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

| Strains as in text | Original name | Characteristics | Source or Ref. |
|--------------------|---------------|-----------------|---------------|
| WT                 | ATCC14028s    |                 | 36            |
| tolC-lac           | EG15109       | ∆tolC-lacZY+ Km+ | 25            |
| acrAB-lac          | NKS505        | ∆acrAB-lacZY+ Km+ | This study   |
| acrEF-lac          | EG15114       | ∆acrEF-lacZY+ Km+ | 25            |
| acrD-lac           | EG15120       | ∆acrD-lacZY+ Km+ | 25            |
| mdtABC-lac         | EG15124       | ∆mdtABC-lacZY+ Km+ | 25            |
| mdsABC-lac         | NKS517        | ∆mdsABC-lacZY+ Km+ | This study   |
| emrAB-lac          | NKS522        | ∆emrAB-lacZY+ Km+ | This study   |
| mdtK-lac           | EG15132       | ∆mdtK-lacZY+ Km+ | 25            |
| macAB-lac          | NKS530        | ∆macAB-lacZY+ Km+ | This study   |
| ∆baeSR cpXR/acrAB-lac | NSES56   | ∆baeSR ∆cpXR-Cm+ ∆acrAB-lacZY+ Km+ | This study   |
| ∆baeSR cpXR/acrD-lac | NSES26   | ∆baeSR ∆cpXR-Cm+ ∆acrD-lacZY+ Km+ | This study   |
| ∆emrAB-mdtABC     | NSES20        | ∆emrAB-Cm+ ∆mdtABC-lacZY+ Km+ | This study   |
| ∆sosS/acrA-lac     | NSES28        | ∆sosS-Cm+ ∆acrA-lacZY+ Km+ | This study   |
| ∆rob/acrB-lac      | NSES29        | ∆rob-Cm+ ∆acrB-lacZY+ Km+ | This study   |
| ∆sdiA/acrB-lac     | NSES35        | ∆sdiA-Cm+ ∆acrB-lacZY+ Km+ | This study   |
| ∆acrR/acrB-lac     | NSES48        | ∆acrR-Cm+ ∆acrB-lacZY+ Km+ | This study   |
| ∆acrEF/acrAB-lac   | NSES55        | ∆acrEF-Cm+ ∆acrAB-lacZY+ Km+ | This study   |
| ∆acrD/acrAB-lac    | NSES58        | ∆acrD-Cm+ ∆acrAB-lacZY+ Km+ | This study   |
| ∆acrD/acrEF-lac    | NSES65        | ∆acrD-Cm+ ∆acrEF-lacZY+ Km+ | This study   |

Plasmids

| pKD3              | repP630-Ap*FRT Cm*FRT | 39          |
| pKD4              | repP630-Ap*FRT Km*FRT | 39          |
| pCP20             | repP630-Ap*FRT Km*FRT | 39          |
| pMALc2X           | Vector, AmpR         | New England Biolabs |
| pMALc2X ramA-His6 | 5’-Terminal (69 bp) deleted ramA-His6 gene cloned into pMALc2X, AmpR | This study |
| pNN387            | Single copy vector, CmR, NotI-HindIII cloning site upstream of promoter-less lacZ | 38          |
| pNNramA           | 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) 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 μg/ml in LB. After incubation for 10 min, the number of colony-forming units was measured.

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 (A600 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 A600 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 fluorometer (PerkinElmer Life Sciences).

**RESULTS**

**Indole Induces Four Multidrug Efflux Pumps and Drug Tolerance of Salmonella enterica serovar Typhimurium**—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. 1A). A survival assay using benzalkonium (500 mg/ml) for 10 min. The survival levels of the indole-treated cells were normalized to 100%, and untreated cells were 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 ΔbaeSR cpxAR mutant, the expression of acrAB was not significantly
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**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**, \( \beta \)-galactosidase activity in WT or \( \Delta \text{baeSR cpxAR} \) strains, carrying acrAB-lac and acrD-lac transcriptional fusions, grown in LB medium with (solid bars) or without (open bars) 2 mM indole. **B**, \( \beta \)-galactosidase activity in strains carrying the acrAB-lac transcriptional fusion in the WT (NKS505), \( \Delta \text{marA} \) (NES20), \( \Delta \text{rob} \) (NES29), \( \Delta \text{soxS} \) (NES28), \( \Delta \text{sdiA} \) (NKS55), \( \Delta \text{acrR} \) (NKS48), \( \Delta \text{marA rob soxS sdiA acrR} \) (NKS55), and \( \Delta \text{ramA} \) (NKS58) 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. 2A). 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. 2B). The stimulatory effect of indole on acrAB expression was completely eliminated in the \( \Delta \text{ramA} \) mutant (Fig. 2B). The results indicate that the RamA regulator is required for indole induction of acrAB in Salmonella.

**FIGURE 3.** Requirement of RamA for induction of acrAB by bile. \( \beta \)-Galactosidase levels were assayed in WT (NKS505) or \( \Delta \text{ramA} \) (NKS58) 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 Typhi- murium induced by an E. coli conditioned medium. The toIC-lac (EG15109), acrAB-lac (NKS505), acrEF-lac (EG15114), acrD-lac (EG15120), mdtABC-lac (EG15124), mdsABC-lac (NKS517), emrAB-lac (NKS522), mdfA-lac (NKS524), mdfK-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 \( \beta \)-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.

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

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 toIC 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
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*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 ΔramA (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. 6A); however, the truncated RamA did not induce *acrAB* (Fig. 6A) 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

### 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* (NK505), ΔramA/acrAB-lac (NESS58), *tolC-lac* (EG15109), or ΔramA/tolC-lac (NESS65) grown in LB medium or conditioned medium from *E. coli* MG1655 (Fig. 4). 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.

### Table 2

| Primer | Sequences (5’ to 3’) |
|--------|----------------------|
| **For gene deletion** | |
| marA-P1 | TACTGGCTTCAGAAAGTTACCTGGGCTAGAAGAAAGAGGAGGTGAGCTGAGCCTGGCTTC |
| marA-P2 | CGCGATATTCCAGGTTGCGCTCCAGTGAGATTCATGATTTATTTG |
| spoX-P1 | GCGGCTATTCTGCTCTGGTCACTGAGAGGAGGAGGTGAGCTGAGCCTGGCTTC |
| spoX-P2 | CGCGATATTCCAGGTTGCGCTCCAGTGAGATTCATGATTTATTTG |
| rob-P1 | TGTTATCGCGGCTCTGGTCACTGAGAGGAGGAGGTGAGCTGAGCCTGGCTTC |
| rob-P2 | CGCGATATTCCAGGTTGCGCTCCAGTGAGATTCATGATTTATTTG |
| sidA-P1 | ATGATTGATTGACTTAAGAGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| sidA-P2 | CGCGATATTCCAGGTTGCGCTCCAGTGAGATTCATGATTTATTTG |
| acrA-P1 | AGCGAGCACGAAAATTTTTCCTGCTTTTGGTTCTG |
| acrA-P2 | AGCGAGCACGAAAATTTTTCCTGCTTTTGGTTCTG |
| ramA-P1 | CTCAGGATTGCAAGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| ramA-P2 | CGCGATATTCCAGGTTGCGCTCCAGTGAGATTCATGATTTATTTG |

| **For plasmid construction** | |
| ramA-F-EcoRI (for histidine-tagged RamA) | GCGAATTCCATGAGCCAGGGCAAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| ramA-F-EcoRI (for N-terminal truncated RamA) | GCGAATTCCATGAGCCAGGGCAAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| ramA-R-HindIII | GACGAGCTTGCAAGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| ramA-R-HindIII (for pNN387ramA) | GACGAGCTTGCAAGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |

| **For EMSA** | |
| acrA-PR (for pAcrA) | CCGCCGCTGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| acrA-PR1 (for pAcrA1) | TCGCCGCTGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| acrA-PR2 (for pAcrA2) | TCGCCGCTGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| acrA-PR3 (for pAcrA3) | TCGCCGCTGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| acrA-PR4 (for pAcrA4) | TCGCCGCTGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| acrA-PR5 (for pAcrA5) | TCGCCGCTGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| acrA-PR6 (for pAcrA6) | TCGCCGCTGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| acrA-PR7 (for pAcrA7) | TCGCCGCTGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| acrA-PR8 (for pAcrA8) | TCGCCGCTGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| acrA-PR9 (for pAcrA9) | TCGCCGCTGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| acrA-PR10 (for pAcrA10) | TCGCCGCTGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| acrA-PR11 (for pAcrA11) | TCGCCGCTGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| acrA-PR12 (for pAcrA12) | TCGCCGCTGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| tolC-PF1 | TCGCCGCTGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| tolC-PF2 (for pToIC1) | TCGCCGCTGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| tolC-PF3 (for pToIC2) | TCGCCGCTGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
| tolC-PF (for pToIC) | TCGCCGCTGAGGAGGTGACAGAATAAAATTTGAGGAGGTGAGCTGAGCCTGGCTTC |
truncated RamA did not (Fig. 6B). However, RamA did not bind to pAcrA2, indicating that the RamA-binding site is located between –795 and –142 upstream of acrA (Fig. 6B). RamA did bind to the upstream region of tolC, whereas the truncated RamA did not (Fig. 6C). 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. 7A). 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. 7B). 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. 7C). 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 ATGGCACAGNNWNNRYAAAYN (N = any base; R = A/G; W = A/T; Y = C/T) (50, 51). A DNA sequence resembling this consensus soxbox sequence, ATGGCAGAATTTGCCAAAACA, was located at –161 to –142 (Fig. 7D).

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. 8A) indicating that RamA binds between –99 to –80 and contains the ATGGCAGAATTTGCCAAAACA consensus sequence (Fig. 8B).

**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-
lic acid was added to 75 nM RamA, there was a strong blue shift in the emission spectrum (Fig. 10A). The blue shift was also slightly seen with 10 μM cholic acid and 75 nM RamA (Fig. 10B). In contrast, a blue shift was not observed when 50 μM cholic acid was added to 75 nM truncated RamA (Fig. 10B). 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 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 Y181-2 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,
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. 6A). 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-ToIC 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-ToIC system, resulting in excretion of toxic compounds by Salmonella into surrounding environments, such as the intestine.

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