Differential Gene Expression by RamA in Ciprofloxacin-Resistant Salmonella Typhimurium

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Abstract

Overexpression of ramA has been implicated in resistance to multiple drugs in several enterobacterial pathogens. In the present study, Salmonella Typhimurium strain LTL with constitutive expression of ramA was compared to its ramA-deletion mutant by employing both DNA microarrays and phenotype microarrays (PM). The mutant strain with the disruption of ramA showed differential expression of at least 33 genes involved in 11 functional groups. The study confirmed at the transcriptional level that the constitutive expression of ramA was directly associated with increased expression of multidrug efflux pump AcrAB-TolC and decreased expression of porin protein OmpF, thereby conferring multiple drug resistance phenotype. Compared to the parent strain constitutively expressing ramA, the ramA mutant had increased susceptibility to over 70 antimicrobials and toxic compounds. The PM analysis also uncovered that the ramA mutant was better in utilization of 10 carbon sources and 5 phosphorus sources. This study suggested that the constitutive expression of ramA locus regulate not only multidrug efflux pump and accessory genes but also genes involved in carbon metabolic pathways.

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Introduction

Active efflux by AcrAB-TolC plays an important role in conferring multidrug resistance (MDR) in salmonellae [1]. The AcrAB-TolC efflux system also contributes to Salmonella pathogenesis [2]. Mutants lacking acrA, acrB, or tolC were attenuated via differential expression of major operons and proteins involved in pathogenesis globally [3].

To date, our knowledge on the regulation of expression of AcrAB has mostly come from work carried out in Escherichia coli [4,5,6]. In addition to marA and soxS [7], ramA, a species-restricted and encoding a 113-amino-acid regulatory protein [8], has been implicated in MDR in Salmonella and other bacteria [7,9,10,11,12]. Increased ramA expression has been noted among clinical isolates of Klebsiella pneumoniae [13,14] and S. Typhimurium [15]. The ramA locus, when cloned in E. coli, elicited resistance to multiple antibiotics, decreased expression of the OmpF porin, and increased expression of the efflux AcrAB [16]. Ricci et al. showed that RamA was required to select MDR mutants after exposure to substrates of the AcrAB-TolC pump [17]. However, van der Straaten et al. reported that inactivation of ramA in wild-type S. Typhimurium failed to confer increased susceptibilities to antibiotics [11]. Recently, it has been shown that mutations in ramR, a tetR-like repressor of ramA [18,19] and mutations in ramA promoter region [18,20] resulted in overexpression of ramA, thereby conferring MDR phenotype through induction of acrAB and tolC. Additionally, van der Straaten et al. [21] showed that soxRS was not the only regulon involved in protective response of Salmonella to macrophage-derived oxidative stress and that ramA played an important role in resistance to superoxide and nitrogen intermediates produced by phagocytes as well.

Barbosa and Levy [22] reported differential expression of over 60 chromosomal genes in the MarA regulon of E. coli. The mar and soxRS systems exert overlapping effects on the regulation of efflux pumps and porin syntheses in E. coli [23]. While in S. Typhimurium, Nikaido et al. demonstrated that induction of acrAB by indole was regulated by ramA, independent of marA, soxS, or rob [24]. Furthermore, Bailey et al. [25] recently showed that following disruption of ramR, or artificial overexpression of ramA in S. Typhimurium, global changes in expression of genes involved in MDR efflux, virulence, and amino acid biosynthesis were observed [26]. However, the relationships between ramA and marA, and ramA and soxRS remain unclear in Salmonella.

The goal of this study was to examine both global genotypic and phenotypic changes associated with inactivation of ramA. Data interrogation of DNA microarray and Biolog Phenotype Micro-Array after growth of S. Typhimurium LT2 and a derivative lacking ramA revealed that constitutive expression of ramA affected expression of genes not only involved in multidrug resistance, but genes associated with carbon and phosphorus metabolism.
Materials and Methods

Bacterial strains

S. Typhimurium strain LT2 was whole-genome sequenced and kindly given by The Institute for Genomic Research (now The J. Craig Venter Institute, JCVI). LT2T (ciprofloxacin MIC: 4 μg/ml) with a single point mutation in GyrA was derived from S. Typhimurium LT2 by in vitro-selection using ciprofloxacin [20]. Strain LT2T represented the control, expressing RamA constitutively. Experimental strain LT2ramA::aph was constructed using λ red site-specific recombination, as previously described [27], in which the ramA gene was replaced with an aph cassette expressing kanamycin resistance. Replacement of target gene, ramA, was verified by PCR using the k1 and k2 primers and primers flanking the deleted regions [20].

RNA isolation and mRNA purification

Overnight bacterial cultures were diluted 1:100 in fresh Difco Mueller-Hinton broth (Becton Dickinson and Co., Sparks, MD) and grown to mid-logarithmic phase (A600 = 0.4–0.45) at 37°C. Cells were harvested using an RNAProtect reagent (Qiagen, Valencia, CA) and processed for microarray real-time PCR analyses. Total RNA was isolated using TRIZOL (Invitrogen, Carlsbad, CA) and cleaned using the RNeasy mid-kit (Qiagen) according to the manufacturers’ instructions. RNA preparations were treated with RNase-free DNase (Qiagen) on columns to remove genomic DNA (gDNA) contamination. Additional PCR reaction was conducted to confirm the loss of gDNA. RNA samples were quantified using an ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and further inspected by gel electrophoresis. For microarray study, ribosomal RNA (rRNA) was extracted from total RNA to increase the sensitivity of the DNA microarray using the MicroExpress bacterial mRNA purification kit (Ambion, Austin, TX). The quality and quantity of the mRNA samples were assessed using gel electrophoresis.

cDNA synthesis, dye labeling and array hybridization

Conversion of mRNA to cDNA and cDNA labeling was performed according to a publicly available microbial microarray protocol [http://pigrn.jcvi.org/index.php/microarray/protocols.html]. Briefly, 200 ng of purified mRNA was converted into cDNA in a total volume of 30.1 μl using SuperScriptTM III first-strand synthesis system (Invitrogen), random hexamer primers, and dNTP/aa-UTP labeling mix (Sigma, St. Louis, MO). Amplified aminoallyl-labelled cDNA was then coupled to dye Cy3 or Cy5. Only cDNA samples with >800 pmol of dye incorporation, and <20 in number of nucleotides/dye incorporation ratio (pmol cDNA/pmol Cy dye) was used in hybridization. The Cy3/Cy5 probe mixture was then dried in a speed vac and resuspended in a hybridization buffer. S. Typhimurium/Typhi slides [28] (v.5.0) were obtained from the Pathogen Functional Genomics Resource Center (PFGRC) at the J. Craig Venter Institute. Each array slide consists of 5462 70-mer oligonucleotide spots (approximately 1,200 phenotypes [35]). All materials, media, and reagents for the PM system were purchased from Biolog. PM experiments were conducted using conditions recommended by the manufacturer [36]. The PM plates were incubated at 37°C in an Omnilog incubator and readings were recorded every 15 min for 48 h. Bacterial respiration was assessed within each well by monitoring color formation resulting from reduction of the tetrazolium violet (dye A), and color intensity was expressed in arbitrary units (AU). Kinetic data were analyzed with OmniLog-PM software from Biolog (OL_PM_Par1.10.02, Dec. 08, 2005).
Based upon work by Zhou et al. [36] and Bailey et al. [25], substrates showing ≥1.5-fold (≥15000 area under curve, arbitrary units) difference between LTL and ramA mutant were considered as a different phenotype. The tests were repeated once to confirm phenotypes detected with the metabolic arrays (PM1 to PM8).

Bioscreen C (Growth Curves USA, NJ) was performed using chemically defined M9 minimal medium to determine utilization of selected carbon and phosphorus sources. To confirm the mutant phenotypes detected on the inhibitor sensitivity arrays (PM9 to PM20), 1.33-fold serial dilutions of selected chemicals were used in 96-well microplates. Agar dilution was also used to test susceptibility to several antimicrobial compounds on MH agar [37].

**Results**

**Effects of ramA deletion on the transcriptome**

The microarray data revealed that approximately 0.7% of the genes in the S. Typhimurium LTL genome displayed ≥2-fold differential expression with a P value ≤0.01 when ramA was inactivated by inserting kanamycin resistance gene, aph (Table 1). Notably, the number of genes with decreased expression (n = 21) were more than that of genes with increased expression (n = 12) by ramA knockout.

Genes affected by the ramA inactivation were dispersed throughout the genome (Table 1). The differentially expressed genes with known COG function were categorized into 11 functional groups, mainly including inorganic ion, coenzyme, ...

| Locus tag in S. Typhimurium | Annotation | Adjusted P-value | Gene name | Fold change |
|-----------------------------|------------|------------------|-----------|-------------|
| STM0581                     | putative regulatory protein | 3.09E-16 | ramA | -4.8 |
| STM3179*                    | NADPH specific quinone oxidoreductase (drug modulator) | 4.25E-16 | mdaB | -3.2 |
| STM0874*                    | oxygen-insensitive NADPH nitroreductase | 1.24E-18 | mdaA/insA | -3.2 |
| STM0476*                    | acidine efflux pump | 8.42E-14 | acrA | -3.2 |
| STM0475*                    | RND family, acidine efflux pump | 1.98E-15 | acrB | -3.1 |
| STM3276*                    | putative alkanal monoxygenase | 1.54E-12 | yhbW | -3.0 |
| STM0873                     | putative inner membrane protein | 2.95E-13 | ybjC | -2.9 |
| STM3186*                    | outer membrane channel specific tolerance to colicin | 7.89E-15 | tolC | -2.8 |
| STM3313                     | putative ABC superfamily (atp_bind) transport protein | 1.58E-15 | ybf | -2.5 |
| STM0156                     | putative outer membrane protein | 5.03E-13 | -2.5 |
| STM3312                     | putative ABC superfamily (membr) transport protein | 2.21E-14 | ybe | -2.5 |
| STM0509                     | putative outer membrane protein (porin) | 1.64E-15 | -2.4 |
| STM3180                     | putative cytoplasmic protein | 7.83E-13 | ygiN | -2.3 |
| STM0215*                    | methionine aminopeptidase | 1.15E-13 | map | -2.3 |
| STM0780                     | putative outer membrane or exported | 3.61E-13 | -2.2 |
| STM0492                     | putative CPA2 family transport protein | 3.17E-13 | ybaL | -2.2 |
| STM3311                     | putative ABC superfamily (bind_prot) transport protein | 2.16E-15 | yrbD | -2.1 |
| STM1004                     | nicotinate phosphoribosyltransferase | 4.43E-11 | pncB | -2.1 |
| STM1651*                    | putative pyruvate-flavodoxin oxidoreductase | 5.61E-13 | niJ | -2.0 |
| STM2203*                    | endonuclease IV | 2.11E-14 | nfo | -2.0 |
| STM0875                     | ribosomal protein S6 modification protein | 1.15E-13 | rimK | -2.0 |
| STM4231*                    | phage lambda receptor protein maltose high-affinity receptor | 5.98E-07 | lamB | 2.0 |
| STM4330                     | chaperone Hsp60 with peptide-dependent ATPase activity | 1.54E-08 | groEL | 2.0 |
| STM4329                     | chaperone Hsp10, affects cell division | 2.76E-09 | groES | 2.1 |
| STM0164                     | putative transcriptional regulator (LysR family) | 7.34E-11 | 2.1 |
| STM0162                     | putative inner membrane protein | 1.57E-09 | 2.2 |
| STM2665                     | ribosome associated factor | 9.95E-06 | yflA | 2.3 |
| STM0974                     | putative FNT family, formate transporter | 8.61E-08 | locA | 2.4 |
| STM0543                     | major type 1 subunit fimbrin (pilin) | 7.97E-15 | fimA | 2.4 |
| STM2261                     | ferredoxin-type protein: electron transfer | 1.26E-06 | napF | 2.5 |
| STM0163                     | 4-hydroxythreonine-4-phosphate dehydrogenase | 1.40E-10 | pdxA | 2.5 |
| STM0999*                    | outer membrane protein F precursor | 1.19E-14 | ompF | 3.2 |
| STM2646                     | putative formate acetyltransferase | 8.51E-07 | yflD | 3.6 |

*genes co-regulated by marA and soxS in E. coli.

genes also regulated by soxS in S. enterica serotype Typhimurium.

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and carbohydrate transport and metabolism, energy production and conversion, cell wall/membrane biogenesis, multifunctional, secondary metabolites biosynthesis, transport and catabolism.

Real-time quantitative RT-PCR was used to corroborate selected values from microarrays. The correlation in the expression ratios of 22 genes between the microarray and real-time PCR was measured using Student’s $t$-test. The results showed that there was a high degree of concordance ($r = 0.883$) between data from the two methodologies ($c = 0.87$, $P < 0.05$) (Fig. 1).

Changes in the transcript abundance of genes regulated among MarA, SoxS, and RamA

marA, soxS, and ramA are transcriptional activators from the family of AraC/XylS. There are over 60 known genes under marA regulation and 15 under soxS regulation in E. coli [22,38,39]. Many oxidative stress genes responding to SoxS are also reactive to MarA in E. coli. Our data revealed that at least 10 genes with diverse physiological functions regulated directly or indirectly by both the MarA and SoxS regulators in E. coli [22,40,41] were also affected by RamA in S. Typhimurium (Table 1). In particular, the transcription level of MDR related genes acrA, acrB [42], and tolC [42,43] decreased; while ompF, encoding an outer membrane porin protein, was activated in the ramA mutant. Real-time PCR showed 2.3±0.1-fold down-regulation of micF transcription in the ramA mutant, which confirmed the role of small RNA encoding gene micF in the regulation of OmpF reported in E. coli [22,44,45]. No significant change was observed in the expression of acrR, encoding a local represor of AcrAB [46]. Furthermore, the transcription level of nifJ, a putative oxidoreductase, regulated by SoxS only in S. Typhimurium [47], decreased by 2-fold in the ramA:aph mutant (Table 1). While the abundance of genes that encode proteins important to stress response (e.g., groEL, groES) (Table 1) and are known to be induced by the presence of SylA in S. Typhimurium [48], were significantly higher in the ramA:aph mutant than in the parent strain with over-expression of ramA. And anaerobic metabolism related gene napF (Table 1) was also positively affected by the ramA knockout, which is consistent with the effect of acrA knockout described previously [3].

![Figure 1. Comparison in expression ratios of 22 randomly selected genes between microarray and real time-PCR. The array fold-changes (in dark grey box) were based on averages from six biological replications. The fold differences ($\Delta C_T$) in expression levels of the genes tested using real time-PCR (in light grey box) were calculated from triplicate reactions against $C_T$ value of housekeeping gene (rrsG) of S. Typhimurium. acrA, tolC and ompF are genes showing significant difference ($\geq$2-fold with $P \leq 0.01$) in microarray data. doi:10.1371/journal.pone.0022161.g001](image)

Table 2. Changes in carbon, and phosphate source utilization greater than or equal to 1.5-fold due to ramA inactivation comparing S. Typhimurium LTLramA:aph to its parental strain S. Typhimurium LTL.

| Mode of action | Compound | Difference in fold (A.U.)* |
|----------------|----------|----------------------------|
| C-source       | N-Acetyl-Neuraminic Acid  | 1.8 (18447) |
|                | D-Fructose                  | 2.0 (19846) |
|                | L-Fucose                    | 2.4 (23929) |
|                | D-Galactose                 | 2.5 (24681) |
|                | x-D-Glucose                 | 2.1 (21423) |
|                | N-Acetyl-D-Glucosamine      | 2.0 (20490) |
|                | D-Mannose                   | 1.8 (17994) |
|                | D-Mannitol                  | 1.7 (16768) |
|                | L-Rhamnose                  | 1.8 (18377) |
|                | Fumaric Acid                | 1.9 (19730) |
| P-source       | Adenosine 3'-Monophosphate  | 3.1 (30703) |
|                | Adenosine 2',3'-Cyclic Monophosphate | 3.2 (32083) |
|                | Guanosine 2',3'-Cyclic Monophosphate | 2.4 (24537) |
|                | Thymidine 3'-Monophosphate  | 2.8 (27588) |
|                | Uridine 3'-Monophosphate    | 2.0 (19659) |
|                | Uridine 5'-Monophosphate    | 1.5 (15399) |

*fold equals to arbitrary unit (A.U.)/10,000. doi:10.1371/journal.pone.0022161.t002
Phenotypic characterization of ramA mutant

Of the 1,200 phenotypes screened, 114 phenotypes showed ≥1.5-fold difference in tetrazolium dye reduction in LTLramA::aph compared to parental strain LTL. Resistance to aminoglycoside antibiotics in the ramA mutant was expected due to the presence of the aph cassette used to inactivate ramA. Among 98 metabolite analogs and antibiotics to which the ramA mutant showed increased sensitivity/susceptibility, with 78 were antimicrobials and toxic compounds (Table S2). The PM assay of the ramA mutant verified susceptibility to compounds in the classes of β-lactam, organic solvent, phenicol, and tetracycline as previously reported [20,25]. Increased susceptibility to biocides was also

Figure 2. Growth kinetics of S. Typhimurium LTL and its ramA mutant grown in M9 minimal medium containing 1×M9 salts, 2 mM MgSO₄, and 0.1 mM CaCl₂ with 0.2% N-Acetyl-D-Glucosamine, 0.2% D-galactose, and Adenosine-2′,3′-cyclic monophosphate, as carbon source or phosphate source, respectively. Growth curves were performed with a Bioscreen C Microbiology Reader from Labsystems. Cell growth was monitored at 37°C with shaking. Absorbance at OD 600 nm (A₆₀₀) was measured and recorded every 20 min for 24 hrs. doi:10.1371/journal.pone.0022161.g002
observed. Furthermore, the strain became hypersensitive to DNA intercalating agents including acriflavine (−3.6-fold), 9-Aminoacridine (−3.9-fold), 2-Phenylphenol (−7.8-fold), and proflavin (−4.0-fold) after ramA disruption. Interestingly, the mutant exhibited better metabolism of 10 carbon and 6 phosphorus sources (Table 2).

Several findings in the PM analysis were subsequently confirmed by additional assays (Fig 2 and Table 3). For example, in growth studies, parental strain LTL was defective (P<0.5) in using 0.2% N-Acetyl-D-glucosamine or 0.2% D-galactose as a carbon source, and 20 mM adenosine 2’,3’ cyclic monophosphate (cAMP) as a phosphorus source (Fig 2). By determining MICs using agar dilution, the ramA mutant showed increased susceptibility to promethazine (160 μg/ml), a cyclic nucleotide phosphodiesterase inhibitor [19], propranolol (240 μg/ml), a non-selective beta-adrenergic blocker, and acriflavine (16 μg/ml), a DNA intercalating agent.

### Discussion

The role of RamA as a transcriptional regulator has been associated with MDR in Salmonella and other Enterobacteriaceae [9,11,25,50]. The present study sought to explore the role of ramA using total genome transcription analysis and phenotypic array analysis as a global regulator. The transcriptomic experiments in this study revealed that in response to the ramA inactivation there were considerable changes in gene expression. These included changes in genes related to MDR, genes co-regulated by other regulators such as marA, and soxS, as well as genes involved in the metabolic pathways. Such changes were also reflected in phenotype microarray analysis. The inactivation of ramA caused changes in the response of Salmonella to at least 100 compounds.

We previously confirmed that both AcrAB-TolC efflux pump activity and the S83F substitution in gyrA contributed to resistance to nalidixic acid and fluoroquinolones in S. Typhimurium strain LTL (unpublished data). The increased expression of acrB was associated with the activation of ramA. In the present study, decreased expression of acrA, acrB, tolC, and micF, and increased expression of ompF were observed in LTLramA::aph. Our data clearly showed that acrAB-tolC, ompF and micF were regulated by ramA at the transcriptional level. No change in the expression of marA, soxS, and rob indicated lack of involvement of these regulators in the development of MDR. It is likely that ramA activates the MDR cascade independently of marA. Consistent with results from a study by Ricci et al [17], our phenotypic microarray data demonstrated that the inactivation of ramA increased Salmonella’s susceptibility to an array of antimicrobials, confirming an important role of ramA in conferring MDR. Among those compounds, many are known or recently identified substrates of AcrAB efflux [18,25].

Bailey et al. [26] recently reported that inactivation of ramA led to altered expression of 223 genes in S. Typhimurium SL1344, including increased expression of 14 SPI-1 genes and decreased expression of 3 SPI-2 genes. Although none of genes involved in pathogenicity was eligible to be listed in Table 1 (ratio less than 2-fold), real-time PCR showed genes including flaA, pgfK, and sipB (Fig 1) had over 1.5-fold enrichment when compared to strain LTL. Moreover, logFC value used to determine genes with significantly altered expression was much higher (at least 2 fold) than B value (log odds value) that was used in the Bailey’s study [26]. If the same criteria were used, the disruption of ramA would result in significantly increased expression of at least 9 SPI-1 genes encoding pgfHJFk and sgABCDF in this study. Similarly, both studies showed the disruption of ramA was associated with changes in expression of genes involved in virulence (such as those in SPI-1). However, the expression of genes responsible for amino acid biosynthetic pathways [26] except his operon was not significantly affected. Nevertheless, these results shed light to the complex of regulation network under ramA.

It is interesting to note that at least 10 of the differentially expressed genes in this study were regulated by MarA and SoxS in E. coli as well [22,38,39] (Table 1). Additionally, this study showed an overlapping regulation between soxS and ramA in Salmonella (Table 1); nifA, encoding oxidoreductase that shuttles electrons from pyruvate to reduce nitrogenase [47]. In line with data from other studies [21], it is likely that ramA also plays a role as a transcriptional regulator of antioxidant defense in Salmonella. Previous studies showed decreased expression of rob in S. Enteritidis was likely due to down-regulation by soxS and marA [51,52]. We also found a putative transcriptional regulator (LysR family) was down-regulated due to the inactivation of ramA. Clearly, there is a cross talk between ramA and other global regulators. One caveat of this transcriptomic study was that the ramA knockout mutant was compared to its parent strain with Gyra S83F mutation rather than to a strain with a clean genetic background. However, based on previous studies [53,54], S83 mutation in Gyra alone has no or least influence on global supercoiling. Our data also showed no statistically significant difference in both doubling time between LT2-wt (31.2±2.3 min, n = 6 generations) and LTL (32.0±3.4 min, n = 6 generations), and biofilm formation between LT2-wt (OD550 = 0.120±0.005) and LTL (OD550 = 0.123±0.008) [55], suggesting little impact on supercoiling by the S83 Gyra mutation. We felt confident that the genetic background of the experimental strains could not have introduced significant confounding factors for data interpretations.

The inactivation of ramA affected the response of Salmonella to many different chemicals. Of these compounds, acriflavine, β-lactams, chloramphenicol, fusidic acid, macrolides, novobiocin, puromycin, sulfonamides, tetracyclines and trimethoprim were known substrates of AcrAB-TolC system [1,56,57,58]. Recently, Zhou et al. [36] and Bailey et al. [25] substantially expanded the range of compounds as substrates of AcrAB-TolC system using phenotype microarray. In this study, PM1, instead of PM11A, through PM20 were used to identify phenotypic differences exhibited by ramA mutant. The disruption of ramA conferred the susceptibility to a wide range of compounds but also led to a better utilization of 10 carbohydrate carbon sources and 6 phosphorus sources. Additionally, the carbon and phosphorus metabolic differences due to the inactivation of ramA occurred only after 24-h growth in these arrays. We sought to resolve the altered phenotypes by comparing to the

| Table 3. Antimicrobial activities of acriflavin, promethazine, and propranolol against S. Typhimurium strain LTL and its ramA mutant. |
|---------------------------------|-----------------|-----------------|-----------------|
| Antimicrobial Agent             | MIC (μg/ml)     | LTLramA::aph    |                 |
| Acriflavin                      | >64             | 16              |                 |
| Promethazine                    | 640             | 160             |                 |
| Propranolol                     | >480            | 240             |                 |

| doi:10.1371/journal.pone.0022161.t003 |
microarray data. It is worthy to note that the transcription level of *marP*, which is in MFS family and responsible for galactose transportation had increased by 0.6-fold (B value: 0.5–0.7) and that the transcription level of *nasE*, which partly comprises the PTS system N-acetyl glucosamine specific transporter subunit II and is responsible for N-acetyl glucosamine transportation, had increased by 0.9-fold (B value: 0.3–0.9) in the *ramA* mutant. Both array findings may provide possible genetic basis for the altered phenotypes.

In the summary, data generated by the transcripomic and phenotypic arrays suggested that *ramA* be a global transcriptional regulator that controls a set of genes with diverse physiological functions. It is possible that RamA plays a role in virulence regulation as well. Further studies on cross talk between global regulators including *marA*, *soxRS* and *ramA*, and the genes under their control in *Salmonella* may provide important details related to the mechanisms that govern how *Salmonella* enhance their fitness in new and challenging environmental landscapes.

**References**

1. Baucherouen S, Tyler S, Boyd D, Mulvey M, Chaslus-Dancla E, et al. (2004) AcrAB-ToLC directs efflux-mediated multidrug resistance in *Salmonella enterica* serovar typhimurium DT104. Antimicrob Agents Chemother 48: 3729–3735.
2. Buckley A, Webber M, Coosles S, Randall L, La Ragione R, et al. (2006) The AcrAB-ToLC efflux system of *Salmonella enterica* serovar Typhimurium plays a role in pathogenesis. Cell Microbiol 8: 847–456.
3. Farrow D, Baucheron S, Tyler S, Boyd D, Mulvey M, Chaslus-Dancla E, et al. (2004) AcrAB-ToLC induces resistance to organic solvent solutes in *Salmonella enterica* serovar Typhimurium. J Bacteriol 186: 5332–5338.
4. Piddock L, Boyd D, Mulvey M, Chaslus-Dancla E, et al. (2006) Multidrug resistance in *Salmonella enterica* serovar Typhimurium may be a global transcriptional program. Antimicrob Agents Chemother 50: 3080–3087.
5. White D, Goldman J, Demple B, Levy S (1997) Role of the *marP* regulatory pathway associated with high-level ciprofloxacin and multidrug resistance in *Salmonella enterica* serovar enteritidis: involvement of RamA and other global regulators. Antimicrob Agents Chemother 41: 2158–2163.
6. Piddock L (2006) Clinically relevant chromosomally encoded multidrug resistance in *Salmonella enterica* serovar Typhimurium. Microbiology 152: 1269–1278.
7. van der Straaten T, Zulianello L, van Diepen A, Granger D, Janssen R, et al. (2006) RamA, a novel gene, confers a multidrug resistance phenotype in *Escherichia coli*. J Bacteriol 188: 2726–2733.
8. Ricci V, Piddock L (2009) Ciprofloxacin selects for multidrug resistance in *Salmonella enterica* serovar Typhimurium mediated by at least two different pathways. J Antimicrob Chemother 63: 909–916.
9. Zhou L, Lei X, Bochner B, Wanner B (2003) Phenotype microarray analysis of *Salmonella enterica* serovar Typhimurium LTLramA::aph to its parental strain *Salmonella enterica* serovar Typhimurium LTL. PLoS ONE 8: e22161.

**Supporting Information**

**Table S1** Real-Time-PCR Primers used in validation of microarray data. (DOC)

**Table S2** Compounds with increased susceptibilities greater than or equal to 3-fold due to *ramA* inactivation in comparing *S. Typhimurium* LTLramA::aph to its parental strain LTL. (DOC)

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**Author Contributions**

Conceived and designed the experiments: JZ. Performed the experiments: JZ. Analyzed the data: FT. Contributed reagents/materials/analysis tools: SHC JS SHZ EWB JHM. Wrote the paper: JZ JHM.

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38. Hidalgo E, Ding H, Demple B (1997) Redox signal transduction: mutations shifting [2Fe-2S] centers of the SoxR sensor-regulator to the oxidized form. Cell Biol 11: 121–129.

39. Liochev SI, Hausladen A, Fridovich I (1999) Nitroreductase A is regulated as a member of the soxRS regulon of Escherichia coli. Proc Natl Acad Sci U S A 96: 3557–3559.

40. Ariza R, Cohen S, Bachhawat N, Levy S, Demple B (1994) Repressor mutations in the marRAB operon that activate oxidative stress genes and multiple antibiotic resistance in Escherichia coli. J Bacteriol 176: 143–148.

41. Greenberg J, Chou J, Monach P, Demple B (1991) Activation of oxidative stress genes by mutations at the soxQ/cfxB/marA locus of Escherichia coli. J Bacteriol 173: 4433–4439.

42. Nishino K, Latifi T, Groisman EA (2006) Virulence and drug resistance roles of multidrug efflux systems of Salmonella enterica serovar Typhimurium. Molecular Microbiology 59: 126–141.

43. Nishino K, Yamada J, Hirakawa H, Hirata T, Yamaguchi A (2003) Roles of TolC-dependent multidrug transporters of Escherichia coli in resistance to beta-lactams. Antimicrob Agents Chemother 47: 3030–3033.

44. Cohen S, McMurry L, Levy S (1988) marA locus causes decreased expression of OmpF porin in multiple-antibiotic-resistant (Mar) mutants of Escherichia coli. J Bacteriol 170: 5416–5422.

45. Devlin N (1997) Antisense marF RNA and 5’-UTR of the target ompF RNA: phylogenetic conservation of primary and secondary structures. Nucleic Acids Symp Ser. pp 33–35.

46. Olliver A, Valle M, Chaslas-Dancla E, Cloeckaert A (2002) Role of an marR mutation in multidrug resistance of in vitro-selected fluoroquinolone-resistant mutants of Salmonella enterica serovar Typhimurium. FEMS Microbiol Lett 238: 267–272.

47. Pomposiello PJ, Demple B (2000) Identification of SoxS-regulated genes in Salmonella enterica serovar typhimurium. J Bacteriol 182: 23–29.

48. Spory A, Bosserhoff A, von Rhein C, Goebel W, Ludwig A (2002) Differential regulation of multiple proteins of Escherichia coli and Salmonella enterica serovar Typhimurium by the transcriptional regulator ShlA. J Bacteriol 184: 3549–3559.

49. Levin RM, Weiss B (1976) Mechanism by which psychotropic drugs inhibit adenosine cyclic 3’,5’-monophosphate phosphodiesterase of brain. Mol Pharmacol 12: 581–589.

50. Komatsu T, Ohta M, Kido N, Arakawa Y, Ito H, et al. (1990) Molecular characterization of an Enterobacter cloacae gene (romA) which pleiotropically inhibits the expression of Escherichia coli outer membrane proteins. J Bacteriol 172: 4062–4069.

51. Schneider T, Levy SB (2006) MarA-mediated transcriptional repression of therob promoter. J Biol Chem 281: 10049–10055.

52. Mehan C, Manchado M, Puero C (2002) SoxRS down-regulation ofrob transcription. J Bacteriol 184: 4733–4738.

53. Bajer S, Hullen V, Wiedermann B, Heinz P (1999) Impact of gacA and parC mutations on quinolone resistance, doubling time, and supercoiling degree of Escherichia coli. Antimicrob Agents Chemother 43: 808–873.

54. Alexandre V, Urios A, Herrera G, Blanco M (2009) New Escherichia coli gacA and gacB mutations which have a graded effect on DNA supercoiling. Mol Genet Genomics 219: 306–312.

55. O’Toole GA, Kolter R (1998) Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Mol Microbiol 30: 295–304.

56. Ma D, Alberti M, Lynch C, Nakaio H, Hearst JE (1996) The local repressor AcrR plays a modulating role in the regulation ofacrAB genes of Escherichia coli by global stress signals. Mol Microbiol 19: 101–112.

57. Ma D, Cook DN, Alberti M, Poff NG, Nakaio H, et al. (1993) Molecular cloning and characterization of acrA and acrE genes of Escherichia coli. J Bacteriol 175: 6299–6313.

58. Sulavik MC, Houseward C, Kramer C, Jiwani N, Murgolo N, et al. (2001) Antibiotic susceptibility profiles ofEscherichia coli strains lacking multidrug efflux pump genes. Antimicrob Agents Chemother 45: 1126–1136.