

# Overexpression of RamA, which regulates production of the multidrug resistance efflux pump AcrAB-TolC, increases mutation rate and influences drug resistance phenotype

Grimsey, Elizabeth M; Weston, Natasha; Ricci, Vito; Stone, Jack W; Piddock, Laura J V

DOI:  
[10.1128/AAC.02460-19](https://doi.org/10.1128/AAC.02460-19)

License:  
None: All rights reserved

Document Version  
Peer reviewed version

*Citation for published version (Harvard):*  
Grimsey, EM, Weston, N, Ricci, V, Stone, JW & Piddock, LJV 2020, 'Overexpression of RamA, which regulates production of the multidrug resistance efflux pump AcrAB-TolC, increases mutation rate and influences drug resistance phenotype', *Antimicrobial Agents and Chemotherapy*, vol. 64, no. 4, e02460.  
<https://doi.org/10.1128/AAC.02460-19>

[Link to publication on Research at Birmingham portal](#)

## General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

## Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact [UBIRA@lists.bham.ac.uk](mailto:UBIRA@lists.bham.ac.uk) providing details and we will remove access to the work immediately and investigate.

**Over-expression of RamA, which regulates production of the MDR efflux pump AcrAB-TolC, increases mutation rate and influences drug-resistance phenotype.**

Elizabeth M. Grimsey<sup>†</sup>, Natasha Weston<sup>†</sup>, Vito Ricci, Jack W. Stone and Laura J.V. Piddock<sup>\*</sup>.

Antimicrobials Research Group, School of Immunity and Infection and Institute of Microbiology and Infection, University of Birmingham, Birmingham B15 2TT, UK

<sup>†</sup>Elizabeth Grimsey and Natasha Weston contributed equally to this work. Author order was determined alphabetically.

<sup>\*</sup>Corresponding author. Antimicrobials Research Group, School of Immunity and Infection and Institute of Microbiology and Infection, University of Birmingham, Birmingham B15 2TT, UK. Telephone: +44 (0)121 414 6966. Fax: +44 (0)121 414 6819. Email: l.j.v.piddock@bham.ac.uk

## Abstract

In Enterobacteriales, the AcrAB-TolC efflux pump exports substrates including antimicrobials from the cell. Over-expression of AcrAB-TolC can occur after exposure to fluoroquinolones leading to multidrug-resistance. Expression of AcrAB-TolC in *Salmonella* is primarily regulated by the transcriptional activator RamA. However, other transcriptional activators such as MarA, SoxRS and Rob can influence AcrAB-TolC expression. This study determined whether over-production or absence of RamA influences mutation rate or the phenotype of mutants selected in *Salmonella* Typhimurium SL1344 after ciprofloxacin exposure. Absence of RamA (SL1344 *ramA::aph*) resulted in mutation frequencies/rates similar to those in wild-type *Salmonella* Typhimurium SL1344. However, over-production of RamA (SL1344 *ramR::aph*), and consequently AcrB, resulted in a significantly higher mutation frequency and rate relative to wild-type *Salmonella* Typhimurium SL1344. Whole genome sequencing revealed that in addition to selecting *gyrA* mutants resistant to quinolones, SL1344 and SL1344 *ramA::aph* also produced MDR mutants, associated with mutations in *soxR*. Conversely, mutations in SL1344 *ramR::aph* occurred in *gyrA* only. Although transcriptional regulators such as SoxRS are believed to play a minor role in AcrAB-TolC regulation when under antibiotic selective pressure, we show that *soxR* mutants can be selected post-exposure to ciprofloxacin, including when RamA is absent. This demonstrates that under selective pressure *Salmonella* can respond to increased efflux pump expression by mutating other AcrAB-TolC regulatory genes allowing for the evolution of MDR. Understanding how *Salmonella* respond to antibiotic pressure in the absence/over-production of RamA is important if targeting transcriptional regulators to alter efflux is to be considered an avenue for future drug discovery.

## Introduction

Antimicrobial resistance is one of the great global challenges facing modern medicine (1). Bacteria can be intrinsically resistant to certain antibiotics but can evolve via chromosomal mutation and can also acquire resistance by horizontal transfer of resistance genes. Mutations that result in antimicrobial resistance typically alter antibiotic activity by one of the following mechanisms: modification of the drug target, reduced membrane permeability and increased efflux. Those mutations that reduce the intracellular accumulation of antibiotics by increasing efflux confer reduced susceptibility to a range of different antimicrobial classes and can cause multidrug resistance (MDR). Therefore, extensive research surrounding the development of compounds capable of neutralising this resistance mechanism has been undertaken.

Listed by the World Health Organisation (WHO) as a high priority pathogen for which new treatments are urgently needed, fluoroquinolone-resistant *Salmonella enterica* cause a significant health burden worldwide (2). Resistance upon exposure to the fluoroquinolone ciprofloxacin frequently results from mutations in the topoisomerase encoding genes *gyrA*, *gyrB*, *parC* and *parE*. However, MDR resulting from ciprofloxacin exposure can occur in Gram-negative bacteria as a result of over-production of efflux pumps (3, 4). In *Salmonella enterica*, increased active efflux is mainly attributed to overexpression of the AcrAB-TolC efflux pump (5). Although subject to multiple levels of regulation, in *Salmonella*, AcrAB-TolC is primarily regulated by RamA, an AraC/XylS transcriptional activator (6). When *ramA* is highly expressed there is a concomitant over-expression of *acrAB* and *tolC* which results in increased translation of the AcrAB-TolC pump proteins, leading to MDR (Figure 1) (7). In the absence of RamA it is difficult to select MDR mutants (8).

In addition to RamA, in Enterobacteriales, the transcriptional activators MarA, SoxRS and Rob are also capable of regulating expression of AcrAB-TolC (Figure 1) (5). Although mutations increasing *ramA* expression are often reported in clinical and veterinary isolates of *Salmonella* and *E. coli*, MDR due to mutations within transcriptional regulators such as *soxR* have been observed (9-12). The *soxRS* regulatory locus is responsible for the response of Enterobacteriales to oxidative stress. In the

77 absence of stressor the [2-Fe-2S] iron clusters of SoxR are reduced and the protein is inactive. Upon  
78 oxidative stress, the iron clusters are oxidised and SoxR is able to stimulate transcription of *soxS* (13).  
79 SoxS, like RamA, is part of the AraC family of transcriptional activators (5, 14, 15). When activated  
80 SoxS is able to cause an increase in transcription all of the genes within its regulon, this includes  
81 *acrAB-TolC* (13). In the absence of AcrB, *soxS* expression increases, probably as a response to  
82 increased oxidative stress caused by the lack of this major efflux pump (14). This suggests that there  
83 are feedback mechanisms by which Enterobacteriales use different transcriptional regulators to  
84 maintain efflux.

85 Capable of increasing bacterial susceptibility to currently available antimicrobials, inhibition of efflux  
86 pumps is an important potential avenue to tackle MDR (16). Targeting transcriptional regulators,  
87 such as RamA in *Salmonella*, may reduce the ability of the organism to develop MDR via over-  
88 expression of AcrAB-TolC. Understanding how *Salmonella* respond to selective pressure in the  
89 absence or over-production of RamA. Furthermore, knowing if in the presence of an AcrAB-TolC  
90 substrate, the bacterium is capable of acquiring mutations allowing it to circumnavigate inhibition  
91 via the RamA-regulated pathways, is important when considering the use of transcriptional  
92 regulators as drug targets and to improve our understanding of the regulation of multidrug efflux.  
93 Antibiotic selective pressure can trigger a plethora of cellular events which can determine the  
94 phenotype of any resultant mutant that evolve during drug exposure; whether this occurs early or  
95 late within the growth of a population may affect mutation rate. Given that bacteria with higher  
96 *acrAB* expression have lower expression of the DNA mismatch repair gene *mutS*, lower growth rates  
97 and higher mutation frequencies, selective pressure that leads to increased expression of the AcrAB-  
98 TolC system may contribute to increased mutation rates (17).

99 In this study, we set out to determine whether different levels of *ramA* expression results in  
100 differences in mutation rate and the mechanism by which resistance to the fluoroquinolone  
101 ciprofloxacin evolves.



## Materials and methods

### Bacterial strains and Mutant selection

*S. enterica* serovar Typhimurium strain SL1344 and its mutants with deletions in RamA (SL1344 *ramA::aph*) or RamR (SL1344 *ramR::aph*) were used throughout. SL1344 *ramA::aph* and *ramR::aph* were constructed by Ricci et al., (3, 8). RT-PCR experiments performed previously determined that the expression levels of *ramA* were undetectable for SL1344 *ramA::aph* and for SL1344 *ramR::aph* were increased 25-fold relative to wild type SL1344 (18). Bacteria were routinely cultured in Lennox broth unless otherwise indicated.

Spontaneous mutants with decreased susceptibility to fluoroquinolones were selected using a fluctuation assay (19). Thirty independent cultures for each parental strain were grown aerobically at 37°C for 16-20 h in antibiotic-free Lennox broth, concentrated by centrifugation, and re-suspended in sterile Lennox broth to give an approximate cell density of 10<sup>9</sup> CFU/mL. Using a spiral plater (Don Whitely Scientific, UK), 50 µl of each suspension was used to inoculate a Lennox broth agar plate containing the MIC of ciprofloxacin for each strain and incubated aerobically at 37°C for up to 3 days (Table 1). To calculate viable counts each overnight culture was diluted to 10<sup>4</sup> CFU/mL and 10<sup>5</sup> CFU/mL; 50 µl of each dilution was sufficient to enable single colony identification; enabling viable counts to be calculated. Each mutant selection experiment was repeated on three separate occasions.

### Calculating mutation frequency and rate of mutations

Mutation frequency was calculated as the average total number of ciprofloxacin-resistant colonies divided by the viable count. The phenotypic mutation rate, ( $\mu$ ), was calculated using the Lea Coulson method of the median (19, 20). The following equations were used:  $(r/m - \ln(m) - 1.24) = 0$  and  $\mu = m/2Nt$ , where  $r$  = average number of colonies obtained,  $m$  = the number of mutants per culture obtained, and  $Nt$  = final number of cells in a culture (20). A one-way ANOVA was used to determine statistical differences in mutation frequency and rate between the wild type *S. Typhimurium* SL1344 and SL1344 *ramR::aph*/SL1344 *ramA::aph*.

## **Susceptibility to antibiotics**

Ten colonies from each fluctuation assay were randomly selected to determine the phenotypes of putative mutants. All antibiotics and dyes were obtained from Sigma (Poole, UK). The susceptibility of putative mutants to six AcrAB-TolC substrates (ciprofloxacin, nalidixic acid, chloramphenicol, tetracycline, ampicillin and ethidium bromide) was determined (21). The minimum inhibitory concentration (MIC) of each agent was determined by the standardised agar doubling-dilution method as described by British Society of Antimicrobial Chemotherapy (BSAC) (22). For ciprofloxacin, a cut-off value of 0.25 mg/L was used to define resistance (8, 23, 24). Mutants were classed as MDR if there was two-fold decreased susceptibility to at least three classes of antimicrobials when compared to the parent strain (8).

## **Whole genome sequencing and PCR**

One mutant of each phenotype (as determined by susceptibility testing) was whole genome sequenced (WGS). Genomic DNA was extracted using a bacterial genomic DNA isolation kit (Norgen Biotek Corporation) according to manufacturer instructions. Paired end sequencing was carried out by Beijing Genomics Institute (BGI; Hong Kong) using the Illumina HiSeq 4000 platform. Raw sequences were assessed for quality with FASTQC. Sequencing depth was 60X. Comparisons were made with the genome of the SL1344 strain from the ensembl database (ASM21085v2) using SNIPPY to determine any single-nucleotide polymorphisms (SNPs). Alignment was performed using bowtie2. BAM files were created and compared using Artemis (Sanger Institute, UK) to confirm any SNPs detected using SNIPPY. Minimum coverage to call a SNP was 10 with a confidence cut off value of 0.9. Where any SNPs were identified, the amino acid sequence was compared using Clustal Omega to identify whether the SNP correlated with a missense mutation and corresponding protein change. PCR and DNA sequencing was performed to confirm single nucleotide polymorphisms (SNPs) within genes of interest. Primers used are described in Table 2. DNA sequencing of PCR amplicons was carried out at the Functional Genomics Laboratory (University of Birmingham, UK).



## Results

### The rate and frequency of mutation upon exposure to ciprofloxacin was dependent on the level of expression of the transcriptional activator RamA

When SL1344 was exposed to ciprofloxacin at the MIC, the average frequency of mutation (proportion of mutant cells in a population) was  $3.82 \times 10^{-8}$  mutations per cell/per generation; the average rate of mutation (rate at which mutation events arise) was  $4.08 \times 10^{-9}$  mutations per cell/ per generation (Table 1, Figure 2 and Figure 3). At the MIC of ciprofloxacin for SL1344 *ramA::aph*, the frequency of mutation was similar to that for the wild-type; mutation frequency and rate was  $7.15 \times 10^{-8}$  and  $5.11 \times 10^{-9}$  mutations per cell/per generation, respectively. Interestingly, SL1344 *ramR::aph*, which overexpresses *ramA* and leads to concomitant overexpression of *acrAB*, had a significantly higher mutation frequency and rate when compared to wild-type SL1344;  $2.54 \times 10^{-7}$  and  $3.03 \times 10^{-8}$  mutations per cell/per generation, respectively.

### Unless RamA is already over-expressed, ciprofloxacin selects for MDR mutants

When SL1344 was exposed to ciprofloxacin, MDR mutants with decreased susceptibility to ciprofloxacin, nalidixic acid, chloramphenicol and ampicillin were obtained (Table 3). WGS of one representative, L1881, revealed a single SNP conferring a missense mutation (D137N) in the transcriptional repressor *soxR* in which aspartic acid was substituted for asparagine. In contrast to the wild-type strain, mutants selected from SL1344 *ramR::aph* were not MDR but had reduced susceptibility to both ciprofloxacin and nalidixic acid; a result of a substitution of aspartic acid for glycine within the quinolone resistance determining region (QRDR) of GyrA. Prior to by WGS, all mutants were passaged on antibiotic-free media; the mutations identified were confirmed by PCR and subsequent DNA sequencing of the amplicons.

### When RamA is absent, ciprofloxacin can still select MDR mutants

In the absence of RamA (SL1344 *ramA::aph*), exposure to ciprofloxacin gave rise to two phenotypically different mutants: those that were MDR and those that were only resistant to quinolones. One mutant, L1880, had decreased susceptibility to ciprofloxacin, nalidixic acid,

chloramphenicol and ampicillin. WGS revealed a single SNP conferring a missense mutation in *soxR* with a substitution of asparagine for threonine at position. Mutants resistant only to quinolones possessed *gyrA* mutations conferring Ser83Phe or Asp87Gly substitutions (mutants L1886 and L1882).

## Discussion

As described by ourselves and others, when ciprofloxacin was used as a selecting agent, both (fluoro)quinolone-resistant and MDR mutants were obtained from wild-type *Salmonella* (3, 25, 26). Antibiotic treatment fluctuation assays were performed at the MIC as mutation selection experiments at sub-MIC concentrations are likely to alter mutation rate and phenotype of mutants selected (27).

The estimated frequency of mutation for *S. Typhimurium* after exposure to ciprofloxacin at the MIC is reported to be  $\sim 10^{-9}$ , which is in the range reported in this study (8, 28). Mutation frequency will measure all the mutants present in a population at a given time, irrespective of whether the mutation event occurred early or later during the growth of that population. Calculating mutation rates can be very complex, but aims to calculate a more accurate frequency of mutational events in a population in the presence of an antibiotic, and is important in predicting the emergence of antibiotic-resistant bacteria under a particular selective pressure. The rate of mutation shown here was also in keeping with previous studies (29).

It has been well documented that *gyrA* mutations at codon 83 and 87 confer ciprofloxacin resistance (30). Selecting bacteria with mutations that interfere with binding to the target site of quinolones is not an unexpected mechanism by which *Salmonella* strains can develop resistance to ciprofloxacin. Mutations that confer MDR typically give rise to “low-level” resistance to a broad spectrum of antibiotics and target site mutations are also necessary to provide high-level resistance (31). Therefore, when ciprofloxacin is present and able to interact with its intracellular target, even at very low concentrations (as is the case when RamA is overexpressed) a selective pressure is exerted that drives for the evolution of target site mutations in *gyrA*. Therefore, *gyrA* mutants are likely to occur in both the absence and over-expression of efflux pumps.

Given that in *S. Typhimurium* RamA is the primary regulator of *acrAB-TolC* transcription, it was interesting to find that that MDR seen for the SL1344 mutant (L1881) did not result from a mutation in *ramA*, rather a mutation in *soxR* was observed. *soxR* is typically upregulated in response to

oxidative stress, leading to increased expression of *soxS* (5, 14). This mutational event is very uncommon but has been described in clinical isolates of *Salmonella* and *E. coli*; these *soxR* mutations were associated with resistance to fluoroquinolones and chloramphenicol (9-12). In the MDR mutant (L1880) selected from SL1344 (*ramA::aph*), a *soxR* mutation was also found. Zheng *et al.* demonstrated that *ramA* inactivation caused altered transcription of genes regulated by *soxS*, suggesting co-regulation between *ramA* and *soxS* (32). When *acrB* is deleted, *soxS* expression increases; it is hypothesised that this is a response to increased oxidative stress caused by the lack of activity of this major efflux pump (14). It is likely, therefore, that in the absence of RamA, mutations enabling increased production of SoxS are selected in order to maintain functional efflux and allow for bacterial survival.

The crystal structure of SoxR from *E. coli* revealed that each monomer consists of an N-terminal DNA-binding domain, a dimerization helix domain and a C-terminal domain with a [2Fe-2S] cluster (33-35). The [2Fe-2S] cluster is vital for SoxR to function, and it is stabilised by  $\alpha 3'$ - and  $\alpha 5'$  helices (33, 34). These areas are highly conserved between all Enterobacteriaceae including *Salmonella* (36). The mutations at locations 134 and 137, in the two mutants (L1880 and L1881) lie very close to the  $\alpha 5'$  helix and the conserved cysteines for [2Fe-2S] cluster binding (37). Mutations in *Salmonella* and *E. coli* within neighbouring regions have been shown to alter redox potential and consequently create conformational changes that interfere with the DNA-binding domain of SoxR (9, 34, 36). We hypothesise that the missense mutations described in the two mutants will have similar effects in *Salmonella*, enabling the mutant to over-express SoxS and result in MDR.

In response to ciprofloxacin exposure, MDR mutants have been shown to occur as a result of RamA overproduction (3). However, in mutant selection experiments using SL1344 (*ramR::aph*) that over-expressed RamA, none of the mutants contained mutations that confer additional increased transcription of efflux pumps or caused MDR. These results suggest that further mutations in efflux regulatory genes would not create an additional fitness advantage. This hypothesis is supported by evidence from clinical isolates demonstrating that fitness costs of a mutation impacts upon the

nature of subsequent second-step mutations, in preference to mutation rate alone (37). It is hypothesised that second step mutations conferring additional increased transcription of an efflux pump would confer a high fitness cost (37). This may also explain why the majority of mutants from SL1344 (*ramA::aph*) had mutations in *gyrA* as opposed to mutations altering efflux pump gene regulation.

We have shown that natural over-expression of *acrAB* via lack of RamR repression of RamA in *Salmonella enterica* affects mutation rate and frequency. This in keeping with results obtained when artificial levels of *acrAB* are produced. El Meouche and Dunlop noted that plasmid-mediated overexpression of *acrAB* resulted in a higher mutation frequency relative to wild-type *E. coli* and *S. Typhimurium* LT2 (17). Here, we show chromosomal mediated overexpression of AcrAB, via deletion of the transcriptional repressor RamR (*ramR::aph*), resulted in a higher rate of mutation and frequency of mutation compared to cells with wild type AcrAB levels; deletion of *ramR* as a means to overexpress *acrAB* was chosen as the experimental strategy as the levels of AcrB produced are more likely to reflect those observed in a clinical isolate. Overexpression of *acrAB* in *E. coli* results in a mutator phenotype because of lower expression of the DNA mismatch repair gene *mutS* (17). This deficiency in *mutS* expression results in an inability to repair mis-incorporation of bases that occur during replication (38). Overexpression of stress response mechanisms, including efflux pumps, can incur a fitness cost by increasing cellular energy requirements and by the removal of metabolites that are essential for bacterial growth (39). We hypothesise that the mutator phenotype occurs in order to compensate for fitness costs that may result from over-expression of *acrAB*.

After exposure to ciprofloxacin, mutations in *soxR* can confer MDR resistance in *S. enterica* serovar Typhimurium in both the presence and absence of RamA. When *ramA* is already over-expressed, further mutations in the genes encoding transcriptional regulators of the AcrAB-TolC pump did not occur. SoxRS is traditionally believed to play a minor role in regulation of AcrAB-TolC in *Salmonella*, however, in response to antimicrobial selective pressure, mutations in the transcriptional regulator *soxR* can confer a survival advantage and confer MDR in the presence of normal and impaired

regulation of the AcrAB-TolC efflux pump. Inhibition of regulatory genes of AcrAB-TolC, including *ramA* and *marA*, is postulated as a method to reduce antibiotic resistance by keeping efflux levels low and thereby increasing the intracellular concentration of antibiotics, and increasing their activity. However, we show that in the absence of RamA compensatory mutations appear within *soxR* that result in MDR. This is important when considering the usefulness of compounds that behave as efflux inhibitors. Future studies evaluating novel approaches to tackling antibiotic resistance by targeting efflux in Enterobacteriales including *Salmonella*, such as inhibition of transcription factors, will need to consider all adaptive response when designing future experiments.

#### **Acknowledgements**

The authors would thank Dr Robert Marshall and Dr Xuan Wang-Kan for helpful discussions and critical appraisal of this manuscript. This work was supported by a research grant from the MRC: MR/P022596/1. Elizabeth Grimsey was supported by an MRC iCASE Studentship: MR/N017846/1.

301

302

## References

1. Organisation WH. 2015. Global action plan on antimicrobial resistance WHO Press:1-28.
2. Cuypers WL, Jacobs J, Wong V, Klemm EJ, Deborggraeve S, Van Puyvelde S. 2018. Fluoroquinolone resistance in *Salmonella*: insights by whole-genome sequencing. *Microb Genom* 4.
3. Ricci V, Piddock LJ. 2009. Ciprofloxacin selects for multidrug resistance in *Salmonella enterica* serovar Typhimurium mediated by at least two different pathways. *J Antimicrob Chemother* 63:909-16.
4. Poole K. 2000. Efflux-Mediated Resistance to Fluoroquinolones in Gram-Negative Bacteria. *Antimicrob Agents Chemother* 44:2233-2241.
5. Weston N, Sharma P, Ricci V, Piddock LJ. 2017. Regulation of the AcrAB-TolC efflux pump in Enterobacteriaceae. *Res Microbiol* S0923-2508:30176-6.
6. Abouzeed YM, Baucheron S, Cloeckaert A. 2008. ramR Mutations Involved in Efflux-Mediated Multidrug Resistance in *Salmonella enterica* Serovar Typhimurium  $\nabla$ . *Antimicrob Agents Chemother* 52:2428-34.
7. Nishino K, Yamaguchi A. 2004. Role of Histone-Like Protein H-NS in Multidrug Resistance of *Escherichia coli*. *Journal of Bacteriology* 186:1423-1429.
8. Ricci V, Tzakas P, Buckley A, Piddock LJ. 2006. Ciprofloxacin-resistant *Salmonella enterica* serovar Typhimurium strains are difficult to select in the absence of AcrB and TolC. *Antimicrob Agents Chemother* 50:38-42.



9. Koutsolioutsou A, Martins EA, White DG, Levy SB, Demple B. 2001. A *soxRS*-constitutive mutation contributing to antibiotic resistance in a clinical isolate of *Salmonella enterica* (Seroovar typhimurium). Antimicrob Agents Chemother 45.
10. Oethinger M, Podglajen I, Kern WV, Levy SB. 1998. Overexpression of the *marA* or *soxS* Regulatory Gene in Clinical Topoisomerase Mutants of *Escherichia coli*. Antimicrob Agents Chemother 42:2089-2094.
11. Kehrenberg C, Institute for Food Quality and Food Safety UoVMH, Bischofsholer Damm 15, 30173 Hannover, Germany, Institute of Farm Animal Genetics F-L-IF, Höltystr. 10, 31535 Neustadt-Mariensee, Germany, Cloeckert A, INRA U, Infectiologie Animale et Sante Publique, IASP, Nouzilly F-37380, France, Klein G, Institute for Food Quality and Food Safety UoVMH, Bischofsholer Damm 15, 30173 Hannover, Germany, Schwarz S, Institute of Farm Animal Genetics F-L-IF, Höltystr. 10, 31535 Neustadt-Mariensee, Germany. 2017. Decreased fluoroquinolone susceptibility in mutants of *Salmonella* serovars other than Typhimurium: detection of novel mutations involved in modulated expression of *ramA* and *soxS*. Journal of Antimicrobial Chemotherapy 64:1175-1180.
12. O'Regan E, Quinn T, Pages JM, McCusker M, Piddock LJV, Fanning S. 2009. Multiple regulatory pathways associated with high-level ciprofloxacin and multidrug resistance in *Salmonella enterica* serovar enteritidis: involvement of *RamA* and other global regulators. Antimicrob Agents Chemother 53:1080-1087.
13. Hidalgo E, Leautaud VD, B. 1998. The redox-regulated SoxR protein acts from a single DNA site as a repressor and an allosteric activator. EMBO 17:2629-2636.

- 346 14. Eaves DJ, Ricci V, Piddock LJ. 2004. Expression of *acrB*, *acrF*, *acrD*, *marA*, and  
347 *soxS* in *Salmonella enterica* serovar Typhimurium: role in multiple antibiotic  
348 resistance. *Antimicrob Agents Chemother* 48:1145-50.
- 349 15. Martin RG, Gillette WK, Rosner JL. 2000. Promoter discrimination by the related  
350 transcriptional activators MarA and SoxS: differential regulation by differential  
351 binding. *Mol Microbiol* 35:623-634.
- 352 16. Piddock LJV. 2014. Understanding the basis of antibiotic resistance: a platform for  
353 drug discovery. *Microbiology* 160:2366-2373.
- 354 17. El Meouche I, Dunlop MJ. 2018. Heterogeneity in efflux pump expression  
355 predisposes antibiotic-resistant cells to mutation. *Science* 9:686-690.
- 356 18. Bailey AM, Ivens A, Kingsley R, Cottell JL, Wain J, Piddock LJV. 2010. RamA, a  
357 Member of the AraC/XylS Family, Influences Both Virulence and Efflux in  
358 *Salmonella enterica* Serovar Typhimurium. *Journal of Bacteriology* 192:1607-1616.
- 359 19. Pope CF, O'Sullivan DM, McHugh TD, Gillespie SH. 2008. A practical guide to  
360 measuring mutation rates in antibiotic resistance. *Antimicrob Agents Chemother*  
361 52:1209-1214.
- 362 20. Foster PL. 2006. Methods for determining spontaneous mutation rates *Methods*  
363 *Enzymol* 409:195-213.
- 364 21. Blair JM, Piddock LJ. 2016. How to Measure Export via Bacterial Multidrug  
365 Resistance Efflux Pumps. *MBio* 7:e00840-16.
- 366 22. Andrews JM, Howe RA, Testing BWPoS. 2011. BSAC standardized disc  
367 susceptibility testing method (version 10). *J Antimicrob Chemother* 66:2726-2757.

- 368 23. Aarestrup FM, Wiuff C, Molbak K, Threlfall EJ. 2003. Is It Time To Change  
369 Fluoroquinolone Breakpoints for *Salmonella* spp.? Antimicrob Agents Chemother  
370 47:827-829.
- 371 24. Wain J, Hoa NT, Chinh NT, Vinh H, Everett MJ, Diep TS, Day NP, Solomon T,  
372 White NJ, Piddock LJV, Parry CM. 1997. Quinolone-resistant *Salmonella typhi* in  
373 Viet Nam: molecular basis of resistance and clinical response to treatment. Clin Infect  
374 Dis 25:1404-1410.
- 375 25. Giraud E, Cloeckaert A, Kerboeuf D, Chaslus-Dancla E. 2000. Evidence for Active  
376 Efflux as the Primary Mechanism of Resistance to Ciprofloxacin in *Salmonella*  
377 *enterica* Serovar Typhimurium. Antimicrob Agents Chemother 44:1223-1228.
- 378 26. Sun Y, Dai M, Hao H, Wang Y, Huang L, A. AY, Liu Z, Yuan Z. 2011. The role of  
379 RamA on the development of ciprofloxacin resistance in *Salmonella enterica* serovar  
380 Typhimurium. PLoS One 6.
- 381 27. Martinez JL, Baquero F. 2000. Mutation Frequencies and Antibiotic Resistance.  
382 Antimicrob Agents Chemother 44:1771-1777.
- 383 28. Webber MA, Randall LP, Cooles S, Woodward MJ, Piddock LJ. 2008. Triclosan  
384 resistance in *Salmonella enterica* serovar Typhimurium. J Antimicrob Chemother  
385 62:83-91.
- 386 29. Ricci V, Loman N, Pallen M, Ivens A, Fookes M, Langridge GC, Wain J, Piddock  
387 LJV. 2011. The TCA cycle is not required for selection or survival of multidrug-  
388 resistant *Salmonella*. Journal of Antimicrobial Chemotherapy 67:589-599.

389 30. Hooper DC. 2001. Emerging mechanisms of fluoroquinolone resistance. *Emerg Infect*  
390 *Dis* 7:337-341.

391 31. Singh R, Swick MC, Ledesma KR, Yang Z, Hu M, Zechiedrich L, Tam VH. 2012.  
392 Temporal Interplay between Efflux Pumps and Target Mutations in Development of  
393 Antibiotic Resistance in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*  
394 56:1680-1685.

395 32. Zheng J, Tian F, Cui S, Song J, Zhao S, Brown EW, Meng J. 2011. Differential gene  
396 expression by RamA in ciprofloxacin-resistant *Salmonella* Typhimurium. *Plos One*  
397 6:1-8.

398 33. Watanabe S, Kita A, Kobayashi K, Miki K. 2008. Crystal structure of the [2Fe-2S]  
399 oxidative-stress sensor SoxR bound to DNA. *Proc Natl Acad Sci U S A* 105:4121-  
400 4126.

401 34. Wu J, Weiss B. 1991. Two divergently transcribed genes, *soxR* and *soxS*, control a  
402 superoxide response regulon of *Escherichia coli*. *J Bacteriol* 173:2864-2871.

403 35. Lee KL, Singh AK, Heo L, Seok C, Roe JH. 2015. Factors affecting redox potential  
404 and differential sensitivity of SoxR to redox-active compounds. *Mol Microbiol*  
405 97:808-821.

406 36. Huseby DL, Pietsch F, Brandis G, Gaross L, Tegehall A, Hughes D. 2017. Mutation  
407 Supply and Relative Fitness Shape the Genotypes of Ciprofloxacin-Resistant  
408 *Escherichia coli*. *Mol Bio Evol* 34:1029-1039.

409 37. Hughes. D, Andersson DI. 2017. Evolutionary Trajectories to Antibiotic Resistance.  
410 *Annu Rev Microbiol* 8:579-596.

- 411 38. Li B, Tsui HC, LeClerc JE, Dey M, Winkler ME, Cebula TA. 2003. Molecular  
412 analysis of mutS expression and mutation in natural isolates of pathogenic  
413 *Escherichia coli*. Microbiology 149:1323-1331.
- 414 39. Du Toit A. 2017. Efflux pumps, fitness and virulence. Nature Reviews Microbiology  
415 15:512-513.
- 416  
417

**Figure 1. Schematic representation of the known regulatory pathways for expression of AcrAB-TolC efflux pump in *Salmonella*.** The genes are represented as arrows and their translated proteins are represented as ovals (transcriptional repressors) and hexagons (transcriptional activators) The AcrAB-TolC pump extrudes drugs across the cytoplasmic and outer membranes. Excessive production of AcrA and AcrB is prevented by the local repressor AcrR. Activation of *acrA*, *acrB* and *tolC* transcription occurs primarily due to the global regulatory protein RamA by binding to the rambox upstream of these genes. As demonstrated the regulatory proteins SoxS and Rob can also activate *acrABtolC* transcription. RamA expression is controlled by RamR which represses activation of *ramA*. Likewise, SoxR controls expression of both *soxR* and *soxS*.

**Figure 2. Mutation frequencies of strains exposed to ciprofloxacin.** Mutation frequency was calculated as the average total number of ciprofloxacin-resistant colonies divided by the viable count. \*  $P < 0.05$  calculated using a one-way ANOVA and is relative to the wild type SL1344.  $n = 30$  independent replicates.

**Figure 3. Mutation rate of strains exposed to ciprofloxacin.** The phenotypic mutation rate, ( $\mu$ ), was calculated using the Lea Coulson method of the median. \*  $P < 0.05$  calculated using a one-way ANOVA and is relative to the wild type SL1344.  $n = 30$  independent replicates.

Figure 1

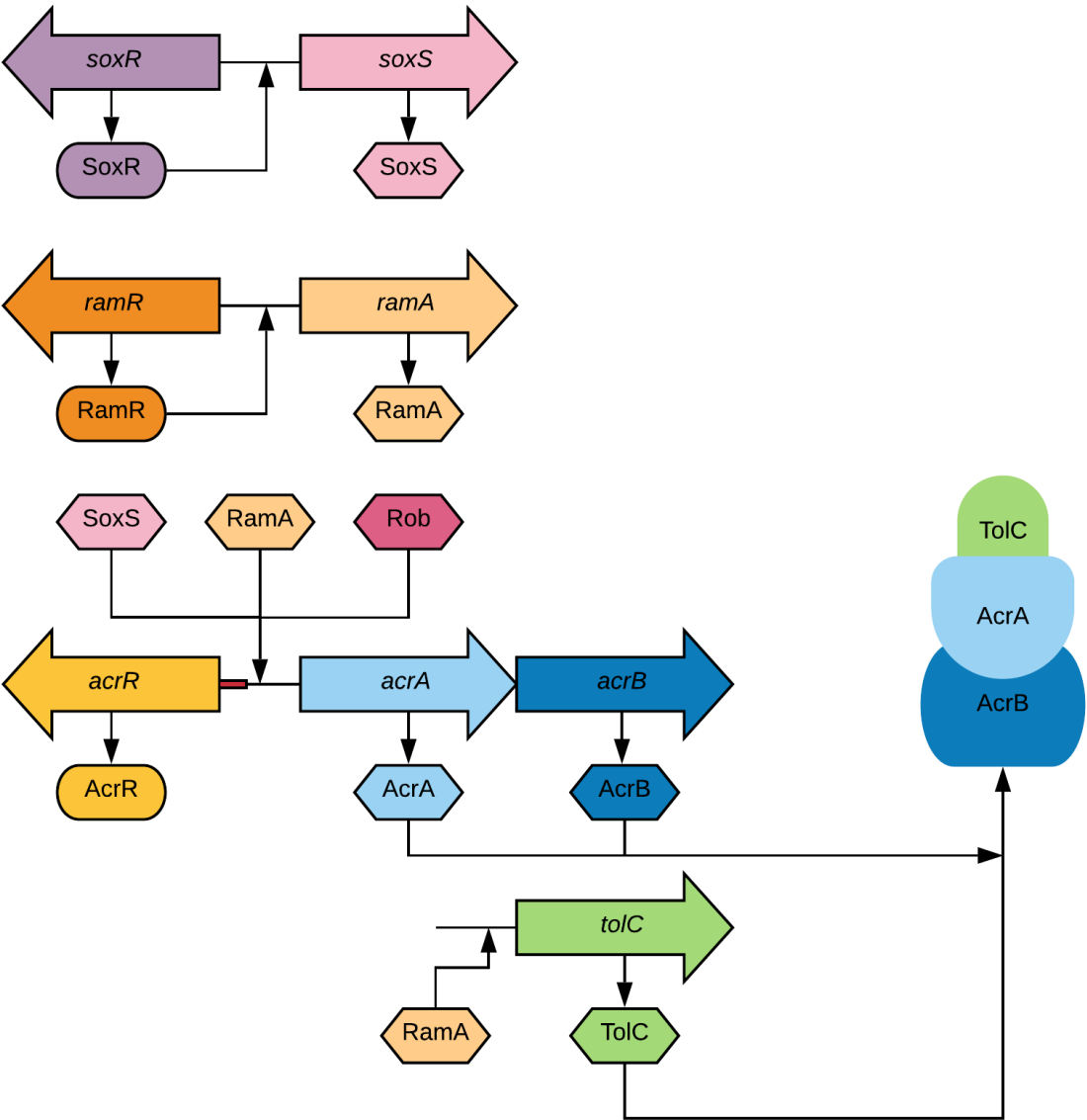
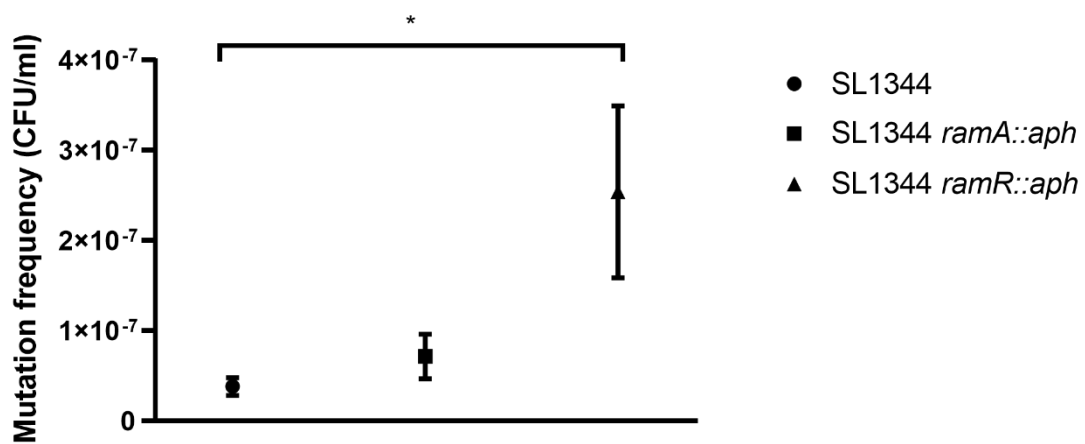
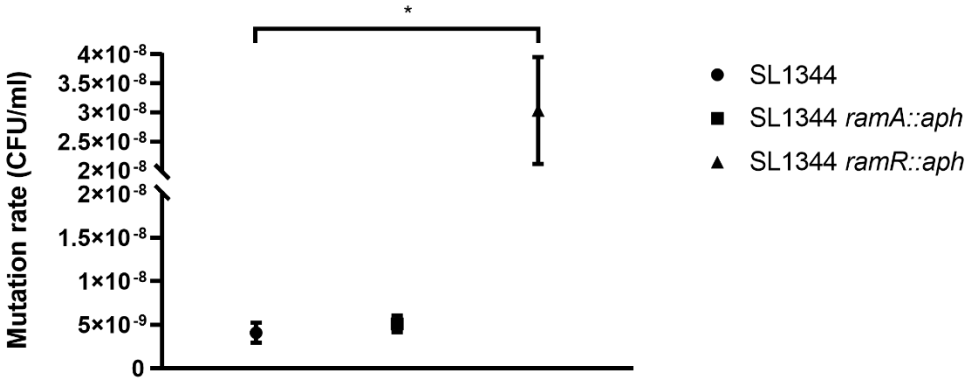


Figure 2





447 **Figure 3**  
448



449

**Table 1.** Average frequency and rate of mutation (per cell/per generation) of *S. Typhimurium* SL1344 wildtype, SL1344 *ramR::aph* and SL1344 *ramA::aph* mutants selection upon exposure to MIC concentrations of ciprofloxacin.

Strain	Genotype	Ciprofloxacin		
		MIC µg/ml	Frequency +/- SD	Mutation rate per cell/ per generation +/- SD
SL1344	wild-type	0.03	$3.82 \times 10^{-8} \pm 9.75 \times 10^{-9}$	$4.08 \times 10^{-9} \pm 1.15 \times 10^{-9}$
L1322	<i>ramA::aph</i>	0.03	$7.15 \times 10^{-8} \pm 2.47 \times 10^{-8}$	$5.11 \times 10^{-9} \pm 9.75 \times 10^{-10}$
L1007	<i>ramR::aph</i>	0.12	$2.54 \times 10^{-7} \pm 9.52 \times 10^{-8}$	$3.03 \times 10^{-8} \pm 9.21 \times 10^{-9}$

The MIC of each parental strain is shown.

455 **Table 2.** Primers used in this study to confirm SNPs identified by WGS.

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')
<i>gyrA</i>	CGTTGGTGACGTAATCGGTA	CCGTACCGTCATAGTTATCC
<i>soxR</i>	CAATGTTTAGCGGTTGGTCG	AATCATCTTCAAGCAGCCGG

456

457 **Table 3.** MIC phenotype of the mutants obtained after exposure to ciprofloxacin. The genotype, determined by WGS of each mutant, is shown.

Strain	Genotype	MIC (µg/ml)					
		CIP	NAL	CHL	TET	Et Br	AMP
<i>S. Typhimurium</i> (SL1344)	WT	0.03	8	4	1	1024	1
<b>L1881</b>	<b>SoxR Asp137Asn</b>	<b>0.12</b>	<b>16</b>	<b>16</b>	<b>2</b>	<b>&gt;2048</b>	<b>8</b>
<i>S. Typhimurium</i> (SL1344)	<i>ramA::aph</i>	0.03	8	4	1	1024	1
L1886	GyrA Ser83Phe	0.5	>64	4	1	2048	2
L1882	GyrA Asp87Gly	0.12	>64	4	1	1024	2
<b>L1880</b>	<b>SoxR Asn134Thr</b>	<b>0.12</b>	<b>16</b>	<b>16</b>	<b>2</b>	<b>2048</b>	<b>8</b>
<i>S. Typhimurium</i> (SL1344)	<i>ramR::aph</i>	0.12	16	16	4	2048	8
L1877	GyrA Asp87Gly	0.5	>64	16	4	2048	8

458

459 CIP, ciprofloxacin; NAL, nalidixic acid; CHL, chloramphenicol; TET, tetracycline; EtBr, ethidium bromide; AMP, ampicillin.

460 Italic values indicate a ≥4-fold increase in MIC in comparison to the parental strain. Bold values indicated SoxR mutants.

461

462

463

464

465

