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.

Received 26 September 2010

Revised 21 November 2010

Accepted 4 December 2010

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

Abbreviation: R6G, rhodamine 6G.

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 *E. coli* and *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 *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 *Salmonella*, indole induces four multidrug efflux pumps, AcrAB, AcrD, MdtABC and EmrAB, and the AcrAB indole induction mechanism is dependent on the RamA regulator (Nikaido *et al.*, 2008). Indole induces RamA expression and activates *acrAB* expression (Nikaido *et al.*, 2008). However, the indole induction mechanisms of *acrAB* and *ramA* are not yet clearly understood.

Here, we report that indole induces AcrAB and RamA via RamR, but that *acrAB* and *ramA* induction is not based on the oxidative effect of indole. On the other hand, the superoxide generator paraquat induces *acrAB* via the SoxS regulator and not via RamA. These results indicate that

there are at least two independent pathways for inducing *acrAB* in response to environmental signals. Our results indicate that *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. *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 *aph*, flanked by F₁p recognition sites, was amplified by PCR using primers *soxS*-FLAG-forward and *soxS*-FLAG-reverse (Table 2). Sequence coding for FLAG-Tag (GACT-ACAAGGACGACGATGACAAG) 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 *ramR* deletion was transferred to the *acrA-lac* strain (NKS505) by P22 transduction (Davis *et al.*, 1980).

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

Strain or plasmid	Original name	Characteristics	Source or references
Strains			
Wild-type	ATCC 14028s	<i>Salmonella enterica</i> serovar Typhimurium wild-type	Fields <i>et al.</i> (1986)
$\Delta ramR$	NKS910	$\Delta ramR::Cm^R$	Horiyama <i>et al.</i> (2011)
<i>acrA-lac</i>	NKS505	$\Delta acrAB-lacZY^+ Km^R$	Nishino <i>et al.</i> (2006)
$\Delta marA/acrA-lac$	NES20	$\Delta marA::Cm^R \Delta acrAB-lacZY^+ Km^R$	Nikaido <i>et al.</i> (2008)
$\Delta soxS/acrA-lac$	NES28	$\Delta soxS::Cm^R \Delta acrAB-lacZY^+ Km^R$	Nikaido <i>et al.</i> (2008)
$\Delta rob/acrA-lac$	NES29	$\Delta rob::Cm^R \Delta acrAB-lacZY^+ Km^R$	Nikaido <i>et al.</i> (2008)
$\Delta sdiA/acrA-lac$	NES35	$\Delta sdiA::Cm^R \Delta acrAB-lacZY^+ Km^R$	Nikaido <i>et al.</i> (2008)
$\Delta acrR/acrA-lac$	NES48	$\Delta acrR::Cm^R \Delta acrAB-lacZY^+ Km^R$	Nikaido <i>et al.</i> (2008)
$\Delta ramA/acrA-lac$	NES58	$\Delta ramA::Cm^R \Delta acrAB-lacZY^+ Km^R$	Nikaido <i>et al.</i> (2008)
$\Delta ramR/acrA-lac$	NKS912	$\Delta ramR::Cm^R \Delta acrAB-lacZY^+ Km^R$	This study
Wild-type/ <i>soxS</i> -FLAG:: <i>Km</i>	NES123	<i>soxS</i> -FLAG:: <i>Km</i> ^R	This study
Plasmids			
pKD46		Red recombinase expression plasmid, Ap ^r	Datsenko & Wanner (2000)
pNN387		Single-copy vector, <i>Cm</i> ^R , <i>NotI</i> - <i>HindIII</i> cloning site upstream of promoter- <i>lacZ</i>	Elledge & Davis (1989)
pNNramA		pNN387 (<i>ramA</i> gene promoter- <i>lacZ</i>)	Nikaido <i>et al.</i> (2008)
pNNramRA		pNN387 (<i>ramA</i> gene promoter and <i>ramR</i> in cloning site)	This study
pNNramRA(C67S)		pNN387 [<i>ramA</i> gene promoter and mutated <i>ramR</i> (C67S) in cloning site]	This study
pNNramRA(C134S)		pNN387 [<i>ramA</i> gene promoter and mutated <i>ramR</i> (C134S) in cloning site]	This study
pNNsoxS		pNN387 (<i>soxS</i> gene promoter- <i>lacZ</i>)	This study

Table 2. Primers used in this study

Primer	Sequence (5' to 3')
For construction of chromosomal epitope	
<i>soxS</i> -FLAG-forward	CGAGTTCGATCGCACTCCCAGCGATTACCGTCACCGCCTGGACTACAAGGACGAC GATGACAAGTAGGTGTAGGCTGGAGCTGCTTCCGAGTTCGATCGCACTCCCAGC GATTACCGTCACCGCCTGGACTACAAGGCGAGTTTCGATCGCACTCCCAGCGATTA CCGTCACCGCCTGGACTACAAGG
<i>soxS</i> -FLAG-reverse	AACAAACGCCCGCGCCTCTGACGATACGCGGGCAGACGCCTTGACATATGAATATC CTCCTTAG
<i>soxS</i> -FLAG-285up	ATTGATGAACATATCGACCAACCGCTAAAC
<i>soxS</i> -FLAG-310down	GGCGTTTCGCCAGGCGGGATACGAGGTGGC
For plasmid construction	
<i>ramA</i> -R- <i>HindIII</i>	GCAAGCTTTCAATGATGATGATGATGATGCGTACGGCCATGCTT
<i>ramA</i> -F- <i>NotI</i> (for pNN387 <i>ramAR</i>)	CGCGCGGCCGCGTTGTTTCAGCCAGCGCC
<i>soxS</i> -R- <i>HindIII</i>	GAGAAGCTTCGTGCTCTCCCTCTATCAA
<i>soxS</i> -F- <i>NotI</i> (for pNN387 <i>soxS</i>)	CGCGCGGCCGCTTATTGCTCCTCGCGAGTC
For plasmid mutagenesis	
<i>ramR</i> C67S-F	TGGCTGAGATCCTGCTTTAAATGCAAATAC
<i>ramR</i> C67S-R	GTCAATGATAATGGAGCTGGATCGA
<i>ramR</i> C134S-F	TGGCTTAAATCGCGCAATTCGGGGAACATA
<i>ramR</i> C134S-R	TGCTTCCGTTTGTATGGTGTATTATG

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-*NotI*, *soxS*-R-*HindIII*, *ramA*-F-*NotI* and *ramA*-R-*HindIII* (Table 2). The PCR fragment was cloned between the *NotI* and *HindIII* sites of the pNN387 vector (Elledge & 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 $\Delta ramR$ 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 $\Delta ramR$ 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.

Fig. 1. Indole activation of *ramA* and *acrAB* expression through RamR. (a) β -Galactosidase levels in wild-type and $\Delta ramR$ 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 $\Delta ramR$ 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 $\pm$ SD from three independent experiments. Asterisks indicate statistically significant differences ($P < 0.01$) in the paired Student's test.

We investigated whether the cysteine (Cys) residues of RamR are required for the induction of RamA induction 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.

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

Fig. 2. The two Cys residues of RamR are not related to *ramA* induction in response to indole. β -Galactosidase levels were assayed in wild-type and $\Delta ramR$ strains carrying the *ramA* reporter plasmid (pNNramA) or *ramA* reporter plasmid with Cys-mutated RamR (C67S and C134S). Cells were grown in LB medium with (solid bars) or without (open bars) 2 mM indole. Data correspond to means $\pm$ SD from three independent experiments. Asterisks indicate statistically significant differences ($P < 0.01$) in the paired Student's test.

& 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 $\Delta ramA$ 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,

Fig. 3. Paraquat does not induce *ramA*, but induces *acrAB*. (a) β -Galactosidase levels in the wild-type strain carrying the *ramA* reporter plasmid (pNN*ramA*). Cells were grown in LB medium with (+) or without (-) 0.2 mM paraquat. (b) β -Galactosidase levels in the wild-type strain carrying *acrAB-lac* transcriptional fusion grown in LB medium with (+) or without (-) 0.2 mM paraquat. (c) Effect of 0.2 mM paraquat on resistance of *Salmonella* to R6G. The wild-type strain was inoculated into LB medium with (+) or without (-) paraquat. Cells were challenged with R6G (25 mM) for 30 min. 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 test.

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 Δ *soxS* strain (Fig. 4b), indicating that paraquat induces *acrAB* via the SoxS regulator.

Paraquat induces *Salmonella* SoxS expression to a high degree

We investigated the interaction between paraquat and SoxS. SoxS is induced by derepression of SoxR (Gaudu *et al.*, 1997). Therefore, we hypothesized that paraquat

Fig. 4. Paraquat induces *acrAB* via SoxS. (a) Paraquat induces *acrAB* by a mechanism independent of RamA. β -Galactosidase levels in wild-type and Δ *ramA* strains carrying *acrAB-lac* transcriptional fusion grown in LB medium with (solid bars) or without (open bars) 0.2 mM paraquat. (b) Differences in β -galactosidase activity of *acrAB* in wild-type, Δ *marA*, Δ *soxS*, Δ *rob* and Δ *acrR* strains. Cells were grown in LB medium with (solid bars) or without (open bars) 0.2 mM paraquat. Data correspond to means $\pm$ SD from three independent experiments. Asterisks indicate statistically significant differences ($P < 0.01$) in the paired Student's test.

may induce *Salmonella* SoxS expression. We constructed a reporter plasmid of the *soxS* promoter and transformed it into the *Salmonella* wild-type strain. We then investigated the level of *soxS* induction in response to paraquat. Paraquat induced the promoter activity of *soxS* to a very high degree (Fig. 5a). This phenotype was also confirmed by determining the protein level by Western blotting (Fig. 5b). These results indicate that paraquat induces *soxS* to overproduce SoxS, causing the increased *acrAB* expression in *Salmonella*.

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-

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 $\pm$ 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.

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 *E. 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 CmeR-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.

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 *tnaA* gene that encodes tryptophanase (McClelland *et al.*, 2001); however, indole is found in various internal human environments, especially in the intestine (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 (AYNGCACNNWNNRYAAAYN: 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 analysis of the relationship between tigecycline resistance and *Salmonella* AcrAB 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 AcrAB 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.

ACKNOWLEDGEMENTS

We thank Eduardo A. Groisman, Barry L. Wanner, and Ronald W. Davis for providing strains and plasmids and Ryosuke Nakashima and

Keisuke Sakurai for communicating unpublished results. Eiji Nikaido was supported by a research fellowship from the Japan Society for the Promotion of Science for Young Scientists. This work was supported by research grants from the Asahi Glass Foundation, Naito Foundation, Research Foundation for Pharmaceutical Sciences, Takeda Science Foundation, Uehara Memorial Foundation, Waksman Foundation of Japan Inc. and Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation. This study was also supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, a Grant-in-Aid from the Japan Society for the Promotion of Science, and PRESTO, the Japan Science and Technology Agency, Japan.

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Edited by: R. J. Maier