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AcrAB Multidrug Efflux Pump Regulation in *Salmonella enterica* serovar Typhimurium by RamA in Response to Environmental Signals*

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***Salmonella enterica* serovar Typhimurium has at least nine multidrug efflux pumps. Among these pumps, AcrAB is effective in generating drug resistance and has wide substrate specificity. Here we report that indole, bile, and an *Escherichia coli* conditioned medium induced the AcrAB pump in *Salmonella* through a specific regulator, RamA. The RamA-binding sites were located in the upstream regions of *acrAB* and *tolC*. RamA was required for indole induction of *acrAB*. Other regulators of *acrAB* such as MarA, SoxS, Rob, SdiA, and AcrR did not contribute to *acrAB* induction by indole in *Salmonella*. Indole activated *ramA* transcription, and overproduction of RamA caused increased *acrAB* expression. In contrast, induction of *ramA* was not required for induction of *acrAB* by bile. Cholic acid binds to RamA, and we suggest that bile acts by altering pre-existing RamA. This points to two different AcrAB regulatory modes through RamA. Our results suggest that RamA controls the *Salmonella* AcrAB-TolC multidrug efflux system through dual regulatory modes in response to environmental signals.**

Salmonella enterica is a bacterial pathogen that causes a variety of diseases in humans, including gastroenteritis, bacteremia, and typhoid fever (1). In the 1990s, the prevalence of multidrug-resistant *Salmonella* increased in the United Kingdom (2, 3), the United States (4, 5), and Canada (6). Many countries documented outbreaks associated with drug-resistant *Salmonella* in poultry, cattle, and swine (4, 7–10). Emerging resistance to antibiotics in *Salmonella* has been found in both humans and animals and is a potentially serious public health problem (11, 12). High level fluoroquinolone resistance in *S. enterica* serovar Typhimurium phage type DT204 has been reported to result from multiple target gene mutations and active efflux by the AcrAB-TolC multidrug efflux pump (13, 14).

Multidrug efflux pumps have important physiological functions, including transport of drugs, bile salts, toxins, and environmental compounds (15, 16). In bacteria, drug resistance is often associated with multidrug efflux pumps that decrease cellular drug accumulation (17, 18). In bacteria, such pumps have been classified into five families on the basis of sequence similarity as follows: the major facilitator, resistance-nodulation-cell division, small multidrug resistance, multidrug and toxic compound extrusion, and ATP-binding cassette families (19–21). In Gram-negative bacteria, resistance-nodulation-cell division pumps are especially effective in generating resistance (17, 22–24). Recent studies have shown that Gram-negative *S. enterica* serovar Typhimurium has nine functional drug efflux pumps (25). Many multidrug pumps have overlapping substrate spectra, and it is intriguing that bacteria, with their economically organized genomes, harbor large sets of multidrug efflux genes (17).

The key to understanding how bacteria utilize these pumps lies in the regulation of their expression (19, 26–28). Currently available data indicate that multidrug efflux pumps are often expressed under precise and elaborate transcriptional control. For example, expression of *acrAB*, which encodes the AcrAB pump, may be subject to multiple levels of regulation. In *Escherichia coli*, it is modulated locally by the repressor AcrR (29). At a more global level, it is modulated by stress conditions and by regulators such as MarA, SoxS, and Rob (30, 31). Olliver *et al.* (32) reported that mutation in *acrR* contributes to overexpression of *acrAB* in *Salmonella* and increases resistance to multiple drugs. Eaves *et al.* (33) reported that *acrB*, *acrF*, and *acrD* are coordinately regulated and that their expression influences expression of transcriptional activators *marA* and *soxS*. Furthermore, integration of IS1 and IS10 elements into the upstream region of the *acrEF* operon has been reported to cause increased expression of *acrEF* (34). These examples illustrate the complexity and diversity of the mechanisms regulating bacterial multidrug efflux pumps. However, few data are available on signals that induce multidrug efflux genes in *Salmonella*.

Previously, it was reported that indole induces the *acrD*, *acrE*, *cusB*, *emrK*, *mdtA*, *mdtE*, and *mdtH* multidrug efflux

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TABLE 1
***S. enterica* strains and plasmids used in this study**

Strain or plasmid	Original name	Characteristics	Source or Ref.
Strains as in text			
WT	ATCC14028s	*S. enterica* serovar Typhimurium wild-type	36
tolC-lac	EG15109	Δ*tolC-lacZY*$^+$ Km^R	25
acrAB-lac	NKS505	Δ*acrAB-lacZY*$^+$ Km^R	This study
acrEF-lac	EG15114	Δ*acrEF-lacZY*$^+$ Km^R	25
acrD-lac	EG15120	Δ*acrD-lacZY*$^+$ Km^R	25
mdtABC-lac	EG15124	Δ*mdtABC-lacZY*$^+$ Km^R	25
mdsABC-lac	NKS517	Δ*mdsABC-lacZY*$^+$ Km^R	This study
emrAB-lac	NKS522	Δ*emrAB-lacZY*$^+$ Km^R	This study
mdfA-lac	NKS524	Δ*mdfA-lacZY*$^+$ Km^R	This study
mdtK-lac	EG15132	Δ*mdtK-lacZY*$^+$ Km^R	25
macAB-lac	NKS530	Δ*macAB-lacZY*$^+$ Km^R	This study
Δ*baeSR cpxAR*/*acrAB-lac*	NES56	Δ*baeSR* Δ*cpxAR*::Cm^R Δ*acrAB-lacZY*$^+$ Km^R	This study
Δ*baeSR cpxAR*/*acrD-lac*	NES26	Δ*baeSR* Δ*cpxAR*::Cm^R Δ*acrD-lacZY*$^+$ Km^R	This study
Δ*marA*/*acrAB-lac*	NES20	Δ*marA*::Cm^R Δ*acrAB-lacZY*$^+$ Km^R	This study
Δ*soxS*/*acrAB-lac*	NES28	Δ*soxS*::Cm^R Δ*acrAB-lacZY*$^+$ Km^R	This study
Δ*rob*/*acrAB-lac*	NES29	Δ*rob*::Cm^R Δ*acrAB-lacZY*$^+$ Km^R	This study
Δ*sdiA*/*acrAB-lac*	NES35	Δ*sdiA*::Cm^R Δ*acrAB-lacZY*$^+$ Km^R	This study
Δ*acrR*/*acrAB-lac*	NES48	Δ*acrR*::Cm^R Δ*acrAB-lacZY*$^+$ Km^R	This study
Δ*marA rob soxS sdiA acrR*/*acrAB-lac*	NES55	Δ*marA* Δ*rob* Δ*soxS* Δ*sdiA* Δ*acrR*::Cm^R Δ*acrAB-lacZY*$^+$ Km^R	This study
Δ*ramA*/*acrAB-lac*	NES58	Δ*ramA*::Cm^R Δ*acrAB-lacZY*$^+$ Km^R	This study
Δ*ramA*/*tolC-lac*	NES65	Δ*ramA*::Cm^R Δ*tolC-lacZY*$^+$ Km^R	This study
Plasmids			
pKD3		rep$_{R6Kg[r]}$Ap^{R}FRT Cm^{R}FRT	39
pKD4		rep$_{R6Kg[r]}$Ap^{R}FRT Km^{R}FRT	39
pCP20		rep$_{pSC101}$^{ts} Ap^R Cm^R *cI857*λP$_R$*flp*	39
pMALc2X		Vector, Amp^R	New England Biolabs
pMALc2X *ramA*-His$_6$		*ramA*-His$_6$ gene cloned into pMALc2X, Amp^R	This study
pMALc2X *ramA*-His$_6$ (truncated)		5′-Terminal (69 bp) deleted *ramA*-His$_6$ gene cloned into pMALc2X, Amp^R	This study
pNN387		Single copy vector, Cm^R, NotI-HindIII cloning site upstream of promoter-less *lacZ*	38
pNN*ramA*		pNN387 (*ramA* gene promoter-*lacZ*)	This study

pump genes in *E. coli* (35). They also reported that indole induction of *acrD* and *mdtA* is mediated by the BaeSR and CpxAR systems. However, the effect of indole on the AcrAB-TolC multidrug efflux pump, which plays a major role in antibiotic resistance, remains unknown. Very few signals inducing multidrug efflux pumps in *Salmonella* have been identified so far. Here we report on induction of *acrAB* in *Salmonella* via the specific regulator RamA in response to indole, bile, and an *E. coli* conditioned medium. This study describes the dual regulatory mode of *acrAB* via RamA in response to environmental signals.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—The bacterial strains and plasmids used in this study are listed in Table 1. The *S. enterica* serovar Typhimurium strains were derived from the wild-type (WT)[4] strain ATCC14028s (36). P22-mediated transductions were performed as described by Davis *et al.*, (37). Bacterial strains were grown at 37 °C in Luria-Bertani (LB) broth or plates. Antibiotics such as ampicillin (100 μg/ml), kanamycin (25 μg/ml), or chloramphenicol (25 μg/ml) were added when required.

Plasmid Construction—*ramA* was amplified by PCR from the genomic DNA of strain ATCC14028s (36) using LA-Taq polymerase (Takara Bio Inc., Otsu, Japan) and the primers listed in Table 2. This process introduced the EcoRI and HindIII restriction sites. The PCR fragment was cloned between the EcoRI and HindIII sites of the pMAL-c2X vector (New England Biolabs Inc., Ipswich, MA). The *ramA* promoter was amplified by PCR, and the PCR fragment was cloned between the NotI and HindIII sites of the pNN387 vector (38). The nucleotide sequences of the recombinant plasmids were determined using an ABI PRISM 3100-*Avant* genetic analyzer (Applied Biosystems Foster City, CA).

Construction of Gene Deletion Mutants—Genes were disrupted as described by Datsenko and Wanner (39). The chloramphenicol resistance *cat* gene, flanked by Flp recognition sites, was amplified by PCR using the primers listed in Table 2. The resulting PCR products were used to transform the recipient ATCC14028s strain harboring the pKD46 plasmid that expresses Red recombinase. Chromosomal structure of the mutated loci was verified by PCR (39). The deletions were transferred to strains by P22 transduction. The *cat* gene was eliminated using plasmid pCP20 (39).

β-Galactosidase Assays—Single colonies of each bacterial strain to be assayed were inoculated into 2 ml of LB containing the appropriate selected antibiotics. After overnight growth at 37 °C, the cultures were diluted to 1:50 into *E. coli* conditioned medium or LB media. The cells were then grown at 37 °C until the optical density of 0.8 at 600 nm. To test the effect of indole or bile on gene expression, 2 mM indole, 0.25 mM bile salts, 0.25 mM cholic acid, or 0.25 mM deoxycholic acid were added to secondary cultures. β-Galactosidase activities were determined as described by Miller (40). All assays were performed in triplicate.

Survival Assay—*Salmonella* WT strain was grown at 37 °C in LB medium, with or without indole, for 7 h. Benzalkonium was added to create a concentration of 150 mg/ml in LB. After incubation for 10 min, the number of colony-forming units was

[4] The abbreviations used are: WT, wild type; LB, Luria-Bertani; EMSA, electrophoretic mobility shift assay.

determined by serial dilutions in phosphate-buffered saline on LB agar. The percentage of cells surviving under benzalkonium was the number of colony-forming units per ml remaining after the benzalkonium treatment divided by the initial number of colony-forming units per ml. Survival levels of the indole-treated cells were standardized to 100%, and untreated cell values were displayed relative to those of the indole-treated cells. All assays were performed in triplicate.

Preparation of Conditioned Medium—Conditioned medium was prepared by inoculating 30 ml of LB broth with 300 μl of a 10^{-3} dilution of an overnight culture of *E. coli* MG1655, followed by shaking (170 rpm) at 37 °C for 24 h (A_{600} of 5.0) (41). The cells were pelleted by centrifugation, and the resulting supernatants supplemented with 20× LB broth to create a final concentration of 0.5× and adjusted to a pH of 7.5. The conditioned medium was then filter-sterilized through a 0.2-μm pore filter.

Purification of Histidine-tagged RamA and Truncated RamA Protein—Full-length *ramA* gene or truncated *ramA* gene (69 bases at 5-terminal was deleted) were amplified from genomic DNA of ATCC14028s by PCR with the primers listed in Table 2. The DNA fragments were cloned into pMALc2x vector (New England Biolabs). Constructed plasmids were transformed into BL21(DE3) to produce histidine-tagged RamA or N-terminal truncated (23 amino acids) His-RamA. For purification of RamA protein, *E. coli* was grown at 37 °C to an A_{600} of 0.5. RamA production was induced by addition of 0.3 mM isopropyl 1-thio-β-D-galactoside. Cultures were incubated for 3 h, and bacterial cells were then disrupted by French press (SLM Instruments, Inc., Urbana, IL). The protein was purified using TALON metal affinity resin (Clontech).

DNA Mobility Shift Assay—Upstream regions of *acrA* and *tolC* were amplified by PCR. The PCR products were purified for a DNA mobility shift assay. Ten microliters of reaction mixture for the DNA mobility shift assay contained 0.15 pmol of DNA and RamA protein. The reaction buffer contained 10 mM Tris-HCl (pH 7.5), 50 mM KCl, and 1 mM dithiothreitol. Reaction mixtures were incubated for 30 min at room temperature and separated on a 5% native polyacrylamide gel at 4 °C. The gel was soaked in 10,000× diluted SYBR Green I nucleic acid stain (Cambrex Corp., East Rutherford, NJ). DNA was visualized under blue incident light at 460 nm (Luminescent Image Analyzer LAS-3000, Fujifilm Life Science, Stamford, CT).

Intrinsic Fluorescence Spectrum of RamA in the Presence of Cholic Acid—Fluorescence spectra of RamA and truncated RamA were measured as described by Rosenberg *et al.* (31). The fluorescence emission spectra were recorded using a LS 55 fluorescence spectrometer, 120 V (PerkinElmer Life Sciences).

RESULTS

Indole Induces Four Multidrug Efflux Pumps and Drug Tolerance of Salmonella—In *E. coli*, indole is produced from tryptophan by tryptophanase and is excreted from the cell (42). However, *Salmonella* does not produce indole because it lacks the *tnaA* gene encoding tryptophanase (43). Indole has also been reported to auto-regulate multidrug efflux genes in *E. coli* (35). We postulated that *Salmonella* multidrug efflux genes may respond to indole. To investigate the effect of indole on the expression of multidrug efflux pumps, *Salmonella* strains, in which the efflux genes were replaced with a reporter gene (*lacZ*), were inoculated into cultures, with or without indole. Expression levels of drug efflux pumps were measured by a β-galactosidase reporter assay. Indole significantly induced expression of the *acrAB*, *emrAB*, *acrD*, and *mdtABC* efflux genes in *Salmonella* (Fig. 1*A*). A survival assay using benzalkonium showed that indole enhanced drug tolerance of *Salmonella* (Fig. 1*B*).

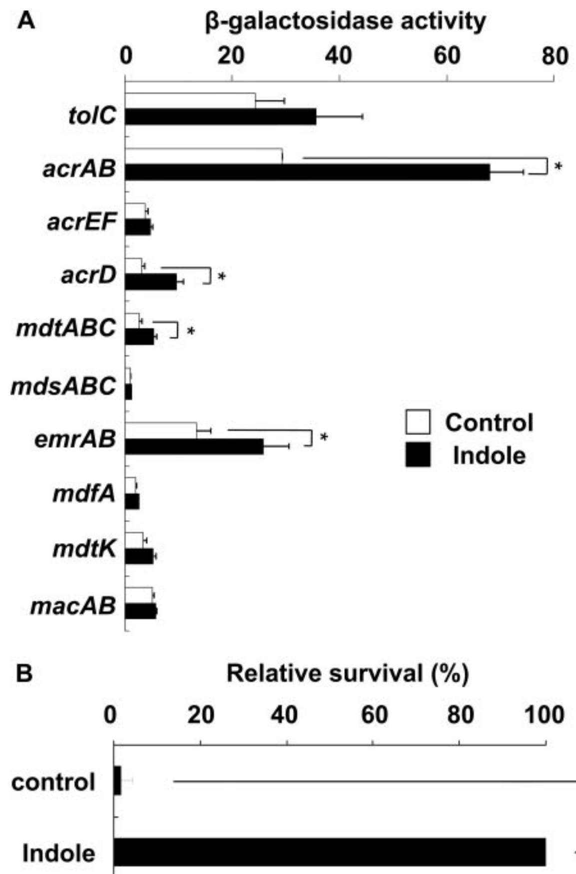

FIGURE 1. **Indole induction of multidrug efflux genes and drug tolerance of *Salmonella enterica* serovar Typhimurium.** The data correspond to mean values from three independent experiments. *Bars* correspond to the standard deviation. *Asterisks* indicate statistically significant differences (*, $p < 0.01$) in the paired Student's *t* test. *A,* differences in β-galactosidase activity in *tolC-lac* (EG15109), *acrAB-lac* (NKS505), *acrEF-lac* (EG15114), *acrD-lac* (EG15120), *mdtABC-lac* (EG15124), *mdsABC-lac* (NKS517), *emrAB-lac* (NKS522), *mdfA-lac* (NKS524), *mdtK-lac* (EG15132), and *macAB-lac* (NKS530) strains grown in LB medium with (*solid bars*) or without (*open bars*) 2 mM indole. *B,* drug tolerance of *S. enterica* serovar Typhimurium induced by indole. WT strain (ATCC14028s) was incubated with or without 2 mM indole. Cells were then challenged to benzalkonium (500 mg/ml) for 10 min. The survival levels of the indole-treated cells were normalized to 100%, and untreated cells are displayed relative to those of the indole-treated cells. The actual survival of indole-treated cells was 0.025%.

Indole Induces acrAB Expression via the RamA Regulator—Among the multidrug efflux pumps, AcrAB plays a major role in the intrinsic resistance of *Salmonella* (25). Also, Hirakawa *et al.* (35) reported that the *baeSR* and *cpxAR* signal transduction system genes are required for indole induction of multidrug efflux pumps in *E. coli*. To identify the regulatory elements that induce *acrAB* in response to indole in *Salmonella*, we constructed a mutant that lacked *baeSR* and *cpxAR*. In the Δ*baeSR cpxAR* mutant, the expression of *acrAB* was not significantly

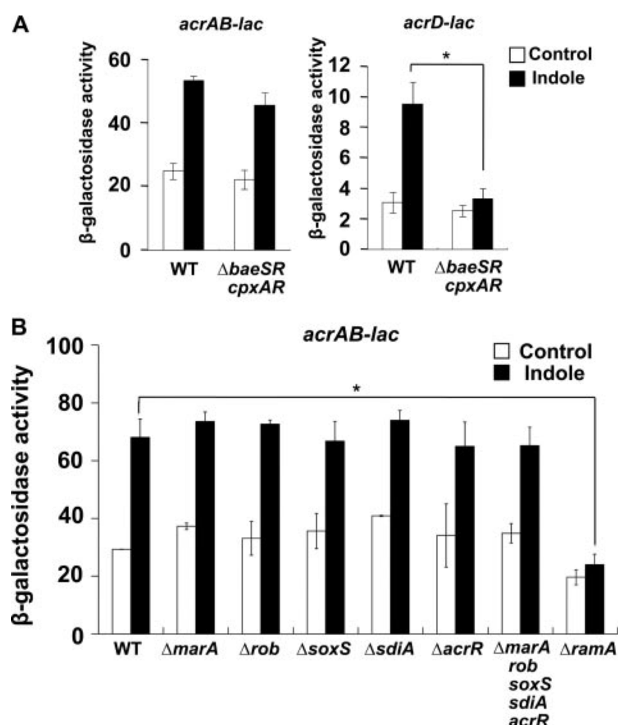

FIGURE 2. **Indole activation of *acrAB* expression through the RamA regulator.** The data correspond to mean values from three independent experiments. *Bars* correspond to the standard deviation. *Asterisks* indicate statistically significant differences (*, $p < 0.01$) in the paired Student's *t* test. *A*, β-galactosidase levels in WT or Δ*baeSR cpxAR* strains, carrying *acrAB-lac* and *acrD-lac* transcriptional fusions, grown in LB medium with (*solid bars*) or without (*open bars*) 2 mM indole. *B*, β-galactosidase levels were assayed in strains carrying the *acrAB-lac* transcriptional fusion in the WT (NKS505), Δ*marA* (NES20), Δ*rob* (NES29), Δ*soxS* (NES28), Δ*sdiA* (NES35), Δ*acrR* (NES48), Δ*marA rob soxS sdiA acrR* (NES55), and Δ*ramA* (NES58) strains. Strains were grown in LB medium with (*solid bars*) or without (*open bars*) 2 mM indole.

different from that in the wild-type (WT) strain; however, indole induction of *acrD* was significantly lower in the mutant compared with the WT strain (Fig. 2*A*). The result indicates that the BaeSR and CpxAR signal transduction systems are not involved in indole induction of *acrAB*, whereas they are required for *acrD* induction. Other regulators, *marA*, *soxS*, *rob*, *sdiA*, and *acrR*, have been previously reported to control *acrAB* expression in *E. coli* (27). With the exception of *ramA*, none significantly altered the indole induction of *acrAB* in *Salmonella* (Fig. 2*B*). The stimulatory effect of indole on *acrAB* expression was completely eliminated in the Δ*ramA* mutant (Fig. 2*B*). The results indicate that the RamA regulator is required for indole induction of *acrAB* in *Salmonella*.

acrAB Activation by Bile Is Dependent on the RamA Regulator—The AcrAB pump is reported to export bile salts and play a role in bile resistance in *E. coli* and *Salmonella* (44–46). Also, *acrAB* is reportedly induced by bile in a Rob-dependent manner in *E. coli* (31). Although *acrAB* is also induced by bile in *Salmonella*, the induction mediating regulator is unknown (47). Prouty *et al.* (47) further reported that *acrAB* activation by bile is independent of MarA, Rob, PhoP/PhoQ, and RpoS. We investigated the possibility that RamA controls *acrAB* expression in response to bile. In agreement with Prouty *et al.* (47), bile salts, cholic acid, and deoxycholic acid significantly induced *acrAB* expression in

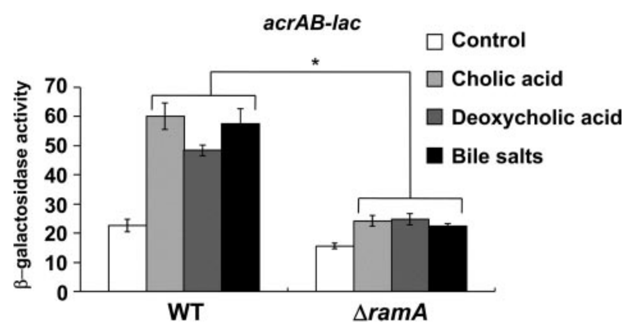

FIGURE 3. **Requirement of RamA for induction of *acrAB* by bile.** β-Galactosidase levels were assayed in WT (NKS505) or Δ*ramA* (NES58) strains carrying the *acrAB-lac* transcriptional fusion. Cells were grown in LB medium (control) or LB medium supplemented with 0.25 mM cholic acid, 0.25 mM deoxycholic acid, or 0.25 mM bile salts. The data correspond to mean values from three independent experiments. *Bars* correspond to the standard deviation. Student's *t* test; *, $p < 0.01$ *versus* WT.

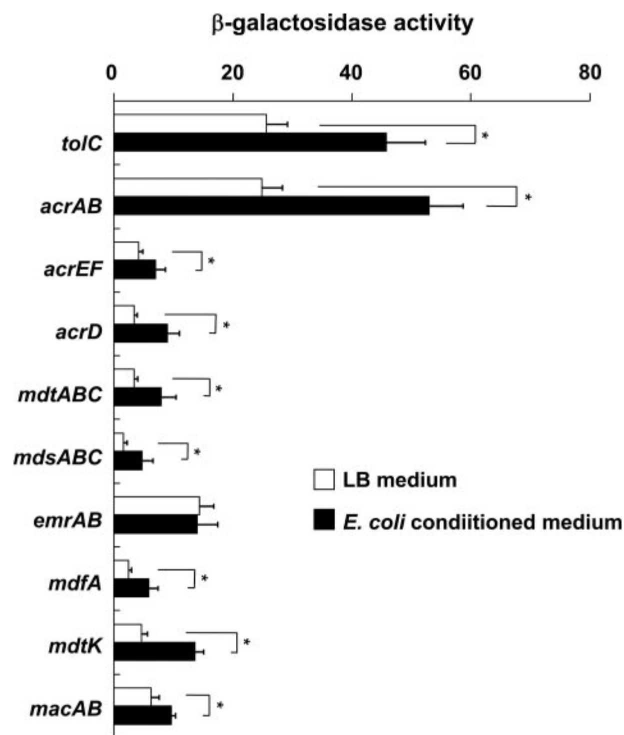

FIGURE 4. **Multidrug efflux genes in *Salmonella enterica* serovar Typhimurium induced by an *E. coli* conditioned medium.** The *tolC-lac* (EG15109), *acrAB-lac* (NKS505), *acrEF-lac* (EG15114), *acrD-lac* (EG15120), *mdtABC-lac* (EG15124), *mdsABC-lac* (NKS517), *emrAB-lac* (NKS522), *mdfA-lac* (NKS524), *mdtK-lac* (EG15132), and *macAB-lac* (NKS530) strains were grown in conditioned medium prepared from *E. coli* culture. Expression levels of multidrug efflux genes were determined by β-galactosidase assay. The data correspond to mean values of three independent experiments. *Error bars* correspond to the standard deviation. *Asterisks* indicate statistically significant differences (*, $p < 0.01$) in the paired Student's *t* test.

Salmonella (Fig. 3). When *ramA* was deleted, *acrAB* induction was eliminated (Fig. 3). These findings indicate a novel RamA-dependent pathway for bile-mediated regulation of the AcrAB efflux pump in *Salmonella*, different from that observed in *E. coli*.

Conditioned Medium from E. coli Induces Salmonella acrAB and tolC Genes via the RamA Regulator—Indole accumulates and MdtEF is induced in stationary phase cultures of *E. coli*, but experiments with a *tnaAB* mutant showed that indole partially contributes to this induction (48). These results indicate that

FIGURE 5. **RamA induction of *acrAB* and *tolC* by conditioned medium of *E. coli*.** The expression of *acrAB* (*A*) and *tolC* (*B*) determined by β-galactosidase assay using strains *acrAB-lac* (NKS505), Δ*ramA*/*acrAB-lac* (NES58), *tolC-lac* (EG15109), or Δ*ramA*/*tolC-lac* (NES65) grown in LB medium or conditioned medium from *E. coli* MG1655. The data correspond to mean values from three independent experiments. *Bars* correspond to the standard deviation. *Asterisks* indicate statistically significant differences (*, $p < 0.01$) in the paired Student's *t* test.

E. coli produces indole as well as other efflux pump inducers. Therefore, we investigated whether an *E. coli* conditioned medium would induce multidrug efflux pumps in *Salmonella*. Conditioned medium, prepared from *E. coli* MG1655, significantly induced eight *Salmonella* multidrug efflux pumps, including *acrAB* and the outer membrane protein gene *tolC* (Fig. 4). Inductions of *acrAB* and *tolC* were significantly decreased in Δ*ramA* (Fig. 5, *A* and *B*), indicating that the *E. coli* conditioned medium induced *acrAB* and *tolC* via the RamA regulator.

RamA Binds to the Upstream Regions of acrA and tolC—The aforementioned results indicate that RamA plays a major role in inducing *acrAB* in response to environmental signals such as indole and bile. To understand the regulation of *acrAB* by RamA, electrophoretic mobility shift assays (EMSA) with the RamA protein were performed. Plasmids encoding the histidine-tagged RamA or the N terminus truncated RamA proteins were constructed (Table 2). Because it was reported that RamA overproduction was related to increased AcrAB expression in clinical *Klebsiella pneumoniae* isolates (49), we investigated the effect of histidine-tagged RamA on *acrAB* expression. Overproduction of histidine-tagged RamA significantly induced the expression of *acrAB* (Fig. 6*A*); however, the truncated RamA did not induce *acrAB* (Fig. 6*A*) and was used as a negative control in subsequent EMSA. Upstream regions of *acrA* were amplified by PCR, and the fragments were incubated with RamA or truncated RamA protein. RamA bound to pAcrA1, whereas

TABLE 2
Primers used in this study

Primer	Sequences (5′ to 3′)
For gene deletion	
marA-P1	TATTTGCTCAAGAAAATTCTGCCGTAGACAAAAAAGAGGTGTGTAGGCTGGAGCTGCTTC
marA-P2	CAGCATTTTCATGGTGCTCTTCGCGTGGCGCATAAACAAACATATGAATATCCTCCTTAG
soxS-P1	GACAAAACGACGAATCGAATACTGTTTAAGAGGCAACAATGTGTAGGCTGGAGCTGCTTC
soxS-P2	GCCTCTGACGATACGCGGGCAGACGCCCTACAGGCGGTGACATATGAATATCCTCCTTAG
rob-P1	TGATATCGGGTGCTCGTTGTTGAAAGGATGGGGATATTTTGTGTAGGCTGGAGCTGCTTC
rob-P2	GCATCTGGACGCCCCTGCATTGGATGAGCTACAGCGTTAACATATGAATATCCTCCTTAG
sdiA-P1	ATAATAATGATTATCAATATCAAAGGCGTGACCATAAAATGTGTAGGCTGGAGCTGCTTC
sdiA-P2	TCAGCCGGTTTCGCGTCTGGCGCGAAGCATCGTCAGCACGCATATGAATATCCTCCTTAG
acrR-P1	AGCGACACAGAAAATGTCCAGGAGTTTTCCTGGAATATTAGTGTAGGCTGGAGCTGCTTC
acrR-P2	TAAATGTATGTAAATCTAACGCCTGTAAATTCACCGACATCATATGAATATCCTCCTTAG
ramA-P1	CCATGCGATAAGCTGTCTCACAATTTGTGTGGTTATTACTGTGTAGGCTGGAGCTGCTTC
ramA-P2	CGATTAAACATTTCAATGCGTACGGCCATGCTTTTCTTTACATATGAATATCCTCCTTAG
For plasmid construction	
ramA-F-EcoRI (for histidine-tagged RamA)	GCGAATTCATGACCATTTCCGCTCAGG
ramA-F-EcoRI (for N-terminal truncated RamA)	GCGAATTCCGTATTGATGATATTGCC
ramA-R-HindIII	GCAAGCTTTCAATGATGATGATGATGATGCGTACGGCCATGCTT
ramA-F-NotI (for pNN387*ramA*)	CGCGCGGCCGCGTTGTTTCAGCCAGCGCC
ramA-R-HindIII (for pNN387*ramA*)	GAGAAGCTTCGTGCTCTCCCCTCTATCAA
For EMSA	
acrA-PF	CCATATGTCGGTGAATTTACAGGCG
acrA-PR1 (for pAcrA1)	TCAGGGGGAGCCGTTGACCGTC
acrA-PR2 (for pAcrA2)	CCTCTGTTTTTGTTCATATGTAAACCTCG
acrA-PR3 (for pAcrA3)	TGTTTGGTTTTTCGTGCCATATGTCG
acrA-PR4 (for pAcrA4)	TCTGATAGCTCCCAGATCTCACTG
acrA-PR5 (for pAcrA5)	AGTAGCTTCAAGAATATGAACTAAAATTTC
acrA-PR6 (for pAcrA6)	AAGGCTACGTTGCGCCTGTTGAACC
acrA-PR7 (for pAcrA7)	ATCAGTATCGCCGCACGACGG
acrA-PR8 (for pAcrA8)	AGGATGTGTTGTCGTGTCTCCAGCGC
acrA-PR9 (for pAcrA9)	TTCGTGCCATATGTCGGTGAATTTACAG
acrA-PR10 (for pAcrA10)	TGTTTGGTTTTTCGTGCCATATGTCG
acrA-PR11 (for pAcrA11)	CAGCGCTTGTTGTTTGGTTTTTCG
acrA-PR12 (for pAcrA12)	GTCGTGTCTCCAGCGCTTGTTGTTTGG
tolC-PF1	CTTTACCTCGCCACTCATTTCTCCG
tolC-PF2 (for pTolC1)	ATGGCACGTAACGCCAACTTTTTGC
tolC-PF3 (for pTolC2)	GTGCTGCCCTGCTAGCAATAATG
tolC-PR	TCCTTGTTGTGAAGCAGTATTTAGCG

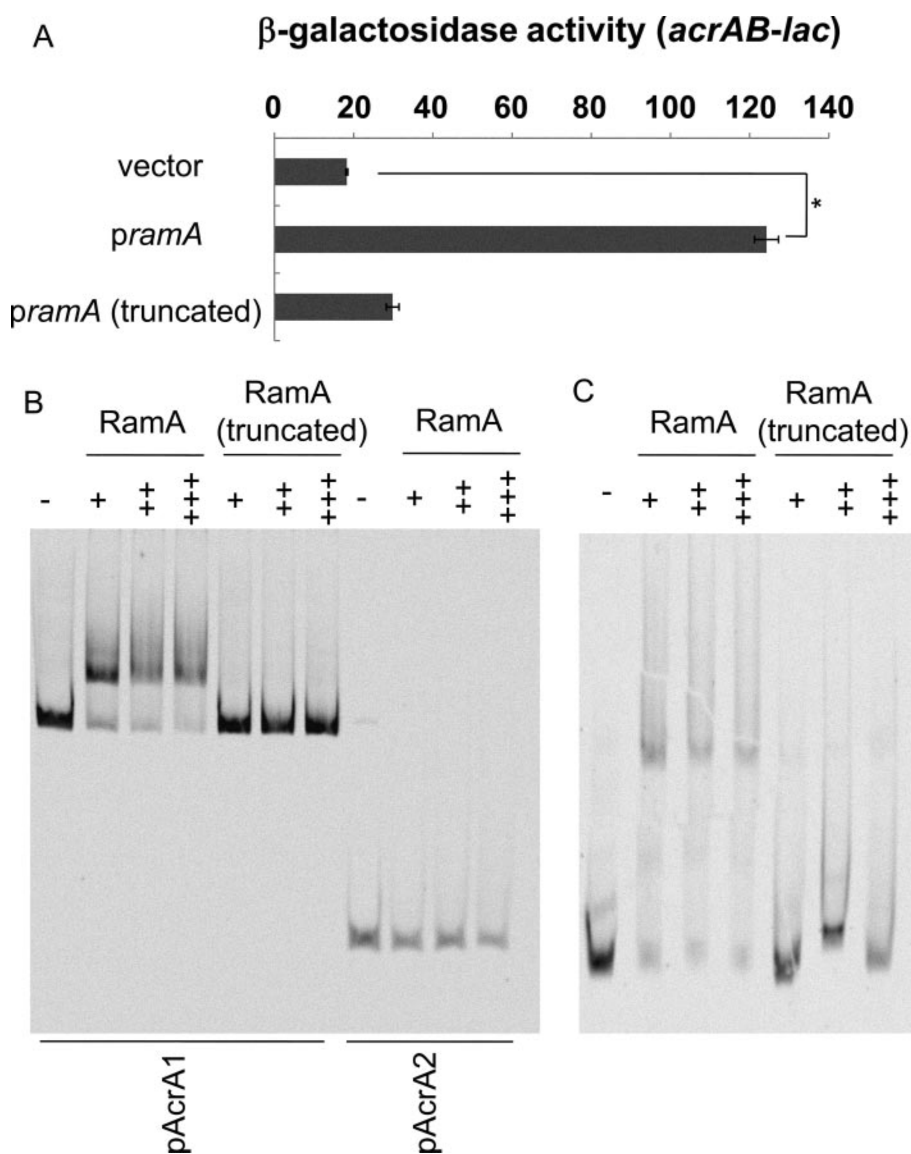

FIGURE 6. **RamA binds to the upstream region of *acrA* and *tolC*.** *A*, β-galactosidase activity measured with *acrAB-lac* (NKS505) harboring a plasmid expressing *ramA*, truncated *ramA*, or the vector control (pMALc2X). The data correspond to mean values from three independent experiments. *Error bars* correspond to the standard deviation. Student's *t* test; *, $p < 0.01$ *versus* control. *B* and *C*, EMSA images for RamA binding to the upstream regions of *acrA* (*B*) and *tolC* (*C*). Upstream regions of *acrA* (*pAcrA1*, −795 to +16 region relative to the start codon of *acrA*; *pAcrA2*, −141 to +16) (*A*) and *tolC* (−250 to −1 region relative to the start codon of *tolC*) (*C*) were incubated with various concentrations of RamA or N-terminal truncated RamA. Protein concentrations are as follows: −, without protein; +, 1.0 μM; ++, 1.5 μM; +++, 2.0 μM.

truncated RamA did not (Fig. 6*B*). However, RamA did not bind to pAcrA2, indicating that the RamA-binding site is located between −795 and −142 upstream of *acrA* (Fig. 6*B*). RamA did bind to the upstream region of *tolC,* whereas the truncated RamA did not (Fig. 6*C*). These results indicate that RamA directly controls the expression of *acrAB* and *tolC*.

Determination of RamA-binding Sites for acrA and tolC—To determine the RamA-binding site for *acrA*, we prepared different lengths of DNA fragments for EMSA. The fragments used were as follows: pAcrA3 (−241 to +16, the numbering is relative to the start codon of *acrA*), pAcrA4 (−341 to +16), pAcrA5 (−441 to +16), pAcrA6 (−541 to +16), and pAcrA7 (−641 to +16). RamA bound to pAcrA3–7, but it did not bind to pAcr2 (Fig. 7*A*). These results indicate that the RamA-binding site was between −241 and −142. We then examined fragment pAcrA8 (−191 to +16); RamA bound to this fragment, indicating a binding site between −191 and −142 (Fig. 7*B*). Further examination with pAcrA9 (−151 to +16), pAcrA10 (−161 to +16), pAcrA11 (−171 to +16), and pAcrA12 (−181 to +16) revealed that RamA bound to pAcrA10–12 but not to pAcrA9 (Fig. 7*C*). These results indicate that the −161 to −152-bp region is required for RamA binding. It was previously reported that RamA bound to a 20-bp asymmetric sequence with a degenerate consensus soxbox of A*YN*GCAC*NNWNNRYY*AAA*YN* (*N* = any base; *R* = A/G; *W* = A/T; *Y* = C/T) (50, 51). A DNA sequence resembling this consensus soxbox sequence, ATGGCACGAAAAACCAAACA, was located at −161 to −142 (Fig. 7*D*).

We also determined a RamA-binding site located upstream of *tolC*. In a *tolC* promoter, we found a soxbox sequence between −99 and −80 (the numbering is relative to the start codon of *tolC*). Therefore, we prepared fragments of pTolC1 (−79 to −1) and pTolC2 (−99 to −1) to determine binding location. RamA bound to pTolC2 but did not bind to pTolC1 (Fig. 8*A*) indicating that RamA binds between −99 to −80 and contains the ATGGCACGTAACGCCAACTT consensus sequence (Fig. 8*B*).

Indole Induces ramA Expression but Bile Does Not—The effects of indole and bile on *ramA* expression levels were investigated because increased *ramA* expression has been reported to cause increased production of the AcrAB-TolC efflux system (49). Using a reporter plasmid of *ramA*, a β-galactosidase assay showed that indole enhanced the promoter activity of *ramA* (Fig. 9). This suggests that indole induces *acrAB* through increased expression of *ramA*. Bile salts, cholic acid, and deoxycholic acid did not affect the expression level of *ramA* despite its requirement for induction of *acrAB*. This indicates an *acrAB* regulatory mode other than through increased production of RamA.

Binding of Bile to RamA Protein—The failure of bile to affect the expression level of *ramA* suggests that RamA may detect the presence of a bile acid component such as cholic acid. This was explored using the intrinsic (tryptophan) spectrum of RamA, as described by Rosenberg *et al.* (31). When 50 μM cho-

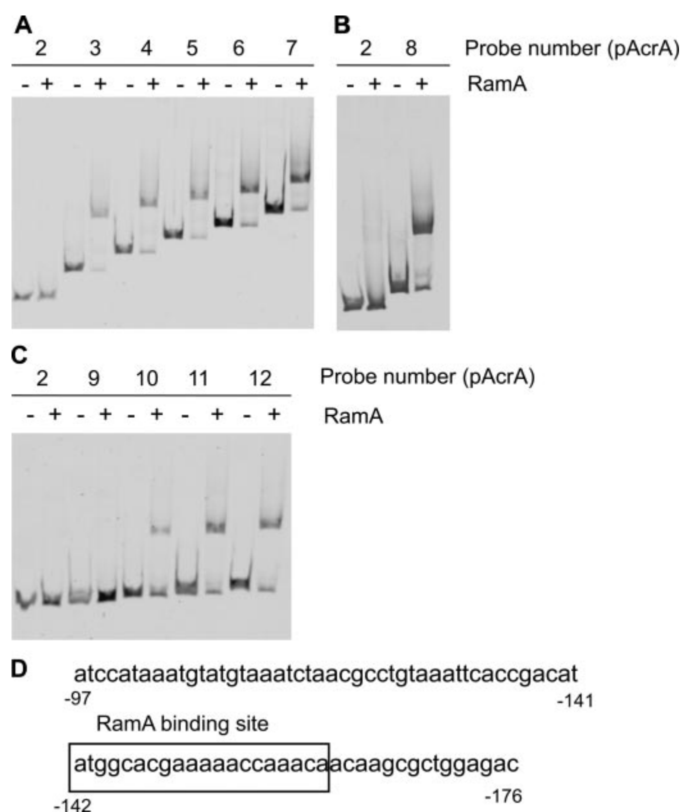

FIGURE 7. *A–C*, determination of RamA-binding site for *acrA*. EMSA of RamA binding to the upstream regions of *acrA* is shown. DNA fragments, including upstream regions of *acrA,* were incubated without (−) or with (+) purified RamA (1.0 μM). DNA fragments are as follows: pAcr2 (−141 to +16, the numbering is relative to the start codon of *acrA*), pAcrA3 (−241 to +16), pAcrA4 (−341 to +16), pAcrA5 (−441 to +16), pAcrA6 (−541 to +16), pAcrA7 (−641 to +16) (*A*), pAcrA8 (−191 to +16) (*B*), pAcrA9 (−151 to +16), pAcrA10 (−161 to +16), pAcrA11 (−171 to +16), and pAcrA12 (−181 to +16) (*C*). *D,* nucleotide sequence in the upstream region of *acrA*. *Boxed* sequence corresponds to the RamA-binding site. The *numbers* indicate the positions from the start codon of *acrA*.

lic acid was added to 75 nM RamA, there was a strong blue shift in the emission spectrum (Fig. 10*A*). The blue shift was also slightly seen with 10 μM cholic acid and 75 nM RamA (Fig. 10*B*). In contrast, a blue shift was not observed when 50 μM cholic acid was added to 75 nM truncated RamA (Fig. 10*C*). These results indicate that bile binds to RamA to induce *acrAB* expression in *Salmonella*.

DISCUSSION

Very few signals inducing multidrug efflux pumps in *Salmonella* have been identified so far (47, 52). In this study, we found that indole, bile, and an *E. coli* conditioned medium induced several multidrug efflux genes in *Salmonella*. We found that *acrAB* induction by these three signal sources is completely dependent on the *Salmonella*-specific regulator RamA, indicating that RamA plays a major role in inducing *acrAB*. RamA belongs to the AraC transcriptional activator family, and activation of RamA is reported to confer drug resistance on *Salmonella* (53). In *Salmonella*, RamA is also involved in resistance to superoxide (54) and in paraquat induction of the flavohemoglobin gene (50).

The *ramA* gene appears to be specific for *Salmonella* serovars and is absent in many other Gram-negative microorganisms; notable exceptions are *K. pneumoniae* and *Enterobacter*

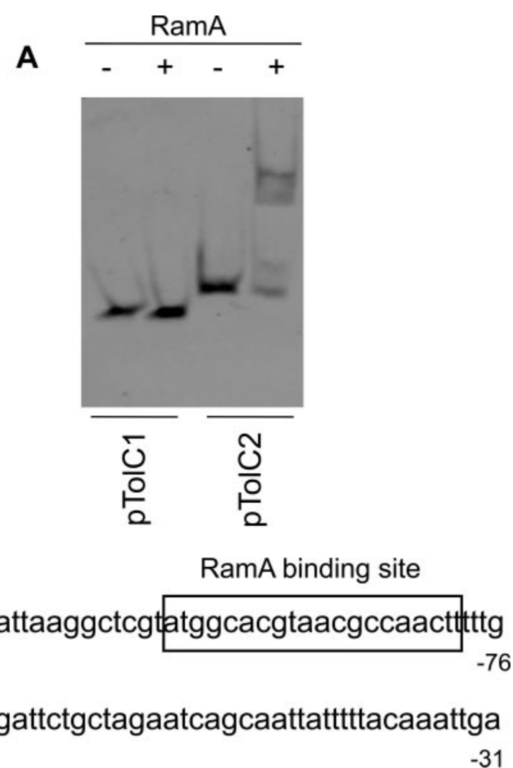

FIGURE 8. **RamA-binding site for *tolC*.** *A,* DNA fragments, including *tolC* promoter regions pTolC1 (−80 to −1; the numbering is relative to the start codon of *tolC*) and pTolC2 (−100 to +1), were incubated without (−) or with (+) purified RamA (1.0 μM). *B,* nucleotide sequence upstream region of *tolC*. *Boxed* sequence corresponds to the RamA-binding site. The *numbers* indicate the positions from the start codon of *tolC*.

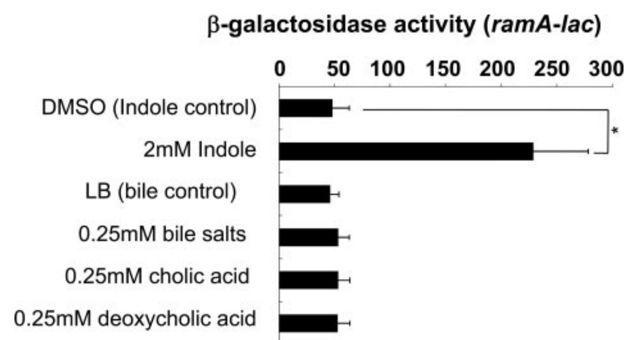

FIGURE 9. **Effect of indole and bile on *ramA* transcription.** β-Galactosidase levels were assayed in the WT strain carrying the *ramA* reporter plasmid (pNN*ramA*) (NES84). Cells were grown in LB medium supplemented with 2 mM indole, 0.25 mM cholic acid, 0.25 mM deoxycholic acid, or 0.25 mM bile salts. The data correspond to mean values from three independent experiments. *Bars* correspond to the standard deviation. Student's *t* test; *, $p < 0.01$ *versus* control.

spp. (54–56). The results of genomic comparison indicate that the gene organization surrounding *ramA* gene and the corresponding region in *E. coli* are similar, with two exceptions as follows: the absence of *ramA* and the presence of *Yi81-2* in *E. coli* (57). We suggest that the AcrAB induction pathway in *Salmonella* is different from that in *E. coli*. Bile induces AcrAB in both *Salmonella* and *E. coli*. In *E. coli*, the transcriptional factor Rob plays a major role in inducing *acrAB* expression in response to bile (31). However, our data indicate that bile induction of *acrAB* in *Salmonella* is completely dependent on RamA, not Rob. Other regulators, including MarA, SoxS, SdiA,

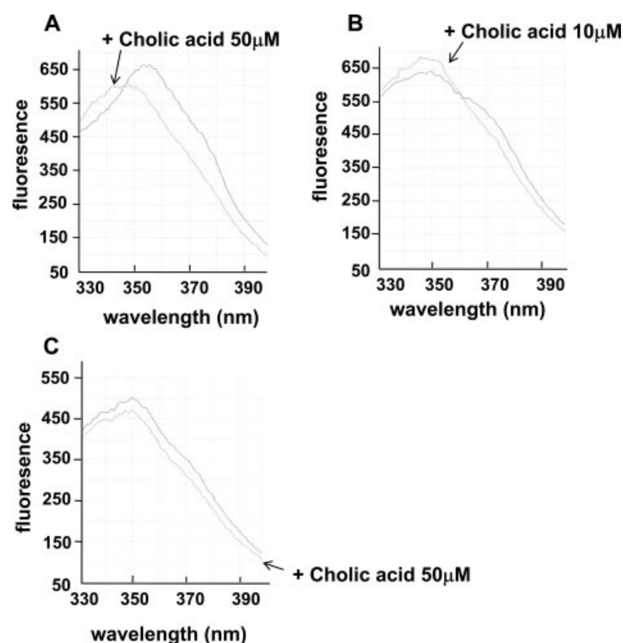

FIGURE 10. **Binding of bile to the RamA protein.** *A,* cholic acid (50 μM) produces a blue shift in the intrinsic spectrum of RamA. Intrinsic fluorescence of RamA (75 nM) was measured in the absence or presence of cholic acid. *B,* cholic acid (10 μM) also produces a blue shift in the intrinsic spectrum of RamA. *C,* blue shift in the intrinsic spectrum of truncated RamA was not produced by 50 μM cholic acid.

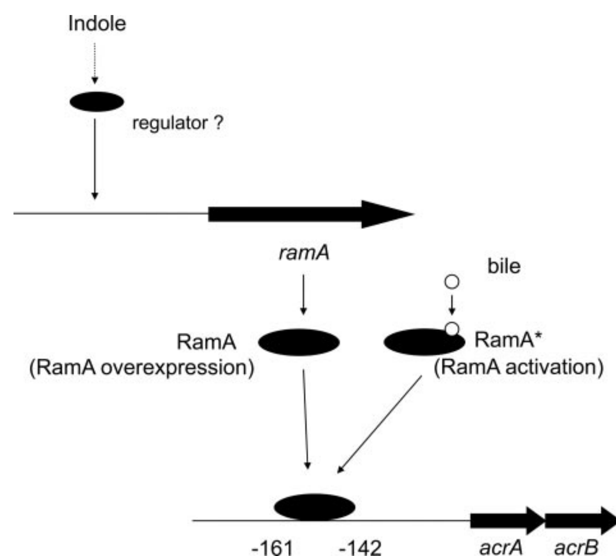

FIGURE 11. **Proposed model for the expression of *acrA* and *acrB* multidrug efflux genes by RamA.** In one pathway, bile may bind to the RamA protein, which is then converted from a low to a high activity state. In the other path, indole may activate *ramA* transcription to directly induce *acrA* and *acrB*.

and AcrR, are not involved in AcrAB induction by indole and bile. These results suggest that RamA is a master regulator of *Salmonella acrAB* and may mask the contributions of any other *acrAB* regulators. In *E. coli*, it was reported that multiple regulators, including MarA, Rob, SoxS, and SdiA, work coordinately in controlling *acrAB* expression in response to *acrAB* inducers. This may be related to the lack of RamA in *E. coli*. Indeed, overproduction of RamA has induced the drug resistance level of *E. coli* (53, 57). A recent report suggests that RamA and RamR, not SoxS and MarA, are involved in AcrAB-mediated multidrug resistance in *Salmonella* (58). Based on our results and these other studies, RamA appears to be the master regulator of *acrAB* in *Salmonella*.

We also suggest the existence of a different induction mechanism for *acrAB* via the RamA regulator (Fig. 11). Indole was shown to induce *ramA* expression (Fig. 9), and such an increased expression of *ramA* can induce *acrAB* (Fig. 6*A*). On the other hand, bile did not affect expression of *ramA* (Fig. 9), but it did bind to RamA (Fig. 11). This is reminiscent of the binding of bile to the Rob protein involved in regulation of *acrAB* in *E. coli* (31). We also suggest that the N-terminal domain of RamA may be required for binding of bile because cholic acid did not bind to the truncated RamA (Fig. 10). Our results suggest a mechanism in which RamA can change between an "activated state" and an "overexpressed state" in response to environmental signals, thereby inducing the AcrAB-TolC system (Fig. 11). Thus, RamA can be converted from a low activity state to a high activity state in response to bile. We also suggest that *Salmonella* may have an additional sensor for indole that controls *ramA* expression (Fig. 11).

Indole and bile are found in various internal human environments, especially in the intestine (59, 60). Indole is produced by many enteric bacterial species (60), and bile is often present at high concentration in the intestinal tract (59). Therefore, RamA may be required for *Salmonella* to detect environmental signals and for subsequent induction of the AcrAB-TolC system, resulting in excretion of toxic compounds by *Salmonella* into surrounding environments, such as the intestine.

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