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RamA Confers Multidrug Resistance in *Salmonella enterica* via Increased Expression of *acrB*, Which Is Inhibited by Chlorpromazine[▽]

Andrew M. Bailey,[1] Ian T. Paulsen,[2]† and Laura J. V. Piddock[1]*

Antimicrobial Agents Research Group, Department of Immunity and Infection, The Medical School, The University of Birmingham, Birmingham B15 2TT, United Kingdom,[1] and The Institute for Genomic Research, 9712 Medical Center Drive, Rockville, Maryland 20850[2]

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Salmonella enterica **serovar Typhimurium SL1344, in which efflux pump genes (*acrB*, *acrD*, *acrF*, *tolC*) or regulatory genes thereof (*marA*, *soxS*, *ramA*) were inactivated, was grown in the presence of 240 antimicrobial and nonantimicrobial agents in the Biolog Phenotype MicroArray. Mutants lacking *tolC*, *acrB*, and *ramA* grew significantly worse than other mutants in the presence of 48 agents (some of which have not previously been identified as substrates of AcrAB-TolC) and particularly poorly in the presence of phenothiazines, which are human antipsychotics. MIC testing revealed that the phenothiazine chlorpromazine had antimicrobial activity and synergized with common antibiotics against different *Salmonella* serovars and SL1344. Chlorpromazine increased the intracellular accumulation of ethidium bromide, which was ablated in mutants lacking *acrB*, suggesting an interaction with AcrB. High-level but not low-level overexpression of *ramA* increased the expression of *acrB*; conferred resistance to chloramphenicol, tetracycline, nalidixic acid, and triclosan and organic solvent tolerance; and increased the amount of ethidium bromide accumulated. Chlorpromazine induced the modest overproduction of *ramA* but repressed *acrB*. These data suggest that phenothiazines are not efflux pump inhibitors but influence gene expression, including that of *acrB*, which confers the synergy with antimicrobials observed.**

In *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, resistance-nodulation-division (RND) efflux pumps such as AcrAB-TolC demonstrate a broad substrate range, including antimicrobials, dyes, and detergents (5, 14, 22, 23, 33). Efflux pumps also confer resistance to biliary salts in *E. coli* and *Salmonella* serovar Typhimurium in vitro (18, 20, 29), suggesting that a physiological function of active efflux is the export of intracellular solutes and protection against a variety of substances in this environment. Several studies have shown that efflux pumps are involved in the survival of bacteria within their ecological niches, as mutants lacking components of efflux pumps are attenuated within their host (9, 10, 24, 27). The deletion or inactivation of *acrB* in *E. coli* and *Salmonella* serovar Typhimurium confers hypersusceptibility to tetracycline, fusidic acid, ampicillin, ciprofloxacin, nalidixic acid, chloramphenicol, erythromycin, novobiocin, bile salts, sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide, crystal violet, acridine orange, and ethidium bromide (14, 20, 24, 28). In the presence of AcrAB, the role of other efflux pumps in conferring antimicrobial resistance in both species seems to be minor, with the deletion of *acrD* and *acrF* resulting in hypersusceptibility only to ampicillin and ethidium bromide (14, 24, 28). Lee et al. (19) reported that combinations of different efflux pump superfamilies contribute additively to antimicrobial resistance, decreasing susceptibility to a greater extent than a single superfamily of pumps. A lack of *tolC* in both *E. coli* and *Salmonella* serovar Typhimurium also confers hypersusceptibility to a wide range of compounds, including chloramphenicol, fusidic acid, erythromycin, novobiocin, SDS, acridine orange, and ethidium bromide, and increases in susceptibility to ciprofloxacin, norfloxacin, tetracycline, nalidixic acid, and bile salts (10, 13, 24, 28). While the AcrAB-TolC efflux system is considered the major efflux pump complex in both *E. coli* and *Salmonella* serovar Typhimurium, the larger range of substrates to which hypersusceptibility is conferred in Δ*tolC* mutants compared to the range of substrates to which hypersusceptibility is conferred in Δ*acrB* mutants is due to the promiscuous nature of TolC, which, by associating with other efflux pump proteins, effluxes substrates independently of AcrB. It has also been demonstrated in *E. coli* that a functional AcrAB-TolC complex is required for plasmid-mediated tetracycline resistance, even though *tetA* is the major resistance determinant (13), and in *Salmonella*, it has been demonstrated that functional forms of *acrB* and *tolC* are required for florfenicol resistance (8).

ramA, a member of the AraC-XylS family of transcriptional regulators, is found in *Salmonella* serovar Typhimurium, *Enterobacter cloacae*, and *Klebsiella pneumoniae* but not *E. coli* (16, 17, 26, 30). The expression of *ramA* from *Salmonella* serovar Paratyphi or Typhimurium in *E. coli* conferred decreased susceptibility to nalidixic acid, ciprofloxacin, chloramphenicol, and tetracycline (31, 36). However, inactivation of *ramA* in wild-type *Salmonella* serovar Typhimurium did not confer a concurrent increase in antibiotic susceptibility, and inactivation in clinical isolates with multidrug resistance (MDR) conferred only a modest decrease in MICs (25, 30). Additionally, whereas spontaneous mutants selected from

* Corresponding author. Mailing address: Antimicrobial Agents Research Group, Division of Immunity and Infection, University of Birmingham, Birmingham B15 2TT, United Kingdom. Phone: (44) (0)121-414-6966. Fax: (44) (0)121-414-6819. E-mail: l.j.v.piddock@bham.ac.uk.

† Present address: Department of Chemistry & Biomolecular Science, Macquarie University, Sydney, NSW, Australia 2109.

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TABLE 1. Strains and plasmids used in this study

Genotype	Strain or plasmid	Source or reference
Salmonella serovar Typhimurium	14028S	ATCC 14028
Salmonella serovar Typhimurium	LT2	ATCC 19585
Salmonella serovar Typhimurium	SL1344	36
SL1344 Δ*acrB*	L101	3
SL1344 *acrD*::*aph*	L103	10
SL1344 Δ*acrF*	L106	3
SL1344 *tolC*::*aph*	L107	10
SL1344 *marA*::*aph*	L108	28
SL1344 *soxS*::*aph*	L561	28
SL1344 *ramA*::*aph*	L644	28
SL1344 transduced *acrB*::*aph*	L130	10
SL1344 transduced *acrD*::*aph*	L131	10
SL1344 transduced *acrF*::*aph*	L132	10
SL1344 transduced *tolC*::*aph*	L133	10
SL1344 transduced *marA*::*aph*	L135	10
SL1344 transduced *soxS*::*aph*	L109	10
SL1344 transduced *ramA*::*aph*	L110	10
L133-pTRC*chisA*::*ramA*	L786	This study
Salmonella serovar Binza	8269	HPA[a]
Salmonella serovar Enteritidis	5188	HPA
Salmonella serovar Haifa	9878	HPA
Salmonella serovar Heidelberg	5171	HPA
Salmonella serovar Mbandaka	7892	HPA
Salmonella serovar Montevideo	5747	HPA
Salmonella serovar Newport	129	HPA
Salmonella serovar Virchow	5742	HPA
IPTG-inducible *ramA* overexpression	pTRC*chisA*::*ramA*	32
IPTG-inducible *ramA* overexpression	pTRC*chisA*	Invitrogen, United Kingdom

[a] HPA, Health Protection Agency, Colindale, United Kingdom.

marA::*aph* or *soxS*::*aph* strains after exposure to ciprofloxacin can be either MDR or resistant to quinolones alone, those selected from *ramA*::*aph* strains were resistant only to quinolones (25). Recently, it has been shown that *ramR*, a *tetR*-like repressor, represses *ramA*, and when *ramR* is inactivated, *ramA* overexpression is observed and MDR is conferred (1).

In this study, we demonstrate that the Phenotype MicroArray (PM) system can be used to identify compounds in which growth is better or worse when genes that encode components of RND efflux pumps or genes that regulate their expression are inactivated. Chlorpromazine and similar compounds with activities against *Salmonella* serovar Typhimurium were identified. Further experiments suggested that chlorpromazine is an inducer of *ramA* and represses the expression of *acrB*. We also demonstrate that in isogenic strains, the overexpression of *ramA* confers MDR and organic solvent tolerance through the overexpression of *acrB*.

MATERIALS AND METHODS

Strains and media used. All mutants were derived from *S. enterica* serovar Typhimurium SL1344 (34), as described previously (12, 14), and various efflux pump genes and genes that regulate their expression were inactivated (Table 1). In strains L561 (Δ*acrF*) and L644 (Δ*acrB*), the *aph* gene used to disrupt the target gene had been removed by using the pCP20 helper plasmid (12). The gene disruptions were transduced into SL1344 by using P22 to minimize the risk of bacteriophage λ red recombinase-mediated mutations. Luria-Bertani (LB) broth was used throughout with no alterations other than changes to the sodium chloride concentrations where indicated. Specialized medium (Biolog, Inc.) was used when growth investigations were performed with the PM system. To overexpress *ramA*, pTRC*chisA*::*ramA* (31) was transformed into L133 (*ramA*::*aph*). The level of overexpression was determined by reverse transcription-PCR, as described previously (14), with 20-mer primer sequences (Invitrogen, United Kingdom) internal to *Salmonella* serovar Typhimurium *ramA* (forward primer 5′-TCCGCTCAGGTTATCGACAC-3′ and reverse primer 5′-AGCTTCCGTT CACGCACGTA-3′) and *acrB* (forward primer 5′-CGTGTTATGACGGAAG AAGG-3′ and reverse primer 5′-GCCATACCGACGACGATAAT-3′). The growth of the mutants was assayed by inoculating 4% of an overnight culture of the relevant strain into 180 μl fresh LB broth in a 96-well microtiter tray. The trays were subsequently incubated at 37°C in a Fluostar Optima spectrophotometer (BMG Labtech, United Kingdom) with regular shaking, and the optical density at 600 nm (OD_{600}) was recorded every 45 s. The data presented are from at least three independent experiments.

PM system. All tests with the PM system were performed as described previously (37). All fluids, agar media, and arrays are commercially available from Biolog. *Salmonella* serovar Typhimurium SL1344 and the mutants were grown overnight at 37°C on specialized Biolog agar. Colonies were harvested from the surface of an agar plate with a sterile cotton wool swab and suspended in 15 ml of Inoculating Fluid-10 until the cell density equaled 42% transmittance (T) on a Biolog turbidimeter and was then diluted further to give a density of 85% T (an A_{420} of approximately 0.12). A total of 600 μl of the 85% T suspension was diluted 200-fold into 120 ml of Inoculating Fluid-10; and 100 μl per well was used to inoculate plates PM11A to PM20, which measure sensitivity to a wide variety of antibiotics, antimetabolites, and other growth inhibitors. No growth supplements were added to any of the inoculating fluids. All PM plates were incubated at 37°C in an OmniLog plate reader and were monitored for color changes in the wells. Readings were recorded for 36 h for all PM plates. Kinetic data were analyzed with OmniLog PM software (Biolog). On the basis of the work of Zhou et al. (37), a testwide median value of growth of approximately ±10,000 the area under the curve was used, and substrates showing a ±1.5-fold (±≥15,000 area under curve, arbitrary units) difference in the results between strain SL1344 and the mutant being tested were considered significantly different. For analysis, on the basis of their average growth over the entire array, the strains were divided into two groups, one consisting of *marA*::*aph*, *ramA*::*aph*, *acrD*::*aph*, and Δ*acrF* strains and the other consisting of *tolC*::*aph* and Δ*acrB* strains. The average growth value for each strain and concentration were taken, and individual values were plotted against the average value for the group. A trend line was applied for each strain, and clusters with outlying individual growth values were examined further. This process was termed "cluster analysis."

Quantification of gene expression. Gene expression analysis was conducted by comparative reverse transcription-PCR and denaturing high-pressure liquid chromatography, as described previously (6, 14).

Susceptibility to antibiotics and phenothiazines. The MICs of phenothiazines for the mutants were determined by the British Society for Antimicrobial Chemotherapy standardized broth microdilution method (4). The lowest concentration of antimicrobial that caused no visible growth was determined to be the MIC of that compound. The compounds tested were amitriptyline, thioridazine, trifluoperazine, orphenadrine, and chlorpromazine (Sigma-Aldrich, United Kingdom). MICs were determined independently three times. Furthermore, the MICs of nalidixic acid, norfloxacin, tetracycline, ethidium bromide, ciprofloxacin, chloramphenicol, acridine orange, SDS, bile salts, sodium deoxycholate, rhodamine 6G, and crystal violet (all from Sigma-Aldrich, United Kingdom) and triclosan (Ciba, Switzerland) were determined in the presence of chlorpromazine, thioridazine, amitriptyline, and trifluoperazine (at concentrations of 100 μg/ml and 200 μg/ml) to examine whether the phenothiazines had synergistic and/or additive effects on the activities of these agents. When the MICs for L786 (*ramA*::*aph*-pTRC*chisA*::*ramA*) were determined, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 50 μg/ml ampicillin were added to the growth medium.

Accumulation and efflux of ethidium bromide with or without phenothiazines. The accumulation of ethidium bromide was determined as described previously (21). Cultures were grown at 37°C until an OD_{600} of 0.7 was obtained. In the case of *ramA*::*aph*-pTRC*chisA*::*ramA*, IPTG was added to 1 mM at an OD_{600} of ~0.6, and then the culture was reincubated for 30 min until the OD_{600} reached 0.7 ± 0.02. The cells were centrifuged, washed with potassium phosphate buffer to remove all traces of external ethidium bromide, recentrifuged, and then resuspended in potassium phosphate buffer to an OD_{660} of 0.2. The cells were incubated in a sterile tube with stirring at 37°C for 10 min to equilibrate. The cultures were then spilt into two aliquots, ethidium bromide was added to both aliquots at a final concentration of 1 μg/ml, and chlorpromazine was added to one aliquot at a final concentration of 100 or 200 μg/ml. Aliquots of 1 ml were taken at 30 and 60 s and 2, 3, 4, 5, 7, and 10 min. Each aliquot was diluted 1:10 and measured on a FS45 fluorospectrometer (Perkin-Elmer, United Kingdom) at an excitation wavelength of 530 nm and an emission wavelength of 600 nm.

The efflux of ethidium bromide was measured by inoculating 3 ml of fresh LB broth with 4% of an overnight culture of strain SL1344, and the culture was incubated until the OD_{600} reached 0.7 ± 0.02. Ethidium bromide (25 μM; final concentration, 1 μg/ml) with or without 100 μM carbonyl cyanide *m*-chlorophenylhydrazone (Sigma-Aldrich) was added to each aliquot (to inhibit any efflux and to ensure a maximum intracellular concentration of ethidium bromide), and the aliquots were incubated at 20°C with stirring for 20 min. After centrifugation

at 1,500 × *g* at 4°C, the supernatant was decanted and the pellet was resuspended in 1 M sodium phosphate buffer with 5% glucose to energize the cells. A total of 180 μl of each aliquot was added to six wells in a black microtiter 96-well tray, and after 140 s chlorpromazine was added at 200 μg/ml to three of the six biological repeats. The fluorescence at 600 nm was measured with a Fluostar fluorescent spectrometer (BMG Labtech, United Kingdom). The mean values from each biological and technical replicate were determined. The data were analyzed with Microsoft Excel software. The standard deviation was calculated and two-tailed paired Student's *t* tests were performed to assess error and significance.

Organic solvent tolerance assay. Organic solvent tolerance assays were adapted from a method described previously (5). Briefly, 5 μl of a 1:100 dilution of an overnight culture of the test strain was spotted onto a dried LB agar surface and overlaid with 5 mm of hexane or cyclohexane (both from Sigma). The plates were sealed to prevent evaporation and were incubated for 24 h at 30°C before they were scored for growth.

RESULTS

The PM system reveals that disruption of *acrB* and *tolC* confers susceptibility to a hitherto unsuspected wide range of compounds. The PM system measures the ability of bacteria to grow under a range of different conditions. In this study, strains lacking a component of an RND efflux pump or a transcriptional activator previously indicated to be involved in MDR in *E. coli* or *Salmonella* serovar *enterica* were exposed to a variety of different compounds, including some known antimicrobials, in plates PM11a to PM20. The PM system allows the parallel testing of whether the growth of any of these strains compared to that of the parental strain, SL1344, was different. This technique was chosen as it allowed comparison of the growth of eight strains in parallel in the presence of 240 compounds. Before tests were performed with the PM system, no significant differences in the growth kinetics of any of the strains were observed. The mean generation time of each strain in LB medium was 31.7 ± 1.4 min. No overgrowth of any strain at stationary phase was seen (data not shown). The most striking observation seen in the PM system was the "clustering" of the responses of four of the mutants, the *marA*::*aph* (strain L101), *ramA*::*aph* (strain L103), *acrD*::*aph* (strain L106), and Δ*acrF* (strain L561) strains, to a large proportion of the compounds tested, in which all grew better than the parental (wild-type) strain, strain SL1344 (Fig. 1). In the presence of the same compounds, L108 (*tolC*::*aph*) and L644 (Δ*acrB*) consistently grew more poorly than SL1344. While the presence of the *aph* gene used to disrupt the target gene conferred the expected resistance to kanamycin and related antibiotics, the wide range of other compounds in the presence of which the strains showed altered growth was surprising. Of the 240 agents screened, the *marA*::*aph*, *ramA*::*aph*, *acrD*::*aph*, and Δ*acrF* strains grew better than SL1344 in the presence of 56 (23%) agents over two or more concentrations. Of those 56 agents, the *tolC*::*aph* and Δ*acrB* strains grew more poorly in the presence of 48 (86%) (Table 2). These included antibiotics of different classes, dyes, detergents, and biocides, 35 of which a literature search indicated have not previously been described as substrates of the AcrAB-TolC efflux pump complex. Furthermore, the *tolC*::*aph* strain grew extremely poorly in the presence of more compounds; of the 48 compounds in the presence of which both the *tolC*::*aph* and the Δ*acrB* strains grew poorly, the *tolC*::*aph* strain grew particularly poorly in the presence of 11 (Table 3). It was also observed that the *ramA*::*aph* strain grew particularly poorly in the presence of several similar agents: amitriptyline, chlorpromazine, sanguinarine, and thioridazine.

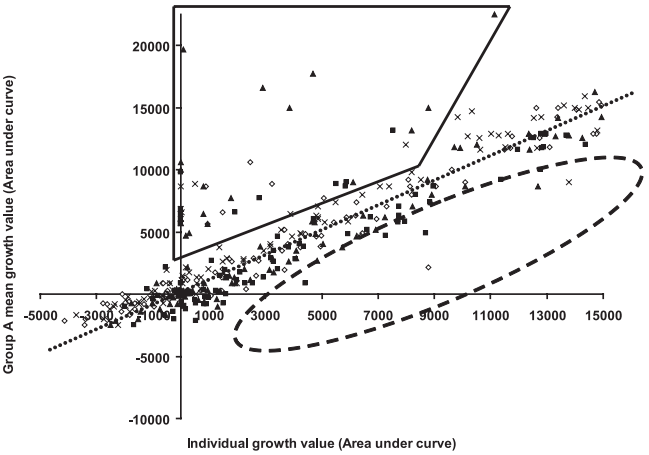

FIG. 1. PM growth data showing clustering of the strains which grew better (enclosed by black lines) or more poorly (enclosed by dashed lines). The dotted line indicates the mean growth of these four strains (group A) compared with of SL1344 in the presence different compounds; ◇, L561 (Δ*acrF*); ■, L130 (*marA*::*aph*); ▲, L133 (*ramA*::*aph*); ×, L106 (*acrD*::*aph*).

Phenothiazines are synergistic with some common antimicrobial agents. The five phenothiazines tested, selected on the basis of the results obtained with the PM system, had poor activities against strain SL1344 (Table 4), other strains of *Salmonella* serovar Typhimurium, and the NCTC type strains of 10 other serovars of *Salmonella* serovar *enterica* (data not shown). The MICs ranged from 512 to ≥1,024 μg/ml. The hypersusceptibilities to phenothiazines of mutants of SL1344 in which *acrB* or *tolC* was inactivated were confirmed. Both strains were 16- to 32-fold more susceptible to thioridazine, trifluoperazine, and chlorpromazine (Table 4). Mutants in which *acrD*, *acrF*, or *ramA* had been inactivated were fourfold more susceptible to chlorpromazine; and those in which *marA* and *soxS* had been inactivated were twofold more susceptible.

Four of the phenothiazines were tested in combination with six antimicrobial agents for synergy against *Salmonella* serovar Typhimurium SL1344. At 100 μg/ml, chlorpromazine had no discernible effect. However, at 200 μg/ml, the MICs of nalidixic acid, norfloxacin, ciprofloxacin, chloramphenicol, tetracycline, and ethidium bromide were significantly reduced. No synergistic effect was observed with the other three phenothiazines tested, except for amitriptyline (200 μg/ml) and norfloxacin (Table 5). The synergy of chlorpromazine with two of the antimicrobial agents, norfloxacin and ethidium bromide, was confirmed with the other strains of *Salmonella* serovar Typhimurium and the NCTC type strains of 10 other Salmonella serovars (Table 6).

Chlorpromazine appears to have efflux pump inhibitor properties. The hypersusceptibilities to chlorpromazine of mutants in which *acrB* or *tolC* had been inactivated and the synergy of this agent with antimicrobial agents suggested that chlorpromazine could be an efflux pump inhibitor and interact with a component(s) of the AcrAB-TolC tripartite efflux pump. The levels of accumulation of ethidium bromide by these strains compared with that by parental strain SL1344 were

TABLE 2. Compounds in which *marA*::*aph*, *ramA*::*aph*, *acrD*::*aph*, and Δ*acrF* strains demonstrated better growth than wild-type *Salmonella* serovar Typhimurium SL1344, whereas *tolC*::*aph* and Δ*acrB* showed hypersusceptibility

Compound class	Name[a]
Aminoglycoside	**O/129**[b]
	Kanamycin
	Neomycin
	Paromomycin
β-Lactam	**Amoxicillin**
	Azlocillin
	Cefamandole
	Cefotaxime
	Cefuroxime
	Cloxacillin
	Nafcillin
	Oxacillin
	Phenethicillin
Coumarin	Novobiocin
Folate inhibitor	Trimethoprim
Glycopeptide	Bleomycin
Macrolide	Erythromycin
	Josamycin
	Lincomycin
	Oleandomycin
	Rifamycin SV
	Spiramycin
	Troleandomycin
	Tylosin
Nitroamidazole	**Ornidazole**
Nitrofuran	**Furaltadone**
	Nitrofurazone
Peptidyl nucleoside	Puromycin
Phenicol	Chloramphenicol
Quinolone	Ciprofloxacin
	Lomefloxacin
	Norfloxacin
Steroid	Fusidic acid
Tetracycline	Chlortetracycline
	Demeclocyline
Dye	Crystal violet
	Iodonitro tetrazolium violet
	Tetrazolium violet
	Acriflavine
Detergent	**Diaminobenzidine**
Phenothiazine	**Thioridazine**
	Chlorpromazine
	Trifluoperazine
Benzophenanthridine	**Sanguinarine**
	Chelerythrine
Biocide	**Dodine**
	Domiphen bromide
	Dequalinium
Cu, Zn ion chelator	**Chloroquinadol**
	Clioquinol
NSAID[c]	**Ketoprofen**
Anticholinergic	**Orphenadrine**
TCA[d]	**Amitriptyline**

[a] Boldface indicates that the compound was not previously described as a substrate of AcrAB-TolC.
[b] Aminoglycoside derivative.
[c] NSAID, nonsteroidal anti-inflammatory drug.
[d] TCA, tricyclic antidepressant.

TABLE 3. Agents in which poor growth of *Salmonella* serovar Typhimurium SL1344 *tolC*::*aph* compared to that for Δ*acrB* was observed

Name	Compound class
O/129[a]	Aminoglycoside derivative
Orphenadrine	Cholinergic antagonist
5-Chloro-7-iodo-8-hydroxyquinoline	Cu, Zn chelator
5,7-Dichloro-8-hydroxyquinaldine	Cu, Zn chelator
Amitriptyline	Norepinephrine and serotonin reuptake inhibitor
Chlorpromazine	Phenothiazine
Trifluoperazine	Phenothiazine
Dequalinium	Ion channel inhibitor
Lincomycin	Lincosamide
Chelerythrine	Protein kinase C inhibitor (benzophenanthridine)
Ketoprofen	NSAID[b]

[a] Aminoglycoside derivative.
[b] NSAID, nonsteroidal anti-inflammatory drug.

determined in the presence and the absence of chlorpromazine (Fig. 2A). Unfortunately, the level of accumulation of ciprofloxacin and norfloxacin (or any other fluoroquinolone) could not be determined, as chlorpromazine quenched the fluorescence of these agents. In the presence of chlorpromazine (200 μg/ml), SL1344 accumulated 3.6-fold more ethidium bromide than it did without chlorpromazine. When *tolC* or *acrB* was inactivated, each mutant accumulated more than double the amount of ethidium bromide than the parental strain, SL1344. Chlorpromazine had no significant effect upon the level of accumulation by the mutant in which *acrB* was inactivated. However, for the mutant in which *tolC* had been inactivated, addition of chlorpromazine increased the level of ethidium bromide accumulation to the same level seen for SL1344 in the presence of chlorpromazine. In an assay determining the efflux of ethidium bromide, chlorpromazine prevented the efflux of this agent from SL1344 (Fig. 3).

Overproduction of *ramA* confers MDR. The data obtained with the PM system also indicated that when *ramA* was inactivated, this mutant was more susceptible to inhibition by phenothiazines. This was confirmed by MIC testing, which revealed that SL1344 *ramA*::*aph* was fourfold more susceptible to chlorpromazine then SL1344. While mutants in which *marA* or *soxS* were inactivated were also more susceptible to chlorpromazine, the decrease in the MIC was modest (Table 4). *ramA* has previously been implicated as having a role in MDR in *Salmonella*, because when this gene was cloned from clinical isolates of *Salmonella* serovar Paratyphi or *Salmonella* serovar Typhimurium into *E. coli*, decreased susceptibility or modest MDR was conferred, and the phenotype was similar to that seen when *marA* or *soxS* is overproduced in *E. coli* (30, 36).

TABLE 4. MICs of phenothiazines and related compounds to the strains in this study

Strain	MIC[a] (μg/ml)				
	THI	TRI	AMI	CPZ	ORP
SL1344	>1,024	1,024	512	512	1,024
acrB::*aph*	**32**	**64**	256	**32**	1,024
acrD::*aph*	>1,024	1,024	1,024	**128**	1,024
acrF::*aph*	>1,024	1,024	512	**128**	1,024
tolC::*aph*	**32**	**32**	256	**32**	1,024
marA::*aph*	>1,024	1,024	512	**256**	1,024
soxS::*aph*	>1,024	1,024	512	**256**	1,024
ramA::*aph*	>1,024	1,024	512	**128**	1,024

[a] Boldface indicates a more than twofold decrease in the MIC. THI, thioridazine; TRI, trifluoperazine; AMI, amitriptyline; CPZ, chlorpromazine; ORP, orphenadrine.

TABLE 5. MICs of various compounds for wild-type *Salmonella* serovar Typhimurium SL1344 exposed to the various compounds and phenothiazines in combination[a]

Compound	Phenothiazine concn (μg/ml)	MIC[b] (μg/ml) of the following compound at the indicated concn (μg/ml):							
		CPZ		AMI		THI		TRI	
		100	200	100	200	100	200	100	200
Nal	4	8	**0.015**	>4	>4	>4	>4	4	4
Nor	0.25	0.5	**<0.007**	0.25	**<0.007**	0.5	0.5	0.5	0.25
Cip	0.12	0.06	**<0.007**	0.06	0.06	0.12	0.12	0.12	0.12
Chl	4	>4	**0.015**	>4	2	2	2	2	2
Tet	2	8	**0.25**	8	8	8	8	8	8
EtBr	1024	1,024	**8**	1,024	1,024	2,048	1,024	1,024	1,024

[a] The synergistic action of orphenadrine was not tested because it was not observed to have antimicrobial action. CPZ, chlorpromazine; AMI, amitriptyline; THI, thioridazine; TRI, trifluoperazine; Nal, nalidixic acid; Nor, norfloxacin; Cip, ciprofloxacin; Chl, chloramphenicol; Tet, tetracycline; EtBr, ethidium bromide.
[b] Boldface indicates a more than twofold decrease in the MIC.

However, inactivation of *ramA* in MDR clinical isolates of *Salmonella* conferred only a modest increase in antibiotic susceptibility (30), and so a clear role for *ramA* in MDR in *Salmonella* has not been defined. This may be because of the confounding influence of other mechanisms of resistance in the clinical isolates, the method of testing of antimicrobial susceptibility, or the agents tested (30, 31). To carefully dissect the role of *ramA* in MDR in *Salmonella* and any effect of chlorpromazine, in the present study isogenic strains were constructed. *ramA* was inactivated in SL1344; and then plasmid-mediated *ramA*, under the control of an IPTG-inducible promoter, was introduced to give strain L786. In the absence of IPTG (minimal induction), L786 expressed *ramA* 1.74-fold more than SL1344 did. In the presence of 1 mM IPTG, the level of expression was 38-fold higher in L786 than in SL1344. The MICs of a wide range of antimicrobial agents for these strains were determined. The MICs of those agents typically associated with the MDR phenotype conferred by the overproduction of *marA* or *soxS* in *E. coli* were unaffected when *ramA* was inactivated. Only for acriflavine was any change in the MIC observed (Table 7). However, it was noted that the inactivated strain became intolerant to hexane. The overproduction of *ramA* in L786 (SL1344 *ramA::aph*-pTR*ChisA*::*ramA*) and induction with IPTG conferred an MDR phenotype (Table 7). In addition, L786 was tolerant to both hexane and cyclohexane. The inactivation or overproduction of *ramA* had no effect on the MIC of SDS, bile, sodium deoxycholate, cetyltrimethylammonium bromide, or rhodamine 6G.

***ramA* affects expression of *acrB*.** It was hypothesized that *acrB* was part of the *ramA* regulon and that the MDR in strain L786 was due to the overproduction of AcrAB-TolC. When *ramA* was inactivated (strain L133), *acrB* was expressed at one-fifth the level it was expressed in the parental strain, SL1344 (Table 8). In the absence of *acrB* (strain L110), *ramA* was overproduced. In the absence of IPTG, *acrB* was expressed in L786 at the same level as it was in SL1344 (data not shown). However, in the presence of IPTG, the level of *acrB* expression was increased by 15-fold. The level of accumulation of ethidium bromide in the mutant in which *ramA* had been inactivated (strain L133) was similar to that in parental strain SL1344 (Fig. 2B). Similarly, upon addition of chlorpromazine, the level of ethidium bromide accumulation increased. When *ramA* was overproduced, the level of accumulation of ethidium bromide was less than that in SL1344, with chlorpromazine having little effect.

Chlorpromazine induces expression of *ramA* but represses *acrB* expression. To determine whether chlorpromazine induced the expression of *ramA* or *acrB*, or both, an SL1344 strain in which *ramA* or *acrB* had been inactivated was exposed

TABLE 6. Synergistic activity of chlorpromazine against various *Salmonella* serovars

Serovar	MIC[a] (μg/ml)			
	Nor	Nor + CPZ	EtBr	EtBr + CPZ
Salmonella serovar Typhimurium SL1344	0.06	**0.008**	1024	**8**
Salmonella serovar Typhimurium LT2	0.06	**0.003**	1,024	**0.5**
Salmonella serovar Typhimurium 14028S	0.06	**0.006**	2,048	**16**
Salmonella serovar Binza	0.06	**0.004**	1,024	**128**
Salmonella serovar Enteritidis	0.06	**0.004**	1,024	**0.5**
Salmonella serovar Hadar	0.06	**0.004**	1,024	**0.5**
Salmonella serovar Haifa	0.06	**0.004**	512	**0.5**
Salmonella serovar Heidelberg	0.06	**0.004**	1,024	**0.5**
Salmonella serovar Kedougou	0.06	**0.004**	512	**256**
Salmonella serovar Mbandaka	0.06	**0.004**	1,024	**0.5**
Salmonella serovar Montevideo	0.06	**0.004**	1,024	**512**
Salmonella serovar Newport	0.06	**0.004**	1,024	**128**
Salmonella serovar Virchow	0.06	**0.004**	1,024	**64**

[a] Boldface indicates a more than twofold decrease in the MIC. Nor, norfloxacin; CPZ, chlorpromazine; EtBr, ethidium bromide.

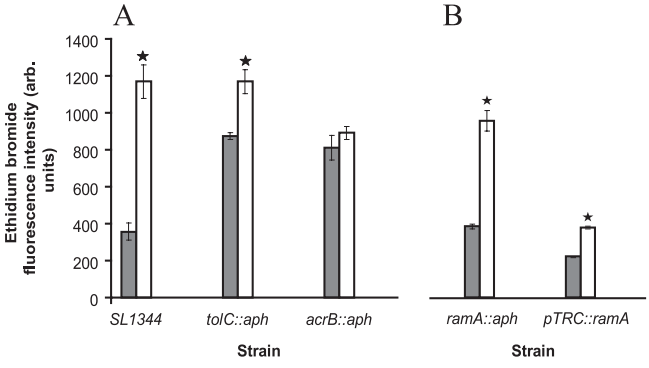

FIG. 2. Accumulation of ethidium bromide in the presence of chlorpromazine at 200 μg/ml (unfilled bars) and in the absence of chlorpromazine (gray bars). *, statistically significant increase in accumulation in the presence of chlorpromazine ($P < 0.05$). All units are arbitrary (arb.).

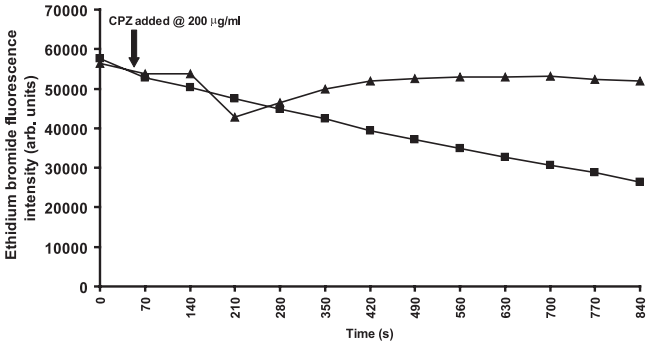

FIG. 3. Efflux of ethidium bromide from *Salmonella* serovar Typhimurium SL1344 in the presence and absence of chlorpromazine (CPZ) at 200 μg/ml. ▲, SL1344; ■, SL1344 plus chlorpromazine at 200 μg/ml. Fluorescence units are arbitrary (arb.).

to chlorpromazine (200 μg/ml) for 30 min at mid-logarithmic phase of growth. In SL1344, 2.6-fold more *ramA* was produced and there was a concomitant decrease in the level of expression of *acrB* (Table 8). When *ramA* was inactivated (strain L133), chlorpromazine exposure increased the level of expression of *acrB* by fourfold compared with that in the absence of chlorpromazine. When *acrB* was inactivated (strain L110), chlorpromazine exposure had little effect on *ramA* expression. In the presence of chlorpromazine, the MICs of ciprofloxacin, nalidixic acid, chloramphenicol, tetracycline, ethidium bromide, triclosan, and crystal violet were reduced by at least twofold (Table 7). However, addition of chlorpromazine to L786 did not restore sensitivity to hexane or cyclohexane.

DISCUSSION

The PM system was used to explore the activities of a wide range of antimicrobial and nonantimicrobial compounds against mutants in which components of an efflux pump had been inactivated or genes previously indicated to control or influence the expression of *acrAB* in *E. coli* had been inactivated. The hypersusceptibility to a wide range of agents of *Salmonella* serovar Typhimurium mutants in which *acrB* or *tolC* had been inactivated or deleted was confirmed previously (8, 14, 24). In addition, in the present study these mutants were also shown to be hypersusceptible to antimicrobials not previously considered to be substrates of AcrAB-TolC and additionally to nonantimicrobial classes of compounds, such as the phenothiazines. The mutant in which *tolC* was inactivated was hypersusceptible to more agents than the mutant in which *acrB* had been deleted, suggesting that these additional agents are exported by pumps other then AcrB and also use TolC as the outer membrane protein channel. We have previously shown that when *acrD* or *acrF* is inactivated, there is an increased level of expression of *acrB*, presumably to compensate for the lack of the transporter (14). This may explain the improved growth of the two mutants seen in the presence of the phenothiazines, whereas the mutant in which *acrB* was inactivated grew more slowly. Inactivation of the transcriptional regulators *marA* and *ramA* typically gave rise to better growth in the presence of compounds in which the *tolC* or *acrB* mutants grew poorly. This may be because the lack of these transcriptional regulators allows the expression of genes which are normally repressed (7). It has been observed in *E. coli* that strains in which efflux pump genes have been deleted can overgrow compared to the level of growth of wild-type strains, and this is postulated to be due to the lack of the export of quorum-sensing factors (35). However, no overgrowth was observed in this study, and no growth differences between SL1344 (the parental strain) and the mutants in which *acrB* or *tolC* had been inactivated were seen. This is likely due to the differences in quorum sensing between *E. coli* and *Salmonella* serovar Typhimurium (2). *Salmonella* serovar Typhimurium mutants in which *tolC*, *acrB*, or *ramA* had been inactivated were particularly susceptible to phenothiazines and compounds with similar modes of action, such as chlorpromazine. Because this class of agents was previously identified to possess potential activity and synergistic activity with some antibiotics against *Staphylococcus aureus* (3, 15), we focused further attention on these compounds. Furthermore, phenothiazines are thought to inhibit NorA, an MFS efflux pump in *S. aureus* (15). More recently, phenothiazines have been shown to have synergistic activity with antimicrobials against *Burkholderia pseudomallei*

TABLE 7. Susceptibility of wild-type *Salmonella* serovar Typhimurium SL1344 and a *ramA*-overexpressing strain to various antibiotics, dyes, detergents, and organic solvents in the presence and absence of chlorpromazine

Strain	MIC[a] (μg/ml)											Sensitivity[b]	
	CPZ	Cip	Nal	Nor	Chl	Tet	EtBr	Tric	Acr	CV	Nov	Hexane	Cyclohexane
SL1344	512	0.03	4	0.25	4	2	2,048	0.12	512	2	512	T[c]	S
SL1344 with CPZ		0.008	0.015	0.008	0.015	0.25	8	0–3	512	1	64	S	S
L133[d]	256	0.03	4	0.25	4	2	1,024	0.12	256	2	256	S	S
L133 with CPZ	ND[e]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	S	S
L786[f] without IPTG	512	0.03	*16*	0.25	8	4	2,048	0.25	256	2	512	T	S
L786 with IPTG	512	0.03	*64*	0.25	*32*	*16*	2,048	*1*	512	2	*1,024*	T	T
L786 without IPTG and with CPZ		**0.015**	**16**	0.25	**16**	**8**	**512**	**0.25**	512	**0.5**	512	T	T

[a] Italics indicate a more than twofold increase in the MIC compared to that for SL1344; boldface indicates a decrease in the MIC compared to that for L786 alone. CPZ, chlorpromazine; Cip, ciprofloxacin; Nal, nalidixic acid; Nor, norfloxacin; Chl, chloramphenicol; Tet, tetracycline; EtBr, ethidium bromide, Tric, triclosan; Acr, acridine orange; CV, crystal violet; NOV, novobiocin.

[b] T, tolerant to organic solvent; S, sensitive to organic solvent.

[c] Addition of chlorpromazine also conferred hexane sensitivity in SL1344.

[d] L133 is a *ramA*::*aph* strain.

[e] ND, MIC not determined as the MIC for chlorpromazine was <200 μg/ml.

[f] L786 is a *ramA*::*aph*-pTRC*hisA*::*ramA* strain.

TABLE 8. Comparison of expression of *ramA* and *acrB* with or without chlorpromazine at 200 μg/ml

Strain	Genotype	Level of expression[a]			
		ramA		*acrB*	
		Without CPZ	With CPZ	Without CPZ	With CPZ
SL1344		1	2.6 ± 0.4	1	0.4 ± 0.04
L133	*ramA*::*aph*	ND	ND	0.2 ± 0.04	1.6 ± 0.3
L786	*ramA*::*aph* pTRC *hisA*::*ramA*	37.6 ± 0.2[b]	ND	14.9 ± 0.2[b]	ND
L110	*acrB*::*aph*	3.9 ± 0.04	0.7 ± 0.04	ND	ND

[a] The data were obtained by comparative PCR. The level of gene expression by the reference strain value was set equal to an arbitrary value of 1, to which the data for the other strains were normalized. Values less than zero reflect the reciprocal of the fold decrease; e.g., 0.2 indicates a fivefold decrease. CPZ, chlorpromazine; ND, not detected.

[b] Values were obtained in the presence of IPTG.

(11). In the present study, chlorpromazine was shown to be synergistic with several agents of different chemical classes, and amitriptyline was shown to be synergistic with norfloxacin against *Salmonella*. These data suggest that phenothiazines could act as efflux pump inhibitors. In support of this hypothesis, it was found that chlorpromazine increased the concentration of ethidium bromide that accumulated in all mutants except the strain in which *acrB* had been inactivated. Likewise, the efflux of ethidium bromide by SL1344 (the parental wild-type strain) was decreased in the presence of chlorpromazine.

The PM system also revealed that the mutant in which *ramA* had been inactivated was hypersusceptible to phenothiazines. As it was previously suggested that when *ramA* is overexpressed it could confer MDR, we first sought to construct a set of isogenic strains which had a defined level of overexpression of *ramA* and then to use the strains to explore the interaction with chlorpromazine. First, the overexpression of *ramA* was shown to confer MDR in *Salmonella*. These data confirm and extend those of van den Straaten et al. (30) and also those most recently obtained by Abouzeed et al. (1). When *ramA* was highly expressed, there was a concomitant overexpression of *acrB*. These data indicate that *acrB* is within the regulon of *ramA* and that the overexpression of *ramA* confers MDR via the overproduction of the AcrB transporter. Of interest, it was observed that when *ramA* was inactivated, the expression of *acrB* was significantly reduced. Likewise, when *acrB* was inactivated, there was an effect upon the expression of *ramA*; in this case, there was a fourfold increase in the level of expression. These data indicate that there is a mechanism within the bacterial cell for detection of both the level of *acrB* expression and the level of *ramA* expression and that regulation is not a one-way process from *ramA* to *acrB*. Second, we showed that the level of expression of *ramA* was important in the determination of whether the strain was MDR or not; for instance, in the absence of IPTG, *ramA* was expressed at low levels and there was no overexpression of *acrB* and very modest MDR.

Although it has previously been suggested that chlorpromazine and other phenothiazine compounds are efflux pump inhibitors, no direct biochemical data have been provided to show an interaction between these compounds and a transporter protein. Another hypothesis that may explain these data is that chlorpromazine affects the expression of the gene(s) involved in regulating the expression of efflux. Therefore, we explored the effect of chlorpromazine upon the expression of *ramA* and *acrB* in the constructs in which *ramA* was either inactivated or overproduced. It was found that chlorpromazine induced the expression of *ramA* in wild-type antibiotic-sensitive strain SL1344 and that there was a concomitant decrease in the level of production of *acrB*. These data suggest that chlorpromazine acts on *ramA* and *acrB* separately and that chlorpromazine induces *ramA* but that it also represses the expression of *acrB*. The repression of *acrB* correlated well with the increased level of accumulation which had previously been hypothesized to be due to efflux inhibition. Our data suggest that, in fact, chlorpromazine acts to repress the expression of the efflux pump gene, and so the efflux pump is produced at a lower level which is insufficient to export the antimicrobial, hence increasing the concentration accumulated. The modest induction of *ramA* by chlorpromazine was at a level that we have shown was insufficient to induce the expression of *acrB* and to confer MDR.

An association between organic solvent tolerance (cyclohexane) and MDR has previously been shown when *marA* and/or *acrB* was overproduced in *E. coli* (5). A similar association has been made for *Salmonella* serovar Typhimurium (32). This has led many to consider that cyclohexane tolerance can be used as a marker for increased efflux via *acrB* and/or that this indicates a *marA* mutant. We observed that the high-level overexpression of *ramA* conferred tolerance to cyclohexane and that the phenotype of this overexpressing *Salmonella* serovar Typhimurium mutant was the same as that previously associated with the overexpression of *marA* in *E. coli* (33). Although addition of chlorpromazine rendered SL1344 sensitive to hexane, it did not affect the hexane or cyclohexane tolerance shown by the overexpressing strain (strain L786) or a previously characterized laboratory-selected cyclohexane-tolerant mutant (unpublished data). Therefore, for *Salmonella*, cyclohexane tolerance may be an indicator of the overproduction of *ramA*.

In summary, this study has revealed that the PM screen is a valuable tool in the search for compounds that can inhibit efflux (in this case, by downregulating the production of the efflux pump) and shows the potential to identify a pharmaceutical compound already in use for the treatment of other human diseases as a possible agent for use in combination with conventional antimicrobial drugs. As the search for ways to reverse antimicrobial resistance continues, the identification of molecules that are already used within human medicine will make the regulatory process for combination treatment more straightforward. The PM screen indicated that the phenothiazines are inhibitors of efflux, and from work done by others

with *S. aureus* and *B. pseudomallei*, they may also act in a similar fashion in these species. Finally, although *ramA* had previously been postulated to confer MDR in *Salmonella* and *K. pneumoniae*, in this study we have both confirmed the hypothesis that MDR is conferred by overproduction of *acrB* and shown that a defined level of overproduction of *ramA* is required to obtain the overproduction of *acrB* at a sufficient level to export antimicrobials.

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