻, dark grey) parental strains to assess their role in ΔacrB-mediated induction of acrABp-lacZ expression; i.e. their effect on the acrB⁺/acrB⁻ ratio, which is shown above each corresponding pair of bars. The experiments were performed using cells grown in LB medium. The results are presented as average ± SEM (n=4) and are shown normalized to acrABp-lacZ expression in the wild-type (acrB⁺ parental) strain. Statistically significant differences between the acrB⁺/acrB⁻ acrABp-lacZ ratio in each mutant compared with the ratio (2.3) of the parental strains are shown as *P<0.02 or **P<0.0001.

shown) and the stationary phase (2.3-fold; Figure 1a). These findings are in agreement with a previous result showing 1.5-fold induction of the acrAB promoter in exponential phase cells grown in L-broth in a strain lacking acrAB. When the cells were grown in 0.2% glucose- or 0.2% glycerol-minimal medium, the basal level of acrAB expression was lower but the induction of the acrAB promoter in the ΔacrB mutant was even larger (3- to 4-fold) than in cells grown in LB (Figure 1a). This finding indicates that the induction of the acrAB promoter found in the ΔacrB mutant was not caused by lack of efflux of any toxic compound specifically present in LB medium. For this reason, and in agreement with earlier studies on the role of efflux on gene expression, the inducer of acrAB expression was eliminated using the efflux pump inhibitor phenylalanine-arginine-β-naphthylamide (PBN) (Figure 1c). When wild-type cells were grown in the presence of increasing concentrations of PBN, we observed dose-dependent induction of acrAB expression. At 250 μM PBN, the expression of the acrAB promoter in wild-type cells increased to a level similar to that of the ΔacrB mutant grown without PBN. Of note, this concentration of PBN is similar to those used by Kern et al. and Coldham et al. (192 μM and 333 μM, respectively) to study the effect of this efflux pump inhibitor on drug accumulation and susceptibility to antibiotics. In ΔacrB cells, however, addition of PBN had no effect on the acrAB promoter (Figure 1c). These results show that it is not its physical presence but its function that makes AcrAB-ToLC influence its own expression.

**AcrR, SoxRS and MarRA mediate feedback regulation of acrAB expression**

Known regulators of acrAB were each deleted in both the wild-type (acrB⁺) and ΔacrB (acrB⁻) strains to determine their effect on ΔacrB-mediated induction of acrAB, which we measured as the ratio of acrB⁺ expression between the ΔacrB⁻ and ΔacrB⁺ strains (Figure 2). Deletion of acrR or soxS eliminated most of the ΔacrB-mediated induction of acrAB. Deletion of marA, and of the soxS regulator soxR, produced a smaller reduction in ΔacrB-mediated induction of acrAB, and the deletion of rob and the marA regulator marR produced no effect (Figure 2). The lack of induction of acrAB in the double ΔacrR ΔacrB mutant compared with the ΔacrB mutant is not because acrAB expression is already maximal in the ΔacrR strain, since we have found 2-fold further activation of the acrAB promoter in both mutants grown in the presence of external inducers (C. Ruiz and S. B. Levy, unpublished results).

We then studied whether the induction of acrAB expression in the ΔacrB strain was accompanied by and could possibly be the result of a change in the expression of these regulators. We found a ~2-fold increase in the expression of soxS and marA in the ΔacrB strain compared with the wild-type (Figure 3). We also found that the induction of soxS expression by the deletion of acrB did not occur in strains in which soxR was also deleted (data not shown). We have previously shown that the inactivation of
acrB does not induce marA expression in strains also inactivated for marR. Finally, we found no change in the expression of acrR in the ΔacrB strain compared with the wild-type (Figure 3). Combined, these results suggest that ΔacrB-mediated induction of acrAB expression is caused by a decrease in AcrR activity, not in acrR expression, and by an increase in soxS and marA expression mostly mediated by soxR and marR, respectively.

Deletion of five different metabolic genes prevents feedback regulation of acrAB expression

Given that acrR, soxRS and marRA are known to respond to specific chemicals, we hypothesized that a change in their activity/expression, and thus the ΔacrB-mediated induction of acrAB expression, was ultimately caused by the accumulation of cellular metabolites normally effluxed by the AcrAB-ToIC pump. To obtain data on the nature of these metabolites, we studied the effect on ΔacrB-mediated acrAB induction of the deletion of genes from metabolic pathways known to affect the expression or activity of acrAB, acrR, soxS or marR.34 We also studied metabolic genes whose expression was dramatically altered in the ΔacrB mutant compared with the wild-type in microarray experiments (Table 2).

Of the 29 genes selected for testing (Figure 4), the deletion of eight of them induced acrAB expression in the wild-type (acrB+) background. Deletion of entA, trpE, metE, acrB, glpB and glpC produced a small induction of acrAB expression (1.2-fold to 1.5-fold). Deletion of entE or glpX induced acrAB expression 2.2-fold (Figure 4). This induction was similar to that found in the strain deleted for acrB (Figure 1a).

Interestingly, the deletion of only three of these genes (entA, entE and glpX), and of two genes whose deletion did not significantly affect acrAB expression in the wild-type background (cysH and purA), decreased the ΔacrB-mediated induction of acrAB expression, i.e. reduced the acrB-ΔacrB+ ratio (Figure 4). Such reduction was partial for entA, cysH and purA, and nearly complete for entE and glpX (Figure 4). The fact that these five genes belong to four different metabolic pathways: enterobactin (entA and entE), cysteine (cysH) and purine (purA) biosynthesis, and gluconeogenesis (glpX), strongly suggests that ΔacrB-mediated induction of acrAB is caused by the accumulation of cellular metabolites from different metabolic pathways.

Addition of 2,3-dihydroxybenzoate (DHB), but not of fructose 1,6-biphosphate (F1,6P), induces acrAB expression via marRA

The two metabolic mutants with the strongest effect on acrAB regulation—entE and glpX—would be expected to accumulate DHB and F1,6P, respectively, based on the step of enterobactin biosynthesis or gluconeogenesis, respectively, that is blocked in these mutants (i.e. EntE uses ATP to catalyse the conversion of DHB into DHB-AMP, which is subsequently used by the enterobactin

### Table 2. Summary of the most significantly up- or down-regulated genes in the ΔacrB mutant relative to the wild-type found by microarray experiments

| Gene/operon/function | Fold change | SEM
|----------------------|-------------|-----
| **Activated** | | |
| motility and flagellar biosynthesis | 58.5/22.9 | |
| flhBAE | 6.8–5.1 | |
| marRA | 2.2–1.5 | |
| predicted protein | 16.8 | |
| Fe-S cluster assembly scaffold complex | 3.7–2.2 | |
| **Repressed** | | |
| predicted conserved protein | 5.1 | |
| yj/E | 8.0/9.1 | |
| glpX | 13.8–3.8 | |

Full lists of up- and down-regulated genes are given in Table S1. The fold activation or repression in gene expression in the ΔacrB mutant relative to the wild-type. Only genes having a 3-fold change or larger that is also statistically significant (P < 0.05) are shown. For genes belonging to the same operon, the range between the gene showing the greatest change (usually the first gene in the operon) and the gene showing the smallest change (usually the last gene in the operon) is provided. When an operon has only two genes, the fold change for both genes is shown separated by ‘/’. Of note, glycerol and glycerol 3-phosphate transport and metabolism genes (glpABCD, glpD, glpFK and glpTQ) were strongly activated (13.8-fold to 3.8-fold) and dipeptide and haem transporter genes (dppABCDF) were strongly repressed (5.7-fold to 3.3-fold) in the ΔacrB mutant relative to the wild-type, although neither of these changes was statistically significant (P > 0.05).

The ΔacrB mutant also showed overexpression of the flagellar biosynthesis and motility genes flhDC (2.5-fold to 2.2-fold) and flIC (1.4-fold), although not statistically significant.

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synthase complex to synthesize enterobactin,\(^{23}\) and GlpX catalyses the hydrolysis of F1,6P to fructose 6-phosphate,\(^{24}\) which is later converted into glucose 6-phosphate.\(^ {25}\) We studied whether these two cellular metabolites added to cultures would induce \(acrB\) expression. DHB is a small, weak acid/salt similar to salicylate, which diffuses spontaneously into the cytosol. F1,6P, like fructose 6-phosphate, is rapidly taken up by \(E.\ coli\) and its concentration within the cell becomes equal to that in the medium in \(<5\) min.\(^ {25}\) Such uptake is likely to be mediated by UhpT, which transports fructose 6-phosphate and other phosphate sugars into the cells.\(^ {26}\)

When the wild-type strain was grown in the presence of DHB, we found a statistically significant 1.6-fold induction of \(acrAB\) expression, whereas no significant increase was found for F1,6P (Figure 5a). Similar results were obtained when wild-type cells were grown to exponential phase in the absence of these compounds, followed by the addition of the metabolites and the measurement after 45 min of their effect on \(acrAB\) expression (data not shown). We also tested which regulator(s) were involved in the DHB-mediated induction of \(acrAB\) expression. Inactivation of \(acrR\), soxS or soxR had little effect (data not shown), whereas inactivation of \(marA\) (completely) and \(marR\) (partially) prevented \(acrAB\) induction by DHB (Figure 5b).

**Inactivation of the AcrAB-TolC pump affects the expression of many different genes and motility**

Given that AcrAB-TolC affects its own expression, potentially via the efflux of multiple cellular metabolites, we asked whether deletion of the pump also affects the expression of other genes and other cellular functions. Microarray experiments comparing gene expression in the \(ΔacrB\) mutant with that in the wild-type strain revealed that inactivation of the AcrAB-TolC pump caused strong induction of genes involved in motility and flagellum biosynthesis, and repression of Fe-S cluster assembly scaffold complex genes (see Table 2 for a summary and see Table S1 for full data). Despite these many changes, we did not find differences between the \(ΔacrB\) and wild-type strains in growth rate (\(μ = 1.46 \pm h^{-1}\) for both strains) or yield, and we did not observe defects in cell morphology or division by light microscopy in the \(ΔacrB\) mutant (data not shown; experiments performed in LB medium). However, in agreement with the strong induction of flagellum biosynthesis genes found in the \(acrB\) mutant, it showed increased motility in 0.3% (w/v) LB agar plates (swimming) (60% larger diameter after 16 h), but not in 0.6% (w/v) LB agar plates (swarming), as compared with the wild-type (Table 3). Inactivation of soxS, but not of \(marA\), prevented the \(ΔacrB\)-mediated increase in motility in 0.3% (w/v) LB agar plates (Table 3).

**Discussion**

Beyond their role in the efflux of antibiotics and other exogenous toxic compounds, there is an increasingly recognized role for TolC-dependent efflux pumps in cellular processes such as cell division and growth,\(^ {9}\) metabolism\(^ {10}\) and regulation of gene expression.\(^ {11}\) However, the mechanisms and individual efflux pumps involved are still poorly understood. Here, we have focused on AcrAB-TolC, the main multidrug efflux pump that mediates multidrug resistance in \(E.\ coli\), and whose overexpression has recently been suggested to precede and facilitate high-level resistance development mediated by target site mutations.\(^ {27}\)
We found that inactivation or inhibition of AcrAB-TolC activated the acrAB promoter (Figure 1). Such activation was mediated by three loci known to regulate acrAB: acrR, soxRS and marRA (Figure 2). Expression of soxS and marA was increased in the absence of the AcrAB-TolC pump (Figure 3). The role of acrR appears to be related to a decrease in the activity of the AcrR protein, since acrR expression was not altered in the ΔacrB mutant. Since AcrR also represses its own expression, it seems that the acrR and acrAB promoters differ in their response to the potentially less active AcrR protein, or to lower amounts of active AcrR. This different response might be related to the combined effect in the acrAB promoter of altered AcrR activity and increased levels of MarA and SoxS, which are not known to affect the acrR promoter.

Activation of the acrAB promoter seemed to be ultimately caused by the accumulation of cellular metabolites from at least four different metabolic pathways: enterobactin, cysteine and purine biosynthesis, and gluconeogenesis (Figure 4). These findings suggest a feedback regulatory circuit involved in the regulation of acrAB expression in response to AcrAB-TolC function. We propose a model (see Figure 6) in which the absence of a functional AcrAB-TolC pump leads to the accumulation of cellular metabolites produced by these four—and maybe other—metabolic pathways, which are usually effluxed by this pump. Such accumulation would then result in the inactivation of AcrR and the induction of soxS and marA expression, ultimately triggering up-regulation of acrAB expression to restore homeostasis.

Table 3. Bacterial motility in LB medium supplemented with 0.3% agar (swimming)

<table>
<thead>
<tr>
<th>Strain</th>
<th>16 h (mm) ± SEM</th>
<th>24 h (mm) ± SEM</th>
<th>48 h (mm) ± SEM</th>
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<tr>
<td>wild-type</td>
<td>7.0 ± 0.2</td>
<td>17.1 ± 1.4</td>
<td>50.2 ± 1.4</td>
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<tr>
<td>ΔacrB</td>
<td>11.0 ± 0.6</td>
<td>21.7 ± 1.3</td>
<td>58.6 ± 1.6</td>
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<tr>
<td>ΔmarA</td>
<td>8.3 ± 0.3</td>
<td>16.8 ± 1.4</td>
<td>47.7 ± 2.3</td>
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<tr>
<td>ΔacrB ΔmarA</td>
<td>12.0 ± 0.6</td>
<td>22.5 ± 1.5</td>
<td>56.4 ± 4.1</td>
</tr>
<tr>
<td>ΔsoxS</td>
<td>12.0 ± 0.7</td>
<td>21.3 ± 1.1</td>
<td>57.3 ± 1.1</td>
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<tr>
<td>ΔacrB ΔsoxS</td>
<td>12.4 ± 0.9</td>
<td>21.6 ± 2.0</td>
<td>61.2 ± 3.8</td>
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<td>Ratio acrB⁻/acrB⁺</td>
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<tr>
<td>ΔacrB/wild-type</td>
<td>1.6—a</td>
<td>1.3—a</td>
<td>1.2—a</td>
</tr>
<tr>
<td>ΔacrB ΔmarA/ΔmarA</td>
<td>1.4—a</td>
<td>1.3—a</td>
<td>1.2</td>
</tr>
<tr>
<td>ΔacrB ΔsoxS/ΔsoxS</td>
<td>1.0</td>
<td>1.0</td>
<td>1.1</td>
</tr>
</tbody>
</table>

*aSignificantly different motility between the pair of acrB⁻ and acrB⁺ strains (P<0.05; n = 3–6).

Figure 5. Effect of externally added cellular metabolites on acrAB expression. (a) Wild-type cells were grown in LB supplemented with the metabolites DHB (4 mM; a similar concentration to that used in Chubiz and Rao19) or F1,6P (5 mM) to measure their effect on acrABp-lacZ expression. (b) The effect of DHB was also measured in strains deleted for marA or marR. The results are presented as average ± SEM (n = 4) and are shown normalized to acrABp-lacZ expression in each strain grown without metabolite. Statistically significant differences for each strain between cells grown with and without metabolite are shown as *P<0.02 or ***P<0.0001. WT, wild-type.
A role of AcrAB-TolC in the efflux of cellular metabolites was first proposed by Helling et al. They found that four different metabolic mutants (icdA, purB, cysH and metE) showed low-level resistance to nalidixic acid and increased levels of acrAB. For icdA and purB, nalidixic acid resistance required soxS plus either marA or rob. The accumulation of unknown cellular metabolites normally excreted by the AcrAB-TolC pump accumulate in the ΔacrB strain, inactivating AcrR and inducing the expression of soxS and marA, ultimately up-regulating the expression of acrAB to restore homeostasis. Functional interactions are represented as arrows for activation/induction or as ‘^’ for repression. Continuous lines indicate known interactions and broken lines indicate hypothetical interactions. DHB and two other putative metabolites are depicted as small shapes.

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Figure 6. Proposed model for the regulation of acrAB expression by cellular metabolites. As detailed more extensively in the Discussion section, our results suggest that DHB and other unknown cellular metabolites normally excreted by the AcrAB-TolC pump accumulate in the ΔacrB strain, inactivating AcrR and inducing the expression of soxS and marA, ultimately up-regulating the expression of acrAB to restore homeostasis. Functional interactions are represented as arrows for activation/induction or as ‘^’ for repression. Continuous lines indicate known interactions and broken lines indicate hypothetical interactions. DHB and two other putative metabolites are depicted as small shapes.
inaA expression in tolC mutants but not in acrB mutants may be additionally dependent on regulators of inaA other than marA and soxS, in the same way that AcrR is important for the regulation of acrAB. In fact, our microarray studies showed no significant increase in inaA expression in the ΔacrB mutant, despite it showing increased expression of soxS and marA.

Alternatively, oxidative stress has been proposed to be responsible for some of the effects found in tolC mutants, i.e. overexpression of the membrane stress-induced protein PsP, and metabolic changes leading to defects in cell division and growth in cells grown in minimal glucose medium but not those grown in LB. Since we found increased soxS expression and an important role for SoxRS in the induction of acrAB expression in the ΔacrB mutant, we cannot discard the possibility that oxidative stress also contributed to our results. The two hypotheses are not mutually exclusive because the accumulation of certain metabolites may lead to oxidative stress. However, our results seem to support the metabolite hypothesis as the more likely explanation for most of the phenotypes we observed. In fact, we have found that at least one cellular metabolite, DHB, induces the acrAB operon. Moreover, we found no altered expression of pspA in the ΔacrB mutant (Table S1); and we also found a 2-fold to 4-fold repression of sufABCDS (Table 2), an operon known to be induced by superoxide generators and hydrogen peroxide.29

Our studies have revealed several metabolic pathways whose inactivation induced acrAB expression and/or affected acrAB induction in the ΔacrB mutant (Figure 4), suggesting that the observed phenotypes are the result of the cumulative effect of more than one metabolite. Two of these pathways, gluconeogenesis and enterobactin biosynthesis, seem to play a particularly important role. Blocking these two pathways by using gene deletions builds up precursors that we hypothesize are capable of inducing acrAB expression. We also hypothesize that such precursors accumulate in the ΔacrB strain, which would explain why this strain also showed increased activation of the acrAB promoter.

Deletion of the gluconeogenesis gene glpX strongly increased acrAB expression in the wild-type strain and also prevented ΔacrB-mediated activation of the acrAB promoter (Figure 4). However, when we tested whether this effect was caused by the accumulation of the GlpX substrate F1,6P, by adding this metabolite to the culture medium, we found almost no induction of the acrAB operon (Figure 5a). As we did for the enterobactin pathway, it will be necessary to study additional gene deletions of the other upstream and downstream enzymes in the pathway to find out whether the induction of acrAB expression found in the glpX mutant is caused by the accumulation of a precursor of F1,6P, by the lack of an F1,6P downstream product or by any inactivation of the pathway itself.

Inactivation of the enterobactin biosynthetic genes entA or entE also strongly increased acrAB expression in the wild-type strain and prevented ΔacrB-mediated activation of the acrAB promoter; however, inactivation of the other genes of the enterobactin pathway had no effect (Figure 4). This finding allowed us to identify the two cellular metabolites whose conversion these two genes mediate—2,3-dihydro-2,3-dihydroxybenzoate and especially its immediate product DHB—as likely to be responsible for the induction of acrAB. DHB is already known to be a MarR ligand capable of inducing marA expression in vivo by inactivating MarR. By adding DHB to the culture medium, we confirmed that this metabolite induces acrAB expression and that such induction is mediated by marA and partially by marR (Figure 5). Salicylate, another MarR ligand structurally very similar to DHB, induces marA expression via both MarR and an unknown MarR-independent regulator.30 A similar situation may explain why lack of acrAB induction by DHB was less severe in the ΔmarR mutant than in the ΔmarA mutant. Because DHB diffuses freely across the membrane, its intracellular and extracellular concentrations tend to equilibrate regardless of the origin of such DHB; i.e. externally added to the culture or internally overproduced because of the inactivation of entE. In either case, marA-mediated induction of acrAB expression by DHB may result in increased efflux of this compound (or its precursors) outside the cell via the AcrAB-TolC pump. An increased efflux rate of DHB that is higher than its diffusion rate across the cell membrane would result in a decrease in the intracellular concentration of this compound compared with its extracellular concentration, thus reducing its toxicity. A similar scenario might also apply to other metabolites that induce acrAB expression.

acrAB seems to be regulated by the cumulative effect of different metabolites. Some of these are yet unknown metabolites may affect AcrR and SoxRS, the two acrAB regulatory systems found to play the strongest role in the induction of acrAB expression in the ΔacrB mutant. In fact, ethidium bromide and other substrates of the AcrAB-TolC pump have been shown to bind to and inactivate AcrR in vitro. SoxS expression is up-regulated by redox cycling compounds that directly oxidize and activate the soxS activator SoxR. Therefore, it seems plausible that some cellular metabolites can inactivate AcrR or activate SoxR.

It remains to be determined whether acrAB induction and the other changes observed in the ΔacrB mutant are a response to DHB and other as yet unknown metabolites being toxic, or whether these metabolites have a signalling role. Since we found no defects in the cell morphology or growth of the ΔacrB mutant, it seems that even if these metabolites are toxic, the gene expression changes that they produce and other changes yet to be found, e.g. the induction of other pumps, may allow the cells to cope with their accumulation. Either way, our findings suggest a strong interconnection between AcrAB-TolC function and gene regulation and metabolism.

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Transparency declarations
None to declare.

Supplementary data
Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).
Feedback regulation of acrAB expression

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