Regulation of the AcrAB multidrug efflux pump in *Salmonella enterica* serovar Typhimurium in response to indole and paraquat

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*Salmonella enterica* serovar Typhimurium has at least nine multidrug efflux pumps. Among these, AcrAB is constitutively expressed and is the most efficient, playing a role in both drug resistance and virulence. The *acrAB* locus is induced by indole, *Escherichia coli*-conditioned medium, and bile salts. This induction is dependent on RamA through the binding sequence in the upstream region of *acrA* that binds RamA. In the present study, we made a detailed investigation of the *ramA* and *acrAB* induction mechanisms in *Salmonella* in response to indole, a biological oxidant for bacteria. We found that *acrAB* and *ramA* induction in response to indole is dependent on RamR. However, the cysteine residues of RamR do not play a role in the induction of *ramA* in response to indole, and the oxidative effect of indole is therefore not related to *ramA* induction via RamR. Furthermore, we showed that paraquat, a superoxide generator, induces *acrAB* but not *ramA*. We further discovered that the mechanism of *acrAB* induction in response to paraquat is dependent on SoxS. The data indicate that there are at least two independent induction pathways for *acrAB* in response to extracellular signals such as indole and paraquat. We propose that *Salmonella* utilizes these regulators for *acrAB* induction in response to extracellular signals in order to adapt itself to environmental conditions.

**INTRODUCTION**

Multidrug efflux pumps are the major agents conferring drug resistance in bacteria. These pumps have been classified into five families on the basis of sequence similarity: the major facilitator, resistance-nodulation-cell division (RND), small multidrug resistance, multidrug and toxic compound extrusion, and ATP-binding cassette (ABC) families (Paulsen *et al.*, 2001; Putman *et al.*, 2000). In Gram-negative bacteria, RND family efflux pumps are especially effective in generating resistance (Murakami *et al.*, 2002, 2006; Nikaido, 1996).

*Salmonella enterica* serovar Typhimurium is a pathogen that causes various diseases in humans, including gastroenteritis, bacteraemia and typhoid fever (Scherer & Miller, 2001). In *Salmonella*, there are at least nine multidrug efflux pumps that confer drug resistance (Nishino *et al.*, 2006). Among these pumps, AcrAB is constitutively expressed and is the most effective in intrinsic drug resistance in *Salmonella* (Nishino *et al.*, 2006). AcrAB is a member of the RND family transporter and cooperates with an outer-membrane component, TolC (Koronakis *et al.*, 2000). AcrAB has a wide substrate spectrum, including antibiotics, dyes and detergents (Nikaido, 1996). It has also been shown to have an important physiological role in bile resistance (Lacroix *et al.*, 1996; Thanassi *et al.*, 1997). A *Salmonella acrB* mutant strain showed decreased invasion and survival in mouse monocyte macrophages (Buckley *et al.*, 2006). *Klebsiella pneumoniae* AcrAB contributes to resistance to antimicrobial peptides, and an AcrB knockout mutant exhibited a reduced capacity to cause pneumonia in a murine model (Padilla *et al.*, 2010). The *acrAB* regulation mechanism must be examined to understand the physiological role of AcrAB. In *Escherichia coli*, AcrAB is regulated by three activators, MarA, SoxS and Rob, and one repressor, AcrR (Ma *et al.*, 1996; Randall & Woodward, 2002; Rosenberg *et al.*, 2003). In *Salmonella*, RamA is involved in inducing *acrAB* in addition to the...
abovementioned regulators (Bailey et al., 2008; Schneiders et al., 2003).

Indole, a bacterial metabolite (Yanofsky et al., 1991), is produced from tryptophan by tryptophanase (TnaA) and excreted outside the cell. Indole is a biological oxidant for bacteria, and it induces antioxidant proteins in \textit{E. coli} and \textit{Brevibacterium flavum} (Garbe et al., 2000). Indole is proposed to dissolve in membrane lipids, causing membrane derangement and enabling direct interaction between redox-cycling isoprenoid quinones and dioxygen, resulting in the generation of superoxide (Garbe et al., 2000). Previously, indole has been shown to induce AcrD and MdtABC in \textit{E. coli} (Hirakawa et al., 2005). The acrD and mdtABC induction mechanisms in response to indole are dependent on the two-component signal system BaeSR and CpxAR (Hirakawa et al., 2005). In \textit{Salmonella}, indole induces four multidrug efflux pumps, AcrAB, AcrD, MdtABC and EmrAB, and the AcrAB induction mechanism is dependent on the SoxS regulator (Nikaido et al., 2008). Indole induces RamA expression and activates \textit{acrB} expression (Nikaido et al., 2008). However, the indole induction mechanisms of \textit{acrB} and \textit{ramA} are not yet clearly understood.

Here, we report that indole induces AcrAB and RamA via RamR, but that \textit{acrB} and \textit{ramA} induction is not based on the oxidative effect of indole. On the other hand, the superoxide generator paraquat induces \textit{acrB} via the SoxS regulator and not via RamA. These results indicate that there are at least two independent pathways for inducing \textit{acrB} in response to environmental signals. Our results indicate that \textit{Salmonella} utilizes these regulators in response to environmental signals and thus adapts itself to different environments.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. \textit{S. enterica} serovar Typhimurium strains were derived from the wild-type strain ATCC 14028s (Fields et al., 1986). Bacterial strains were grown at 37 °C in Luria–Bertani (LB) broth or on plates. Antibiotics [ampicillin (100 mg ml\(^{-1}\)), kanamycin (25 mg ml\(^{-1}\)) and chloramphenicol (25 mg ml\(^{-1}\))] were added as required.

**Epitope-tag insertion at the 3'-terminus of the chromosomal soxS gene.** Insertion of the FLAG-Tag at the 3'-terminus of the soxS gene was performed as described by Datsenko & Wanner (2000). The kanamycin-resistance gene \textit{aph}, flanked by Flp recognition sites, was amplified by PCR using primers soxS-FLAG-forward and soxS-FLAG-reverse (Table 2). Sequence coding for FLAG-Tag (GACT-ACAAAGGACGACGATGACAAG) was introduced in the primer soxS-FLAG-forward. The resulting PCR products were used to transform the recipient ATCC 14028s strain harbouring plasmid pKD46, which expresses Red recombinase (Datsenko & Wanner, 2000). The chromosomal structure of the mutated loci was verified by PCR using primers soxS-FLAG-285up and soxS-FLAG-310down (Table 2). The \textit{ramR} deletion was transferred to the \textit{acrA-lac} strain (NKS505) by P22 transduction (Davis et al., 1980).

### Table 1. \textit{S. enterica} strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Original name</th>
<th>Characteristics</th>
<th>Source or references</th>
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<td>Wild-type</td>
<td>ATCC 14028s</td>
<td>\textit{Salmonella enterica} serovar Typhimurium wild-type</td>
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<tr>
<td>(\Delta\text{ramR})</td>
<td>NKS910</td>
<td>(\Delta\text{ramR}::\text{Cm})</td>
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<tr>
<td>(\text{acrA-lac})</td>
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<td>(\Delta\text{acrB-lacZY}^+\ \text{Km})</td>
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<tr>
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<td>soxS-FLAG::\text{Km}\</td>
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<tr>
<td>pNN387</td>
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<td>Single-copy vector, Cm(^R), NotI–HindIII cloning site upstream of promoter-lacZ</td>
<td>Elledge &amp; Davis (1989)</td>
</tr>
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<td>pNN\text{ramRA(C134S)}</td>
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<td>(soxS gene promoter–lacZ)</td>
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Table 2. Primers used in this study

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<td>soxS-FLAG-forward</td>
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<td>soxS-FLAG-reverse</td>
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</tr>
<tr>
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<td>ATTGATGAACATATCGACCAACCGCTAAAC</td>
</tr>
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<td>soxS-FLAG-310down</td>
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<td>For plasmid construction</td>
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<td>CGCGCCGCCGCGTTCATITGCTTGTCGCAATAGGACG</td>
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<td>ramR-C134S-R</td>
<td>GCGGGCGCGCCTATTGTGTTCGCAATAGGACG</td>
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<tr>
<td>For plasmid mutagenesis</td>
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</tr>
<tr>
<td>ramR-C67S-F</td>
<td>CTCCCTTAG</td>
</tr>
<tr>
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<td>AAAAAACGCGCGCGCTCTGAGATACGCCATATG</td>
</tr>
<tr>
<td>ramR-C134S-F</td>
<td>ATGGATGAACATATCGACCAACCGCTAAAC</td>
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<tr>
<td>ramR-C134S-R</td>
<td>GGGGTTCGCCAGGGGATACGAGGTGC</td>
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**Plasmid construction.** The ramA promoter, including the total ramR sequence, and the soxS promoter were amplified by PCR from the genomic DNA of strain ATCC 14028s using LA-Tag Polymerase (Takara Bio) and primers soxS-F-NoI, soxS-R-HindIII, ramA-F-NoI and ramA-R-HindIII (Table 2). The PCR fragment was cloned between the NoI and HindIII sites of the pNN387 vector (Ellidge & Davis, 1989). The two mutations, RamRC67S and RamRC134S, were obtained by an inverse PCR-based site-directed mutagenesis using the KOD-Plus-Mutagenesis kit (Toyobo) and primers ramR-C67S-F, ramR-C67S-R, ramR-C134S-F and ramR-C134S-R (Table 2).

**β-Galactosidase assay.** Single colonies of each bacterial strain to be assayed were inoculated into 2 ml LB medium containing the appropriate amounts of the selected antibiotics. After overnight incubation at 37°C, cultures were diluted 1 : 50 in LB medium. The cells were then incubated at 37°C until they reached an OD₆₀₀ of 0.8. To examine the effect of indole or paraquat on acrAB, ramA and soxS expression, either 2 mM indole or 0.2 mM paraquat was added to secondary cultures. β-Galactosidase activities were determined as described by Miller (1972). All assays were performed in triplicate.

**Survival assay.** The wild-type Salmonella strain was grown at 37°C in LB medium with or without paraquat. Rhodamine 6G (R6G) was added to a concentration of 25 mM in LB medium. After a 30 min incubation, the number of colony-forming units was determined by performing serial dilutions in PBS and plating on LB agar, and the percentage of cells surviving R6G treatment was calculated.

**Western blot analysis of the epitope-tagged protein.** Bacteria were grown in LB medium with or without 0.2 mM paraquat for 5 h at 37°C, washed with buffer A (20 mM Tris/HCl (pH 8.0), 200 mM NaCl, 1 mM EDTA), resuspended in 1 ml buffer A, and disrupted by sonication using a Branson Sonifier 200 (Branson Sonic Power Co.) on ice for 2.5 min. A whole-cell lysate (8 µg protein) was run on 15% SDS-PAGE with Tris/glycine/SDS running buffer, transferred to PVDF membranes, and analysed by Western blotting using monoclonal anti-FLAG antibody (Sigma-Aldrich). The blot was developed using anti-mouse IgG horseradish-peroxidase-linked antibody and analysed using the ECL detection system (GE Healthcare UK).

**RESULTS**

**Indole induces acrA and ramA via RamR**

We previously reported that indole induces acrA by overexpressing ramA (Nikaido et al., 2008). However, the induction mechanism of ramA in response to indole is unknown. Recently, Abouzeed et al. (2008) reported that RamR is the local repressor of ramA. Therefore, we examined whether indole induces acrA and ramA via RamR. We first investigated the effect of ramR deletion on acrA induction in response to indole. acrA expression increased in the presence of indole, but was absent in the ΔramR strain (Fig. 1a). This result suggests that acrA induction in response to indole is RamR dependent. We next investigated the effect of ramR deletion on ramA induction in response to indole. ramA expression in the wild-type increased in the presence of indole (4.9-fold increase compared to that in the absence of indole), but only slight ramA induction in response to indole was observed in the ΔramR strain (1.4-fold increase) (Fig. 1b). This suggests that RamR is required for the complete ramA induction in response to indole, and that there may be an additional RamR-independent regulatory pathway for ramA induction in response to indole.

**The cysteine residues of RamR are not required for RamA induction**

Indole is a biological oxidant for bacteria and induces antioxidant proteins in E. coli (Garbe et al., 2000). MexR in Pseudomonas aeruginosa is a repressor of the multidrug efflux pump MexAB, and involves a thiol-oxidation-based mechanism for sensing the peroxide stress (Chen et al., 2008). Therefore, we proposed that the oxidant effect of indole may induce ramA and acrAB via RamR oxidation.
We investigated whether the cysteine (Cys) residues of RamR are required for the induction of RamA in response to indole. There are two Cys residues, Cys-67 and Cys-134, in the sequence of RamR. To investigate whether these Cys residues are required for *ramA* induction in response to indole, we constructed two *ramR* mutants, C67S and C134S, with mutation from Cys to Ser. The results showed that the Cys residues of RamR are not necessary for *ramA* induction in response to indole (Fig. 2), suggesting that the oxidative effect of indole is not necessary for *ramA* and *acrAB* induction via RamR.

**Fig. 1.** Indole activation of *ramA* and *acrAB* expression through RamR. (a) β-Galactosidase levels in wild-type and ΔramR strains carrying the *acrAB*-lac transcriptional fusion grown in LB medium with (solid bars) or without (open bars) 2 mM indole. (b) β-Galactosidase levels in wild-type and ΔramR strains carrying the *ramA* reporter plasmid (pNNramA). Cells were grown in LB medium with (solid bars) or without (open bars) 2 mM indole. Data correspond to means ± SD from three independent experiments. Asterisks indicate statistically significant differences (*P*<0.01) in the paired Student's test.

Paraquat does not induce RamA but it induces *Salmonella acrAB* expression

The abovementioned findings suggest that indole induces AcrAB and RamA via RamR. However, the oxidative effect of indole is independent of the *ramA* and *acrAB* induction mechanisms. Paraquat is a superoxide generator (Hassan & Fridovich, 1979); therefore we investigated whether paraquat induces *ramA* in *Salmonella*. We cultured a *Salmonella* wild-type strain carrying the *ramA* reporter plasmid with or without 0.2 mM paraquat and evaluated the *ramA* expression level by β-galactosidase assay. The results show that paraquat does not induce *ramA* in *Salmonella* (Fig. 3a), but it induces *acrAB* 2.5-fold (Fig. 3b).

R6G is recognized by *Salmonella* AcrAB (Nishino et al., 2006); therefore we investigated whether paraquat would enhance R6G resistance in *Salmonella*. We cultured the *Salmonella* wild-type strain with or without paraquat and challenged it with 25 mM R6G. The survival rate in the presence of R6G was 10-fold greater when the cells were treated with paraquat than when untreated (Fig. 3c). These results indicate that paraquat induces *acrAB* and enhances drug resistance in *Salmonella*.

Paraquat induces *acrAB* via the SoxS regulator and not via the RamA regulator

The induction of *acrAB* in response to paraquat suggested that this induction mechanism might be mediated by the RamA regulator. To test this, we measured the level of *acrAB* induction of a *ramA* mutant strain in response to paraquat by β-galactosidase assay. *acrAB* induction by paraquat was observed in the ΔramA strain (Fig. 4a), indicating that RamA was not required for this induction response. Therefore, we investigated the induction pathway of *acrAB* in *Salmonella* in response to paraquat. In addition to RamA, AcrAB is regulated by three activators, MarA, SoxS and Rob,
and one repressor, AcrR (Ma et al., 1996; Randall & Woodward, 2002; Rosenberg et al., 2003). We used deletion mutants of these regulators to investigate whether they were required for \acrAB induction in response to paraquat. AcrAB induction in response to paraquat was reduced in the \docsoxS strain (Fig. 4b), indicating that paraquat induces \acrAB via the SoxS regulator.

### DISCUSSION

Multidrug efflux pumps are the major mechanism of drug resistance in \salmonella. AcrAB is the most effective multidrug efflux pump, exporting various drugs, deter-
gents and dyes. Several investigators have studied the important roles of AcrAB in bacterial drug resistance and virulence; however, the regulation mechanism of \acrAB in \salmonella in response to extracellular signals has not been well characterized. AcrAB regulation is mediated by five regulators: MarA, SoxS, Rob, AcrR and RamA (Bailey et al., 2008; Ma et al., 1996; Randall & Woodward, 2002; Rosenberg et al., 2003; Schneiders et al., 2003). In \salmonella, AcrAB regulation in response to indole, bile salts and \coli metabolites depends on RamA (Nikaido et al., 2008). RamA belongs to the AraC/XylS transcriptional activator family and directly induces \acrAB. Here we report that the induction of \acrAB and \ramA in response to indole requires RamR. \ramA induction in response to indole was significantly decreased in the \Delta\ramR strain, but slight increased expression was still observed in this strain when cells were treated with indole. This result suggests that both RamR-dependent and RamR-independent pathways are involved in induction of \ramA by indole. The CmeABC multidrug efflux pump in \campylobacter jejuni is induced by bile salts; this induction is required for the TetR family regulator CmeR. Lin et al. (2002, 2005) reported that some bile salts (e.g. taurocholate) induced cmeABC via both the CmeR-dependent and CemR-independent pathways, and this regulation resembles the \ramA induction in response to indole. At present, it is unclear which additional factor(s) is required for \ramA induction in response to indole via the RamR-independent pathway.

We showed that the oxidative effect of indole was not related to \ramA induction. Cys mutants of RamR have the ability to sense indole and regulate RamA. RamR is a TetR family regulator that binds to an operator sequence in the upstream region of \ramA (Chinni et al., 2010). TetR family regulators are homodimeric DNA-binding proteins (Yu et al., 2010) that are composed of an N-terminal DNA-binding domain and a C-terminal domain that mediates dimerization and binds to substrates (Yu et al., 2010). Thus, it is hypothesized that indole binds to the C-terminal domain of RamR and derepresses the transcriptional activity of \ramA by causing detachment from the binding site of RamR.

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**Fig. 5.** Paraquat induces SoxS expression. (a) β-Galactosidase levels in the wild-type strain carrying the soxS reporter plasmid (pNNsoxS). Cells were grown in LB medium with (+) or without (−) 0.2 mM paraquat. Data correspond to means ± SD from three independent experiments. The asterisk indicates a statistically significant difference (P<0.01) in the paired Student’s test. (b) Western blotting of SoxS expression in the wild-type strain carrying the epitope-tagged soxS gene. Cells were grown in LB medium with (+) or without (−) 0.2 mM paraquat.

**Fig. 6.** Proposed model for \acrAB multidrug efflux gene expression in \salmonella in response to extracellular signals. There are at least two independent pathways of \acrAB expression in response to extracellular signals, the RamR/RamA-dependent pathway and the SoxS-dependent pathway.
In *E. coli*, indole is produced from tryptophan by tryptophanase and is excreted from the cell (Yanofsky *et al.*, 1991). *Salmonella* does not produce indole because it lacks the *traA* gene that encodes tryptophanase (McClelland *et al.*, 2001); however, indole is found in various internal bacterial species (Sonnenwirth, 1980). Indole is produced by many enteric bacterial species (Sonnenwirth, 1980) and it is detected in human faeces at concentrations of ~250–1100 μM (Karlin *et al.*, 1985). Therefore, RamR may be required by *Salmonella* to detect environmental signals and for subsequent induction of the AcrAB–TolC system, resulting in excretion of toxic compounds by the bacterium into the surrounding environment, such as the intestine.

We showed that paraquat induces *acrAB* via SoxS. Paraquat induces SoxS expression to a very high degree but does not induce *ramA*. These results indicate that RamA and RamR could not detect paraquat, and that SoxS induction is required for *acrAB* induction in response to paraquat. SoxS is a transcriptional activator of the AraC/XylS family and activates several genes in bacteria. The binding sequence of SoxS (AYNGACNNWNNYAAAYN: N=any base; R=A/G; W=A/T; Y=C/T) has been shown to be the same as that of RamA (Martin *et al.*, 2000). Therefore, SoxS is proposed to bind to the upstream region of *acrA* and directly induce *acrAB*. This suggests that RamA and SoxS competitively bind to the upstream region of *acrA*. According to the analysis of the relationship between tigecycline resistance and *Salmonella* AcrA regulators, RamA is the most effective regulator of *Salmonella* *acrAB* (Horiyama *et al.*, 2011). Therefore, the level of SoxS induction in response to paraquat is sufficient to negate the effect of RamA. On the other hand, the oxidative effect of indole is too weak to induce *acrAB* via the SoxS regulator.

We propose a new model for the *acrAB* regulatory network in *Salmonella*. There are at least two *acrAB* induction mechanisms in *Salmonella* in response to extracellular signals (Fig. 6), the RamR/RamA signal pathway and the SoxS signal pathway. Previous studies have suggested that AcrAB is necessary for the survival of *Salmonella* in environments such as the intestine or host cell. Therefore, it is possible that *Salmonella* utilizes appropriate AcrA regulators by sensing extracellular signals.

*Salmonella* has to adapt to various environments in its life cycle. RamA affects not only efflux pump gene expression of *Salmonella* but also the virulence genes, and there are some binding sequences of RamA in the upstream region of both SPI-1 and -2 genes (Bailey *et al.*, 2010). Furthermore, RamA and SoxS are global regulators that affect the expression levels of various genes. Further studies will be required to examine the precise stages in the life cycle of *Salmonella* when RamA and SoxS are required.

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