Effect of transcriptional activators RamA and SoxS on expression of multidrug efflux pumps AcrAB and AcrEF in fluoroquinolone-resistant Salmonella Typhimurium

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Objectives: Multidrug resistance (MDR) including fluoroquinolone resistance in Salmonella Typhimurium can result from overexpression of efflux pumps. We examined the mechanisms of fluoroquinolone resistance among in vitro-induced ciprofloxacin-resistant Salmonella Typhimurium mutants, LTL and LTH, derived from laboratory strain LT2.

Methods: Deletion mutation and RT–PCR techniques were employed to study the role of efflux pumps in fluoroquinolone resistance and their regulation cascades.

Results: In addition to point mutations in DNA gyrase (gyrA, gyrB) and topoisomerase IV (parC, parE) genes, increased expression of efflux pump genes, such as acrAB and acrEF, was observed in fluoroquinolone-resistant Salmonella strains. Constitutive expression of ramA containing a 9 bp deletion in the promoter region was directly associated with the overexpression of acrAB and acrEF and conferred an MDR phenotype in LTL. Inactivation of ramA increased the antimicrobial susceptibility of LTL, whereas complementation with the mutant allele induced an MDR phenotype in drug-susceptible Salmonella Typhimurium LT2, as demonstrated by 2- to 64-fold increases in resistance to fluoroquinolones, tetracycline and chloramphenicol. On the other hand, inactivation of mutant soxRS resulted in a slight increase in the susceptibility of LTH to several fluoroquinolone drugs, and the introduction of the mutant allele had no effect on antimicrobial susceptibility of LT2, indicating that constitutive expression of soxRS played a minimum role in fluoroquinolone resistance.

Conclusions: Mutations in the promoter region of ramA appear to play a role in the up-regulation of RamA and AcrAB, and RamA is an activator of the MDR regulation cascade in Salmonella Typhimurium.

Keywords: multidrug resistance, antimicrobial resistance, regulation

Introduction

Non-typhoidal salmonellae are an important cause of food-borne gastroenteritis worldwide. Each year, an estimated 1.4 million people suffer from salmonellosis in the USA.1 Numerous reports have shown that an increased number of Salmonella have become resistant to antimicrobials including fluoroquinolones.2–4 Since fluoroquinolones are central to the management of severe salmonellosis, the emergence of multidrug-resistant (MDR) Salmonella Typhimurium with reduced susceptibility to fluoroquinolones is a serious public health concern. Although high-level fluoroquinolone resistance in Salmonella remains rare, outbreaks of fluoroquinolone-resistant Salmonella infections have been reported in the USA,5,6 Taiwan7 and Japan.2 Bacterial resistance to antimicrobials can be conferred by horizontal transfer of mobile elements carrying resistance genes, such as plasmids, transposons and bacteriophage, by target gene mutations, and by increased expression of multidrug efflux pumps resulting in reduced intracellular concentrations of various antibiotics, including β-lactams, macrolides, tetracycline, chloramphenicol and quinolones.8–13 Studies in Escherichia coli have shown that transcriptional activators, such as MarA, SoxS and Rob, play a role in antimicrobial resistance by activating transcription of efflux pumps,14–16 including acrAB and tolC.17–21
SoxS is a member of the AraC/XylS family of transcriptional regulators and is positively regulated by SoxR. It can be activated by oxidation or nitrosylation. In both laboratory and clinical strains of E. coli and Salmonella, activation of the soxRS regulon contributes to the increased resistance to quinolone, nalidixic acid and chloramphenicol.

In addition to the MarA/SoxS/Rob family, George et al. identified and characterized RamA, another member of the AraC/XylS family, for its role in conferring MDR in Klebsiella. Most recently, the ramA gene was identified in Enterobacter aerogenes, Enterobacter cloacae and Salmonella enterica Paratyphi B and may also be involved in MDR in these organisms. However, van der Straaten et al. showed that inactivation of ramA did not affect the antimicrobial susceptibility of wild-type and clinical isolates of MDR Salmonella Typhimurium. We have previously reported contributions of target gene mutations and efflux pumps to decreased susceptibility in Salmonella Typhimurium to quinolones/fluoroquinolones and other antimicrobials. In the present study, we determined the effect of the transcriptional activators RamA and SoxS on the expression of the multidrug efflux pumps AcrAB and AcrEF in fluoroquinolone-resistant Salmonella Typhimurium.

Materials and methods

Bacterial strains, plasmids and growth media

Fluoroquinolone-resistant strains LTL and LTH were independently derived from Salmonella Typhimurium LT2 after in vitro induction with ciprofloxacin from 0.015 to 128 mg/L. The fluoroquinolone resistance phenotypes were stabilized by passing the strains in Luria–Bertani (LB) broth 10 times without antibiotics. The MICs of selected antimicrobial agents for Salmonella enterica were determined by agar dilution in order to expand the tested concentrations up to 1024 and 128 mg/L, respectively. E. coli ATCC 25922 and 35218, Enterococcus faecalis ATCC 51299 and Pseudomonas aeruginosa ATCC 27853 were used as quality control organisms in the antimicrobial susceptibility testing.

Construction of marRAB, soxRS and ramA null mutations

Null Salmonella Typhimurium LT2 mutations in marRAB, soxRS and ramA genes were constructed using the methods described by Datsenko and Wanner. Briefly, the kanamycin resistance cassette (kan) of plasmid pKD4 was PCR amplified using oligonucleotides marRAB-KP1 and marRAB-KP2 for marRAB, soxRS-KP1 and soxRS-KP2 for soxRS, or ramA-KP1 and ramA-KP2 for ramA (Table S1, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)). These 62 base oligonucleotides included 22 bases complementary to the kan sequence at the 3' end and 40 bases complementary to regions adjacent to marRAB, soxRS or ramA. Replacement of target gene, marRAB, soxRS or ramA, was verified by PCR using the k1 and k2 primers and primers flanking the deleted regions (Table S1).

Expression analysis of acrB, acrF, marA, soxS, ramA and robA

 Overnight bacterial cultures were diluted 1/100 in MH broth and grown to mid-logarithmic phase at 37°C with vigorous shaking (230 rpm). Total RNA was harvested from 2 mL aliquots of culture using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and cleaned using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA preparations were treated with RNase-free DNase (Qiagen) on columns and assessed for purity by PCR. RNA yield and quality were determined using an ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). At least two independent RNA preparations were isolated for each strain and treatment.

Template RNA (2 μg) and random hexamers in a final volume of 18.5 μL were incubated at 70°C for 10 min and then added to a reverse transcriptase reaction mixture containing 1× first strand buffer, 1 mM dNTPs, 10 mM 1,4-dithiothreitol (DTT), 40 U/μL RNaseOUT and 1 μL of SuperScript III reverse transcriptase (Invitrogen). The 30 μL mixture was incubated at 25°C for 10 min and at 42°C for 90 min. Additional incubation at 70°C for 15 min was done to inactivate the enzyme. The resultant cDNAs were diluted 500-fold, except those of the normalizing gene, rrsG, which underwent a 103-fold dilution. Real-time PCR was performed using the IQ5 multicolour real-time PCR system (Bio-Rad, Hercules, CA, USA) with each specific primer pair described in Table S1. CDNA and iQ SYBR Green Supermix (Bio-Rad). PCR conditions were 3 min at 95°C, followed by 40 cycles of 95°C for 10 s, 60°C for 15 s and 72°C for 30 s. The ΔΔCt method was used to calculate fold induction of transcription of a target gene by comparison with a value relative to the wild-type strain grown in MH broth at log phase.

DNA sequence analysis

PCR primers were designed using chromosomal DNA as templates from LT2, LTL and LTH and specific oligonucleotide primers for the amplification of acrR (acrR-F21 and acrR-R993), marRAB (marRAB-F23 and marRAB-R1179), soxRS (soxRS-F75 and soxRS-R1025), robA (robA-F57 and robA-R1180) and ramA (ramA-F121 and ramA-R690) (Table S1). PCR products were purified using a QIAquick® PCR purification kit (Qiagen). Each amplicon was sequenced using the same primer pairs at Macrogen, Inc. (Seoul, Korea). DNA sequences were analysed with...
Replacement of wild-type ramA with mutant ramA allele in LT2 and LTH

Mutant ramA from strain LTL was introduced into wild-type strain LT2 and strain LTH using plasmid pKD46. The replacement of wild-type ramA was selected by tetracycline (8 mg/L) and confirmed by PCR using primers ramAF10, containing eight of the nine bases that were absent in mutant ramA at the 3′ end, and ramAR416.

Replacement of wild-type soxRS with mutant soxRS allele in LT2

Additionally, mutant soxRS from LTH was introduced into LT2 using a suicide plasmid-mediated approach described previously. Briefly, the mutant soxRS was amplified by PCR using primers soxRS-xbaI-F and soxRS-xbaI-R (Table S1). The PCR product after XbaI digestion was cloned into pCVD442 resulting in plasmid pCVD442/sox. The insertion was confirmed by PCR using primers pCVD442-F and pCVD442-R. The construct pCVD442/sox was then introduced into LT2 via electroporation. The replacement of soxRS was confirmed by

Sequencher 4.0 software (Gene Codes Corporation, Ann Arbor, MI, USA) and aligned using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The predicted amino acid alignments presented in Figure 2 were prepared using the CLUSTALW program (version 1.8; http://www.ebi.ac.uk/clustalw/) and the BOXSHADE program (version 3.21; http://www.ch.embnet.org/software/BOX_form.html).
Table 1. Antimicrobial resistance profiles of Salmonella Typhimurium LT2 and its derived mutants

<table>
<thead>
<tr>
<th>Strains</th>
<th>CIP</th>
<th>NAL</th>
<th>DAN</th>
<th>DIF</th>
<th>ENR</th>
<th>GAT</th>
<th>LVX</th>
<th>MAR</th>
<th>ORB</th>
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<td>4</td>
<td>0.06</td>
<td>0.25</td>
<td>0.06</td>
<td>0.03</td>
<td>0.06</td>
<td>0.03</td>
<td>0.125</td>
<td>8</td>
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</tr>
<tr>
<td>LT2:: ramA</td>
<td>0.25</td>
<td>64</td>
<td>0.25</td>
<td>0.5</td>
<td>0.25</td>
<td>0.03</td>
<td>0.06</td>
<td>0.03</td>
<td>0.125</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>LT2:: soxRS</td>
<td>0.015</td>
<td>4</td>
<td>0.06</td>
<td>0.25</td>
<td>0.06</td>
<td>0.03</td>
<td>0.06</td>
<td>0.03</td>
<td>0.125</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>LTL</td>
<td>4</td>
<td>1024</td>
<td>8</td>
<td>&gt;32</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>16</td>
<td>64</td>
<td>16</td>
<td></td>
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<tr>
<td>ΔacrAB::km</td>
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<td>64</td>
<td>0.125</td>
<td>0.5</td>
<td>0.125</td>
<td>0.125</td>
<td>0.25</td>
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<tr>
<td>ΔramA::km</td>
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<td>64</td>
<td>0.5</td>
<td>0.25</td>
<td>0.25</td>
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<td>0.5</td>
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<td>32</td>
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<tr>
<td>LTH</td>
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<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
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<td>32</td>
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<tr>
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<td>16</td>
<td>8</td>
<td>8</td>
<td>0.5</td>
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<td>2</td>
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<td>0.5</td>
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<td>16</td>
<td>8</td>
<td>&gt;32</td>
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<td>64</td>
<td>512</td>
<td>&gt;32</td>
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<td>32</td>
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<td>&gt;32</td>
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<tr>
<td>ΔmarRAB::km</td>
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<td>512</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
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<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>64</td>
<td>16</td>
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PCR using primers soxRSwt-F and soxRSwt-R containing 10 bases that were absent in mutant soxRS at the 3’ end (Table S1).

**Results**

**Expression of acrB and acrF in Salmonella Typhimurium LT2 series strains**

The role of efflux pumps in fluoroquinolone resistance of *Salmonella* was evaluated by comparing the expression levels of *acrB* and *acrF* in fluoroquinolone-resistant strains LTL and LTH with ciprofloxacin MICs of 4 and 64 mg/L, respectively, with those of LT2. LTL possessed a mutation (Ser83Phe) in GyrA, whereas LTH contained double mutations (Ser83Phe, Asp87Asn) in GyrA and a single mutation (Gly78Asp) in ParC. Based on RT–PCR, LTL and LTH both expressed *acrAB* and *acrEF* at elevated levels compared with LT2 (Figure 1a). The expression of *acrAB* was 15.1- and 5.5-fold greater in LTL and LTH, respectively, compared with their counterpart in LT2. *acrEF* had a relatively lower expression level compared with *acrAB* within the same strains. Noticeably, the expression of *acrAB* and *acrEF* in LTL was 2- to 3-fold greater than that in LTH.

**Expression of marA, soxS, ramA or robA in Salmonella Typhimurium LT2 mutants**

Earlier studies have shown that constitutive expression of either *marA* or *soxS* mRNA is primarily responsible for the activation of *acrAB* transcription, contributing to clinical MDR in *Salmonella Typhimurium*.25,36 In addition to *marA* and *soxS*, we also examined the expression of *robA*, whose product is closely related to MarA and SoxS in *E. coli*, and *ramA*, which is newly identified in *Salmonella* as a possible MDR regulator. LTL and LTH showed highly up-regulated *ramA* and *soxS*, respectively (Figure 1b). However, neither LTL nor LTH expressed *marA* or *robA* at an elevated level compared with the wild-type LT2 (Figure 1b).

**Mutations in ramA, the soxRS region and acrR of Salmonella Typhimurium LT2 mutants**

The DNA sequence of *ramA* of LTL, compared with that of *ramA* of LT2 (accession no. NC_003197), showed a 9 bp (ATGAGTGCT) deletion in the *ramA* promoter – 10 (TATA box) region (Figure 2a). This change might have turned on the transcription of the *ramA* gene constitutively. For the *soxRS* region in LTH that exhibited increased *soxS* expression, the sequence analysis showed LTH had an in-frame 3 bp insertion (T<sub>111</sub>C<sub>117</sub>C<sub>121</sub>), which created two amino acid alterations (His105Ser, Arg106Pro) and one residue (Ala) insertion at the C-terminus (Figure 2b).

**The role of ramA, soxS, acrAB or marRAB in MDR and fluoroquinolone resistance phenotypes of Salmonella Typhimurium**

As *ramA* and *soxS* were highly up-regulated in fluoroquinolone-resistant LT2 and LTH, their role in drug resistance in *Salmonella* was determined by comparing antimicrobial susceptibility of *Salmonella Typhimurium* strains with or without deletion of these genes (Table 1). Inactivation of *ramA* in LTL displayed 16- to 32-fold increased susceptibility to nalidixic acid, ciprofloxacin and other fluoroquinolones, and 8- to 16-fold to tetracycline and chloramphenicol. Unlike the dominant effect of *ramA* inactivation in LTL, inactivation of *soxRS* in LTH exhibited only 2- to 8-fold increased susceptibility to nalidixic acid, ciprofloxacin and several other fluoroquinolones, and the resistance levels to tetracycline and chloramphenicol remained the same. Consistent with changes of antimicrobial phenotypes after gene inactivation, the overexpression of *acrAB* and *acrEF* was reduced to baseline level in LTL ΔramA and LTH ΔsoxRS mutants, respectively (Figure 1a). Furthermore, inactivation of *acrAB* in LTL and LTH resulted in increased susceptibilities to all antimicrobials tested, whereas inactivation of *mar* regulon (*marRAB*) in these strains did not result in detectable changes in the antimicrobial susceptibility profiles (Table 1).
RamA and SoxS on expression of AcrAB and AcrEF in S. Typhimurium

(a) 

\[
\begin{array}{c|c}
\text{ramA} & \\
\hline
\text{LTL} & 1 \\
\text{LTL} & 61 \\
\text{LTL} & 121 \\
\text{LTL} & 172 \\
\text{LTL} & 241 \\
\text{LTL} & 232 \\
\text{LTL} & 301 \\
\text{LTL} & 361 \\
\text{LTL} & 421 \\
\text{LTL} & 412 \\
\text{LTL} & 481 \\
\text{LTL} & 472 \\
\text{LTL} & 541 \\
\hline
\text{LTH} & 1 \\
\text{LTH} & 61 \\
\text{LTH} & 61 \\
\text{LTH} & 121 \\
\text{LTH} & 121 \\
\end{array}
\]

(b) 

\[
\begin{array}{c|c}
\text{soxS} & \\
\hline
\text{LTL} & 1 \\
\text{LTL} & 61 \\
\text{LTH} & 1 \\
\text{LTH} & 61 \\
\hline
\text{soxR} & \\
\hline
\text{LTL} & 1 \\
\text{LTL} & 61 \\
\text{LTL} & 61 \\
\text{LTL} & 121 \\
\hline
\end{array}
\]

Figure 2. DNA and amino acid sequence alignments of ramA and soxSR. (a) Salmonella Typhimurium LTL ramA with the consensus. Nine base deletions in the LTL ramA gene are shown in bold. (b) Predicted amino acid sequences of the soxS and soxR genes from Salmonella Typhimurium strain LTH identified in this study aligned with the consensus. Dots indicate residues identical to those in wild-type strain LT2.

There was also an in-frame 12 bp deletion and eight residue alterations in soxR close to the C-terminus. A deletion of thymine (T457) resulted in a +1 frameshift at stop codon-153, which might delay the termination. However, no mutation was found in marRAB, robA or acrR genes in all strains (data not shown).

Antimicrobial resistance conferred by the ramA or soxRS allele

The role of ramA in conferring MDR phenotypes in Salmonella Typhimurium was unclear. The replacement of the wild-type ramA allele in LT2 with ramA* was performed to determine whether it would confer antimicrobial resistance. The resistance profile of LT2 with the ramA* allele to fluoroquinolones and other unrelated antibiotics showed the MICs of all antibiotics tested were significantly greater (Table 1). Resistance to fluoroquinolones increased by 2- to 64-fold. The MIC of ciprofloxacin was 0.25 mg/L, 16-fold greater than that exhibited by the wild-type allele. MICs of tetracycline and chloramphenicol were raised to 16 and 32 mg/L, which were 16- and 4-fold greater, respectively, compared with those of LT2. The results further confirmed the role of ramA in conferring an
M DR phenotype in Salmonella Typhimurium. In addition, when the ramA allele was introduced into LTH, fluoroquinolone resistance levels were also increased compared with LTH with the wild-type allele. For example, MICs of ciprofloxacin and nalidixic acid increased to 128 and 1024 mg/L, respectively (Table 1). Increase in resistance to tetracycline and chloramphenicol was also observed. The MICs of tetracycline and chloramphenicol were 16 and 64 mg/L, increases of 16- and 8-fold, respectively (Table 1).

Expression of constitutive SoxR protein in laboratory strains of E. coli and Salmonella was shown to lead to an increase in resistance to nalidixic acid, chloramphenicol and tetracycline by 2- to 4-fold. However, the introduction of the soxRS allele in the present study resulted in no change in drug susceptibility compared with LT2 (Table 1), suggesting that SoxRS was not the primary cause of the resistance to nalidixic acid and fluoroquinolones in strain LTH.

**Discussion**

Bacterial resistance to fluoroquinolones is usually mediated by mutations in bacterial DNA gyrase (gyrA, gyrB) and topoisomerase IV (parC, parE) genes, as well as by active efflux. Plasmid-mediated resistance to quinolones in Gram-negative bacteria has also been described. In Salmonella, the most common residues in GyrA, known to confer quinolone resistance, are associated with mutations at serine-83 and aspartate-87, either alone or together. Serine-80 or glycine-78 substitution in ParC has been found in ciprofloxacin-resistant clinical and laboratory-induced strains. In the present study, fluoroquinolone-resistant LTL (MIC 4 mg/L ciprofloxacin) and LTH (MIC 64 mg/L ciprofloxacin) were independently derived from Salmonella Typhimurium LT2 induced with ciprofloxacin. In addition to the decreased susceptibility to nine fluoroquinolones, LTL also displayed resistance to chloramphenicol (MIC 64 mg/L) and tetracycline (MIC 16 mg/L). The findings indicated that, although they had the same ancestor, LTL and LTH use different mechanisms against antimicrobials. The topoisomerase mutations alone were not able to explain the fluoroquinolone resistance observed in LTL and LTH. In LTL, the MDR phenotype was lost after inactivation of the acrAB locus even with the presence of the point mutation in gyrA. In LTH, however, the MICs of most fluoroquinolones were still above the breakpoints, although the inactivation of acrAB increased the susceptibility to all antibiotics tested. These data suggest that increasing expression of efflux pumps together with a single gyrA mutation confers fluoroquinolone resistance in laboratory-induced Salmonella strains and that high-level fluoroquinolone resistance requires additional mutations in topoisomerases, whereas the overexpression of efflux pumps becomes less important. Previous studies showed that there was no direct correlation between the level of acrB mRNA and MICs of ciprofloxacin. Possible explanations for the non-correlative relationship include: (i) the presence of post-transcriptional regulation of acrB; and (ii) efflux pumps are only one of several contributing factors in mediating fluoroquinolone resistance.

In E. coli, MarA, SoxS and RobA, transcriptional activators from the AraC/XylS family, interact with acrAB, thereby increasing the production of AcrAB and effectively enhancing efflux. Constitutive overexpression of these genes due to mutations in both laboratory and clinical isolates of E. coli has been described previously. These global regulators are also present in Salmonella, essentially identical to those in E. coli. It is thought that efflux and influx in salmonellae might be regulated in a similar way as in E. coli. This hypothesis is supported by studies showing that constitutive overexpression of soxRS can contribute to antimicrobial resistance in a clinical isolate of Salmonella Typhimurium. Our findings demonstrated that constitutive overexpression of soxS due to mutations in both soxR and soxS was involved in the fluoroquinolone resistance in LTH by an marA independent pathway. However, inactivation of the soxRS regulon only slightly reduced the resistance to several fluoroquinolones in LTH; the introduction of the soxRS allele from LTH to the drug-susceptible strain LT2 displayed the same susceptibility profile as wild-type LT2. Furthermore, inactivation of acrAB did not abolish resistance to at least five fluoroquinolone drugs, suggesting that efflux pumps may not be the primary mechanism to cause resistance to nalidixic acid and fluoroquinolones in LTH.

In addition to the MarA/SoxS/Rob family, RamA was identified as a potential alternative global regulator. Overexpression of RamA has been associated with MDR in E. aerogenes, Klebsiella pneumoniae and S. enterica serovar Paratyphi B. In Salmonella Typhimurium, increased expression of efflux pumps, conferring MDR in Salmonella Typhimurium DT104 isolate. Our study independently identified the 9 bp deletion in the promoter region of mutant ramA in fluoroquinolone-resistant Salmonella, which includes the 2 bp deletion of Abouzeed et al., suggesting that it might be the binding site for RamR, a local repressor of RamA. The deletion of the binding site of the repressor leads to activation of ramA in LTL. Niakido et al. showed that indole- and bile-mediated regulation of AcrAB in Salmonella is also RamA-dependent, and that induction of ramA is required for overexpression of acrAB, indicating the important role of RamA in the regulation of efflux pumps in Salmonella Typhimurium. However, van der Straaten et al. reported no effect on MDR phenotype among 15 clinical Salmonella isolates after ramA was disrupted.

In summary, we showed that ramA is an activator of the MDR cascade in Salmonella Typhimurium. Increased expression of efflux pumps, conferring MDR in Salmonella Typhimurium, is regulated by multiple transcriptional activators under different environmental pressures. Future studies on interactions among MarA, SoxS and RamA genetic systems are needed to decipher their individual contributions to MDR.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


