

Genetic inactivation of *acrAB* or inhibition of efflux induces expression of *ramA*

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Objectives: The transcriptional activator RamA regulates production of the multidrug resistance efflux AcrAB–TolC system in several Enterobacteriaceae. This study investigated factors that lead to increased expression of *ramA*.

Methods: In order to monitor changes in *ramA* expression, the promoter region of *ramA* was fused to a *gfp* gene encoding an unstable green fluorescence protein (GFP) on the reporter plasmid, pMW82. The *ramA* reporter plasmid was transformed into *Salmonella* Typhimurium SL1344 and a Δ *acrB* mutant. The response of the reporter to subinhibitory concentrations of antibiotics, dyes, biocides, psychotropic agents and efflux inhibitors was measured during growth over a 5 h time period.

Results: Our data revealed that the expression of *ramA* was increased in a Δ *acrB* mutant and also in the presence of the efflux inhibitors phenylalanine-arginine- β -naphthylamide, carbonyl cyanide *m*-chlorophenylhydrazine and 1-(1-naphthylmethyl)-piperazine. The phenothiazines chlorpromazine and thioridazine also increased *ramA* expression, triggering the greatest increase in GFP expression. However, inducers of *Escherichia coli* *marA* and *soxS* and 12 of 17 tested antibiotic substrates of AcrAB–TolC did not induce *ramA* expression.

Conclusions: This study shows that expression of *ramA* is not induced by most substrates of the AcrAB–TolC efflux system, but is increased by mutational inactivation of *acrB* or when efflux is inhibited.

Keywords: antibiotic resistance, efflux inhibitors, phenothiazines

Introduction

AcrAB–TolC, the major multidrug resistance efflux pump in Enterobacteriaceae, is associated with the export of a wide range of substrates, including various classes of antibiotics, biocides and dyes. Strains lacking a functional component of this efflux system become hyper-susceptible to antimicrobials.^{1–4} The control of efflux pump gene expression is highly regulated, presumably to allow adaptation to hostile environments. In *Escherichia coli*, expression of the transcription activators MarA, SoxS and Rob increases upon exposure to a wide variety of signals. MarA is induced following salicylate treatment,⁵ SoxS in response to superoxide and Rob following treatment with bile salts and dipyrindyl.^{6–8} Up-regulation of these transcription factors has been shown to increase the expression of several genes, including *acrAB* and *tolC*.^{5,9,10}

Although MarA, SoxS and Rob are present in *Salmonella*, studies have shown that the transcriptional activator RamA, which is not present in *E. coli*, plays the dominant role in the regulation of AcrAB–TolC in *Salmonella* and other Enterobacteriaceae.^{11–17} To date, few inducers of RamA have been reported; however, Nikaido *et al.*^{18,19} demonstrated increased expression of *ramA* in response to the bacterial metabolite indole, and that *acrAB* induction by indole is dependent on RamA. Further work by Nikaido *et al.*²⁰ also found that *acrAB* and *ramA* induction in response to indole is dependent on RamR, a repressor of *ramA* transcription, encoded by *ramR*, located upstream of *ramA*. Hence, mutations in *ramR*, or the operator target for RamR at the *ramA* promoter, confer multidrug resistance.^{11,21–23}

Bailey *et al.*¹³ showed that *Salmonella* lacking *acrB* and *tolC* had significantly reduced growth in the presence of phenothiazines, psychotropic drugs with efflux inhibitor-like properties.

Table 1. Strains used in this study

Strain	Genotype	Source
L1407	SL1344 pMW82	this study
L1232	SL1344 pMW82 <i>ramA</i>	this study
L1353	SL1344 Δ <i>acrB</i> pMW82 <i>ramA</i>	this study

One such drug, chlorpromazine, showed antimicrobial activity, synergism with common antibiotics against *Salmonella enterica* and increased intracellular accumulation of ethidium bromide. Furthermore, chlorpromazine increased *ramA* expression, suggesting that this agent may be an inducer of *ramA*.¹³ Since *ramA* overexpression is associated with up-regulation of *acrAB* and multidrug resistance, it was hypothesized that the presence of antibiotics that are substrates of the AcrAB–TolC multidrug efflux system might cause an increase in *ramA* expression. In this study, we used a *ramA* promoter–*gfp* fusion reporter to explore this hypothesis. We found that few antibiotics induced *ramA*, whilst deletion of *acrB* or inhibition of efflux increased expression of *ramA*.

Materials and methods

Bacterial strains, plasmids, growth media and chemicals

All bacterial strains and plasmids used in this study are listed in Table 1. Mutant SL1344 Δ *acrB* was derived from the antibiotic-susceptible strain SL1344 and constructed as previously described.¹³ Plasmid pMW82 was kindly donated by Dirk Bumann (University of Basel, Basel, Switzerland). Bacterial strains were grown overnight at 37°C in Luria–Bertani (LB) broth. All chemicals and antibiotics were supplied by Sigma Aldrich with the exception of biocides: Superkill and Trigene were supplied by AFS Animal Care and Medichem International; AQAS and Virkon were both supplied by Du Pont. Triclosan was kindly provided by Ciba Geigy.

Construction of *ramA*::GFP transcriptional fusion

To construct the GFP reporter fusion the promoter region of *ramA*, flanked by BamHI and XbaI restriction sites, using primers 5'-GGGGGATCCAACTCGTCAGCGGCTCCC-3' and 5'-GGGGGATCCTACTCACTTCATGCGGCGAG-3', was cloned into pMW82 as described previously.²⁴ The *ramA* promoter GFP fusion was transformed into SL1344 and SL1344 Δ *acrB*. Empty vector control strain was constructed by transforming the promoterless pMW82 plasmid into SL1344.

Flow cytometry of strains carrying the pMW82*ramA* construct

To ascertain whether production of GFP was homogeneous across the bacterial population, the fluorescence of individual cells carrying the *ramA* GFP reporter construct (L1232) and cells carrying the empty vector (L1405) were grown to late logarithmic phase and induced with chlorpromazine at 50 mg/L and then re-incubated at 37°C for 2 h with agitation. Cells were diluted in PBS before sampling and excitation with a 488 nm blue laser. Strain L1405 was used to set parameter channels for forward scatter and side scatter to exclude background noise and green fluorescence (515–545 nm) on the FACS Aria (Becton Dickinson) before strain L1232 was sampled in the presence and absence of chlorpromazine.

Ninety-six-well plate fluorescence assay of *ramA* induction

To observe induction of *ramA* in our GFP reporter, we measured fluorescence following the addition of different compounds. From overnight cultures, fresh LB broth supplemented with 25 mg/L ampicillin was inoculated and incubated at 37°C with agitation (180 rpm) until the cultures reached an optical density (OD) of 0.9 at 600 nm (late logarithmic phase). Appropriate concentrations of potential inducers were added to 100 μ L aliquots of cultures, which were loaded into black 96-well plates with clear flat bottoms (Corning). Two biological and three technical replicates of each culture were used in each assay. Simultaneous measurement of fluorescence at excitation and emission wavelengths of 492 and 520 nm, respectively, and absorbance (growth kinetics) at a wavelength of 600 nm was carried out in a FLUOstar Optima (BMG Labtech) at an incubation temperature of 37°C. Cultures were agitated before each fluorescence reading, which was taken every 3 min for 5 h. Maximum fluorescence values normalized for growth achieved over the course of the assays were expressed as fold change relative to cultures in the absence of the compound at the same timepoint. To determine statistical significance, Student's *t*-test was performed comparing the maximum fluorescence value achieved in the presence of a compound with the fluorescence value of the culture in its absence, with values of $P < 0.05$ indicating significance.

To determine whether temperature affected induction of *ramA*, cultures were incubated at temperatures of 25, 30, 37 and 42°C with agitation (180 rpm) until an OD of 0.9 at 600 nm was achieved. Chlorpromazine at a final concentration of 50 mg/L was used for induction of *ramA*. Fluorescence was measured as described above. Values were expressed as fold change in fluorescence of cultures in the presence of chlorpromazine relative to cultures at the same temperature in its absence.

To determine whether growth phase influenced induction of *ramA*, cultures were incubated at 37°C with agitation until the OD at 600 nm of cultures reached early (0.3), middle (0.6), late logarithmic (1.1) and stationary phase (1.6). Chlorpromazine at a concentration of 50 mg/L was used to induce *ramA* at different growth phases. Fluorescence values were expressed as fold change of cultures in the presence of chlorpromazine against cultures at the same growth phase in its absence.

To investigate the inducing effect of a variety of antibiotics, biocides, detergents, efflux inhibitors, antipsychotic agents and compounds known to induce *marA* and *soxS* and at a range of concentrations (Table S1, available as Supplementary data at JAC Online), we developed a rapid 96-well assay by modifying the assay described above. This allowed screening of multiple compounds at a range of concentrations in parallel. Chlorpromazine was initially tested at a concentration of 100 mg/L as this concentration produced maximal induction; however, for screening purposes a concentration of 50 mg/L was used as this also produced easily detectable fluorescence with no deleterious effect on growth. The assay was modified by incubating cultures in the presence and absence of the test agent for 2 h at 37°C with agitation (180 rpm); the fluorescence and absorbance of each culture was then measured by a single endpoint read in the FLUOstar Optima as described above. The reduced incubation period identified compounds at concentrations with potential inducing activity for further investigation.

Results

Induction of *ramA* expression by deletion of *acrB* or addition of chlorpromazine

Bailey et al.¹³ reported induction of *ramA* by chlorpromazine. Here we exploited a *ramA* promoter–*gfp* fusion reporter to measure activity of the *ramA* promoter in *Salmonella* Typhimurium

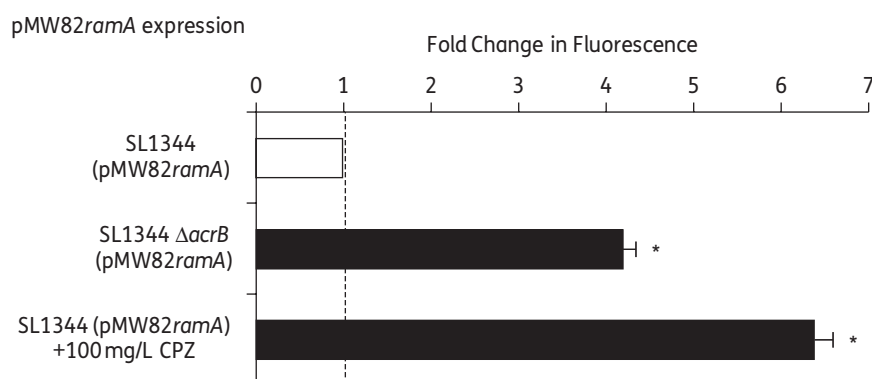


Figure 1. Induction of *ramA* expression by deletion of *acrB* or addition of chlorpromazine. Fold change in fluorescence in SL1344 Δ acrB and SL1344 in the presence of 100 mg/L chlorpromazine relative to SL1344. Fold change was calculated from the maximum fluorescence value achieved for SL1344 after 5 h of exposure relative to SL1344 Δ acrB and SL1344 in the presence of 100 mg/L chlorpromazine at the same point. An asterisk indicates a statistically significant difference ($P < 0.05$). CPZ, chlorpromazine.

SL1344 and the effect of deletion of the *acrB* gene. Compared with wild-type *Salmonella* Typhimurium SL1344, expression of GFP from the reporter fusion in a Δ acrB mutant strain was 4.2-fold higher, and in the presence of chlorpromazine (100 mg/L) this concentration gave maximal induction over the 5 h period; it was 6.4-fold higher (Figure 1). To investigate whether production of GFP was homogeneous across the bacterial population, flow cytometry was used to measure fluorescence in individual cells of wild-type *Salmonella* Typhimurium SL1344 carrying the reporter fusion, in the presence and absence of chlorpromazine (50 mg/L). In the presence of chlorpromazine, the geometric mean of fluorescence (515–545 nm) was increased from 28286 to 45099. This positive shift was monophasic in response to induction with chlorpromazine (Figure 2).

Compounds that inhibit efflux are inducers of *ramA* expression

The efflux inhibitor phenylalanine-arginine- β -naphthylamide (PA β N) (200 mg/L), a competitive substrate of resistance-nodulation-division (RND) family efflux pumps,^{25–27} and 1-(1-naphthylmethyl)-piperazine (NMP) (0.3 mM), a novel efflux inhibitor,²⁸ caused a 2.3- and 1.89-fold increase, respectively, in GFP expression at 5 and 4.7 h, respectively (Figure 3a). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (0.01 mM), which disrupts the proton gradient,^{25–27} and BM-38 (0.2 mM), an efflux inhibitor under development, caused a 1.86- and 1.27-fold increase in GFP expression at 5 and 4.4 h, respectively.

Psychoactive compounds increase the expression of *ramA*

Since chlorpromazine induced *ramA* expression, the effect of other phenothiazines and psychoactive drugs was investigated. Trifluoperazine (50 mg/L), orphenadrine (100 mg/L) and amitriptyline (50 mg/L) caused a 2.7-, 3.4- and 2.4-fold, increase in GFP expression, respectively, at 5 h (Figure 3b). The antipsychotic haloperidol (0.2 mM) and the selective serotonin re-uptake inhibitor sertraline (0.1 mM) also increased the expression of GFP by 2.4- and 2.9-fold, at 4.3 and 5 h, respectively. However,

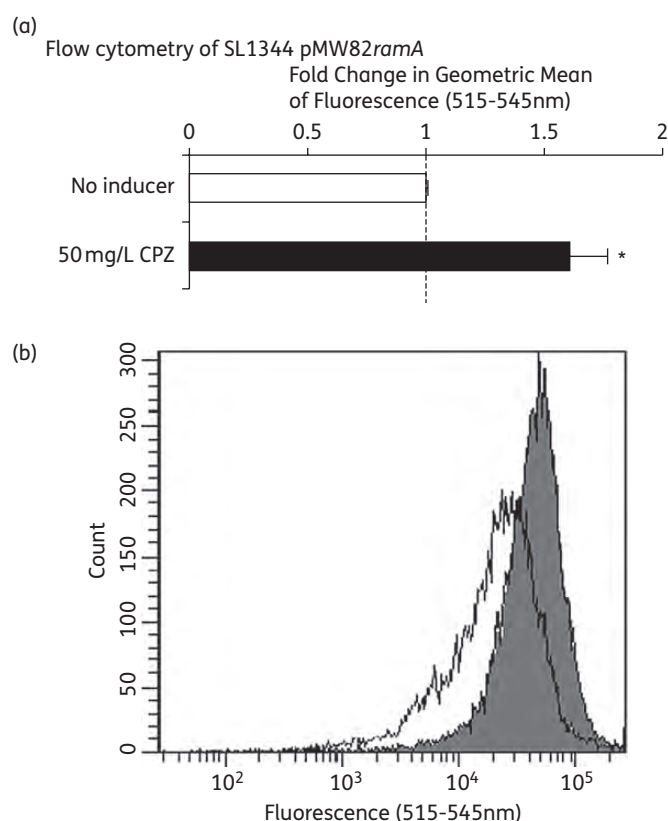


Figure 2. Flow cytometry of strain L1232 in the presence and absence of chlorpromazine. (a) Fold change in geometric mean of fluorescence of strain L1232. An asterisk indicates a statistically significant difference ($P < 0.05$) between values for L1232 and L1232 in the presence of 50 mg/L chlorpromazine after 2 h of exposure. (b) A representative histogram showing the *gfp* fluorescence of L1232 (unfilled) and L1232 in the presence of chlorpromazine (filled). CPZ, chlorpromazine.

chlorpromazine (100 mg/L) and thioridazine (50 mg/L) had the greatest impact on GFP expression, causing a 6.4- and 6.7-fold increase, respectively, at 5 h.

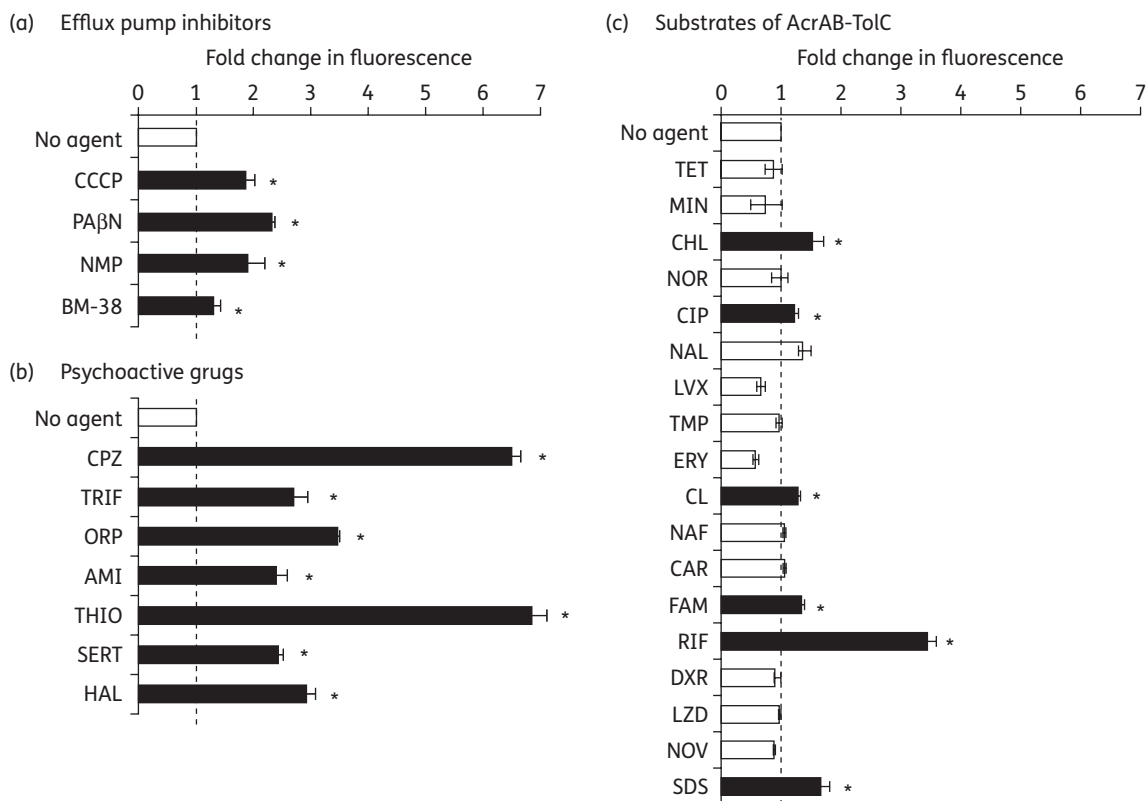


Figure 3. Exposure of strain L1232 to efflux pump inhibitors, psychoactive drugs and substrates of AcrAB–TolC. Fold change in fluorescence after 5 h of exposure after the addition of: (a) CCCP, PAβN, NMP, BM-38 and the control value at the same timepoint; (b) chlorpromazine (CPZ), trifluoperazine (TRIF), orphenadrine (ORP), amitriptyline (AMI), thioridazine (THIO), sertraline (SERT), haloperidol (HAL) and the control value at the same timepoint; and (c) tetracycline (TET), minocycline (MIN), chloramphenicol (CHL), norfloxacin (NOR), ciprofloxacin (CIP), nalidixic acid (NAL), levofloxacin (LVX), trimethoprim (TMP), erythromycin (ERY), cloxacillin (CL), nafcillin (NAF), carbenicillin (CAR), cefamandole (FAM), rifampicin (RIF), doxorubicin (DXR), linezolid (LZD), novobiocin (NOV), SDS and the control value at the same timepoint. Concentrations of each compound are stated in the Results section and Table S1. An asterisk indicates a statistically significant difference ($P < 0.05$) between values for L1232 and L1232 in the presence of compound.

Induction of *ramA* by chlorpromazine was further investigated by adding this compound when the culture was growing at different temperatures or at different growth phases to determine the effect of environmental conditions on induction. The highest induced level of GFP expression (4.5-fold) was observed at 37°C after 5 h of exposure, and not at 42°C (as seen in the absence of inducer). Chlorpromazine caused an increase in GFP expression at every growth phase tested, but the extent of this increase varied—early, middle and late logarithmic phase induction with chlorpromazine produced similar fluorescence levels, but induction in stationary phase was significantly reduced (Figure S1, available as Supplementary data at JAC Online). A growth curve of SL1344 pMW82*ramA* revealed that our 96-well assays were performed before the onset of stationary phase (Figure S2, available as Supplementary data at JAC Online). The growth kinetics of L1232 and L1407 were not affected in the presence of chlorpromazine at 50 mg/L and minimally so at 100 mg/L.

Many exemplar substrates of AcrAB–TolC do not induce *ramA* expression

As the AcrAB–TolC efflux system is essential for survival during antibiotic exposure,²⁹ we hypothesized that the presence of a

substrate^{3,17,30–32} would induce the expression of genes that regulate expression of efflux pump genes. However, 12 of the 17 antibiotics tested had no significant effect on GFP expression. Five antibiotic substrates, chloramphenicol (1.0 mg/L) ciprofloxacin (0.0075 mg/L), cloxacillin (2048 mg/L), cefamandole (512 mg/L) and rifampicin (2.0 mg/L), and one non-antibiotic substrate, SDS (128 mg/L), caused increased expression of GFP by 1.5-, 1.2-, 1.2-, 1.3-, 3.4- and 1.6-fold, respectively, at subinhibitory concentrations, at 5 h for the antibiotics and for SDS after 2.5 h of exposure (Figure 3c).

Indole, but not bile, induces expression of *ramA*

Nikaido et al.^{18–20} have shown, with a *ramA-lacZ* reporter, that indole induces *ramA* expression, whereas the bile salts cholic acid and deoxycholic acid do not. In agreement, we found that indole (2 mM) induced expression of GFP from our *ramA* GFP reporter construct by 2.5-fold. Our experiments also confirmed no significant change in fluorescence in response to the bile components deoxycholic acid and sodium deoxycholate at the concentrations tested (Figure S3, available as Supplementary data at JAC Online).

Inducers of *marA* and *soxS* are not inducers of *ramA* expression

It was hypothesized that inducers of *marRAB* and *soxRS* expression in *E. coli* and *Salmonella* could also induce expression of *ramA* in *Salmonella*. However, no significant induction of GFP was seen in the presence of menadione, paraquat, sodium salicylate or 2,4-dinitrophenol (Figure S4, available as Supplementary data at JAC Online).

Biocides and detergents induce the expression of *ramA*

Exposure to some biocides confers cross-resistance to antibiotics via the increased expression of *acrB*.³³ AQAS, Superkill and Trigene (all at 0.01% v/v) caused a 1.3-, 1.4- and 1.7-fold increase in *ramA* expression at 3.2, 3.1 and 2.6 h, respectively (Figure S5, available as Supplementary data at JAC Online).

Temperature and growth phase influence expression of *ramA*

Expression of GFP from the pMW82*ramA* construct was similar at 25, 30 and 37°C, but was 1.7-fold higher at 42°C compared with that at 25°C. The expression of GFP during different growth phases was also determined; expression peaked at mid-logarithmic phase and significantly decreased from late logarithmic to stationary phase (Figure S6, available as Supplementary data at JAC Online).

Discussion

In this study, we developed a reporter system that exploited an unstable GFP in order to monitor induction of the *ramA* promoter and to measure its activity. Our data revealed that the greatest promoter activity occurred during mid-logarithmic phase and at a temperature of 42°C. In the presence of chlorpromazine, transcription of *ramA* was greatest at late logarithmic phase and at 37°C, but was significantly reduced in stationary phase. This complements the available data for *E. coli marA*, which is more inducible in exponential phase compared with stationary phase,³⁴ and expression of *Salmonella Typhimurium marA* is highest at 37°C.³⁵

Due to the important role of the AcrAB–TolC efflux pump in multidrug resistance and its diverse substrate profile, it was expected that several antibiotic substrates of the AcrAB–TolC efflux system would be inducers of *ramA*. However, 12 of 17 antibiotics used in this study had no significant impact on GFP expression. With the exception of rifampicin, the remaining five antibiotics tested in the present study only caused a modest increase in GFP expression. This is similar to previous reports with *Salmonella Typhimurium marA*, where nine antibiotics tested had no influence on expression.³⁶

The biocides Superkill, AQAS and Trigene caused a significant increase in *ramA* expression, suggesting that increased *ramA* expression may be responsible for increased expression of *acrAB* in response to biocides.³³ Our data show that inducers of other regulators of AcrAB–TolC, such as *marA* and *soxS*, did not induce *ramA*, suggesting that *ramA* regulation of AcrAB–TolC is in response to distinct stimuli. A previous known inducer of *ramA*,

indole, was also investigated and our data confirmed previous observations.^{18–20}

Bailey *et al.*¹³ previously showed that the phenothiazine chlorpromazine increased *ramA* expression and was associated with a phenotype of efflux inhibition. In addition to phenothiazines, the selective serotonin re-uptake inhibitor sertraline and haloperidol have been identified as having efflux inhibitor-like activity;^{37,38} in our study all were identified as inducers of *ramA* expression.

In addition to investigating *ramA* expression in the presence of efflux inhibitors, the disruption of a functional AcrAB–TolC system was also investigated. The deletion of *acrB* caused a greater increase in *ramA* expression than any of the efflux inhibitors tested, suggesting that the removal of a component of the efflux system causes greater expression of *ramA* than a temporary inhibition of efflux by an efflux inhibitor. However, the phenothiazines chlorpromazine and thioridazine increased *ramA* expression to the greatest level, suggesting that these compounds may affect multiple efflux systems. The increased expression of *ramA* in response to the disruption of *acrB* has been previously reported by us^{13,39} and we suggested that the disruption or deletion of *acrB* increased the accumulation of substrates of the AcrAB–TolC efflux pump, and so the bacterium sought to increase export by increasing expression of *ramA*.³⁹ In the present study we now show that the disruption or inhibition of a functional AcrAB–TolC efflux system via the addition of an efflux inhibitor also increased *ramA* expression. Despite the different mechanisms of efflux inhibition, the expression of *ramA* was significantly increased by all efflux inhibitors tested. Taken together, these data suggest that *ramA* expression is induced not by a specific group of chemical compounds but by disruption of the AcrAB–TolC efflux pump.

We propose that in the absence or inhibition of efflux, accumulation of internal metabolites occurs within the cell, and these bind to the repressor of *ramA*, RamR, thereby increasing transcription of *ramA*. Alternatively, the accumulation of internal metabolites may in turn trigger a stress response, which, when recognized by a specific sensor, may affect the expression of *ramA*. For those substrates of the AcrAB–TolC efflux pump in which we observed moderate induction of *ramA*, we hypothesize that this may be due to their slow transport through the system, which may in turn trigger a response similar to inhibition of efflux.

In summary, we have revealed that expression of *ramA* was up-regulated in response to the genetic inactivation of *acrB* or inhibition of efflux by efflux inhibitors. The role of RamA in *Salmonella* is distinct from that of MarA and SoxS, the expression of which is influenced by specific environmental stimuli, and we propose that RamA has an important role in the positive regulation of AcrAB–TolC when the AcrAB–TolC efflux system becomes compromised.

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figures S1 to S6 are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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