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

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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 aquire 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

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

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

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

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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 10^9 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 10^4 CFU/mL and 10^5 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, (µ), 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 amplimers 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 3.82×10^{-8} mutations per cell/per generation; the average rate of mutation (rate at which mutation events arise) was 4.08×10^{-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 7.15×10^{-8} and 5.11×10^{-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; 2.54×10^{-7} and 3.03×10^{-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 amplimers.

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).

196 **Discussion**

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

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

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

220 Given that in *S.* Typhimurium RamA is the primary regulator of *acrAB-TolC* transcription, it was
221 interesting to find that that MDR seen for the SL1344 mutant (L1881) did not result from a mutation
222 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 α3'-and α5' 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 α5' 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

249 nature of subsequent second-step mutations, in preference to mutation rate alone (37). It is
250 hypothesised that second step mutations conferring additional increased transcription of an efflux
251 pump would confer a high fitness cost (37). This may also explain why the majority of mutants from
252 SL1344 (*ramA::aph*) had mutations in *gyrA* as opposed to mutations altering efflux pump gene
253 regulation.
254 We have shown that natural over-expression of *acrAB* via lack of RamR repression of RamA in
255 *Salmonella enterica* affects mutation rate and frequency. This in keeping with results obtained when
256 artificial levels of *acrAB* are produced. El Meouche and Dunlop noted that plasmid-mediated
257 overexpression of *acrAB* resulted in a higher mutation frequency relative to wild-type *E. coli* and *S.*
258 Typhimurium LT2 (17). Here, we show chromosomal mediated overexpression of AcrAB, via deletion
259 of the transcriptional repressor RamR (*ramR::aph),* resulted in a higher rate of mutation and
260 frequency of mutation compared to cells with wild type AcrAB levels; deletion of *ramR* as a means to
261 overexpress *acrAB* was chosen as the experimental strategy as the levels of AcrB produced are more
262 likely to reflect those observed in a clinical isolate. Overexpression of *acrAB* in *E. coli* results in a
263 mutator phenotype because of lower expression of the DNA mismatch repair gene *mutS* (17). This
264 deficiency in *mutS* expression results in an inability to repair mis-incorporation of bases that occur
265 during replication (38). Overexpression of stress response mechanisms, including efflux pumps, can
266 incur a fitness cost by increasing cellular energy requirements and by the removal of metabolites
267 that are essential for bacterial growth (39). We hypothesise that the mutator phenotype occurs in
268 order to compensate for fitness costs that may result from over-expression of *acrAB*.
269 After exposure to ciprofloxacin, mutations in *soxR* can confer MDR resistance in *S. enterica* serovar
270 Typhimurium in both the presence and absence of RamA. When *ramA* is already over-expressed,
271 further mutations in the genes encoding transcriptional regulators of the AcrAB-TolC pump did not
272 occur. SoxRS is traditionally believed to play a minor role in regulation of AcrAB-TolC in *Salmonella*,
273 however, in response to antimicrobial selective pressure, mutations in the transcriptional regulator
274 *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.

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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, (μ), 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.

438 **Figure 1**

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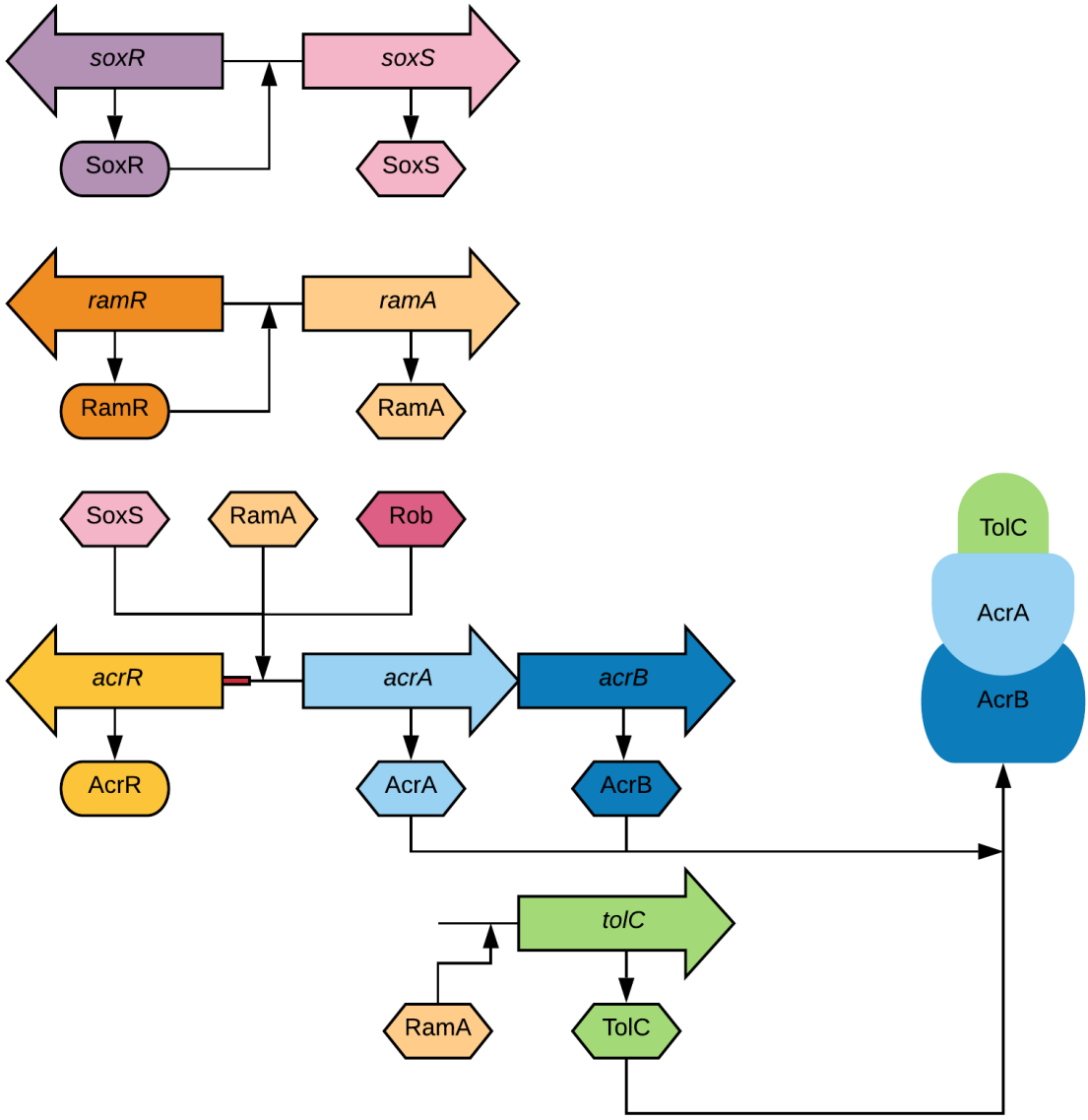

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443 **Figure 2**
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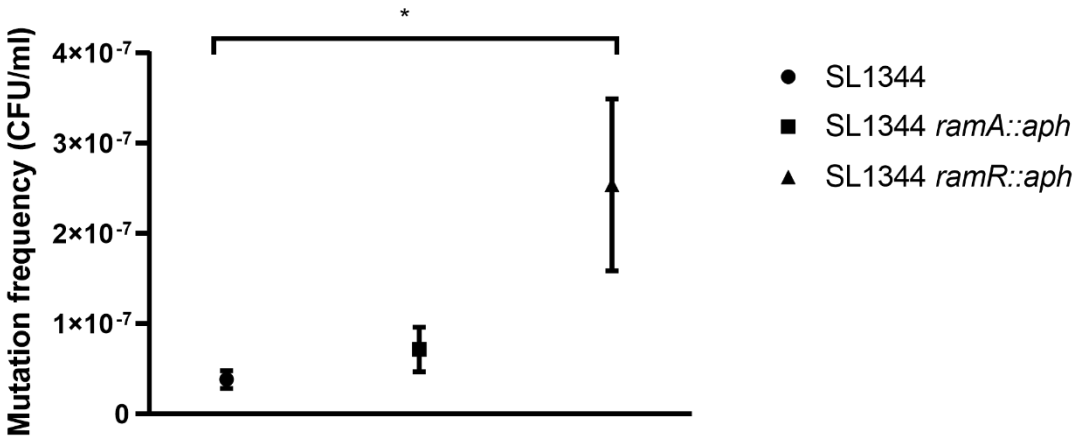

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447 **Figure 3**
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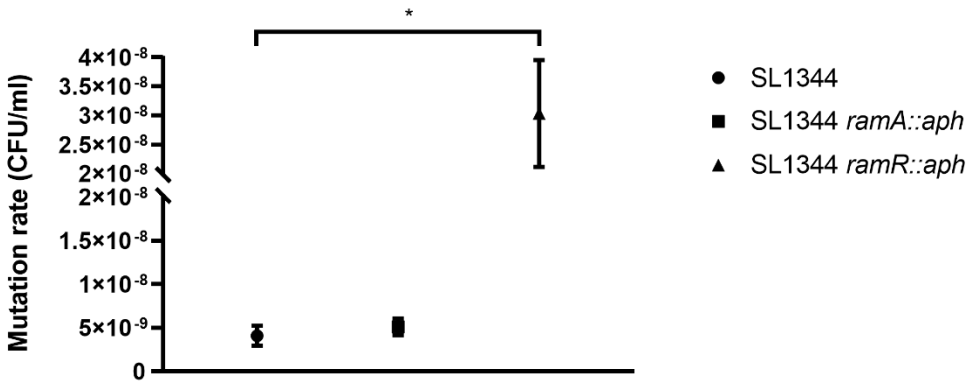

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Table 1. Average frequency and rate of mutation (per cell/per generation) of *S.* Typhimurium SL1344 wildype, SL1344 *ramR::aph* and SL1344 *ramA::aph* mutants selection upon exposure to MIC concentrations of ciprofloxacin.

Strain	**Genotype**	**Ciprofloxacin**		
		MIC µg/ml	Frequency +/- SD	Mutation rate per cell/ per generation +/- SD
SL1344	wild-type	0.03	$3.82x10^{-8}$ +/- $9.75x10^{-9}$	$4.08x10^{-9}$ +/- $1.15x10^{-9}$
L1322	*ramA::aph*	0.03	$7.15x10^{-8}$ +/- $2.47x10^{-8}$	$5.11x10^{-9}$ +/- $9.75x10^{-10}$
L1007	*ramR::aph*	0.12	$2.54x10^{-7}$ +/- $9.52x10^{-8}$	$3.03x10^{-8}$ +/- $9.21x10^{-9}$

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')
gyrA	CGTTGGTGACGTAATCGGTA	CCGTACCGTCATAGTTATCC
soxR	CAATGTTTAGCGGTTGGTCG	AATCATCTTCAAGCAGCCGG

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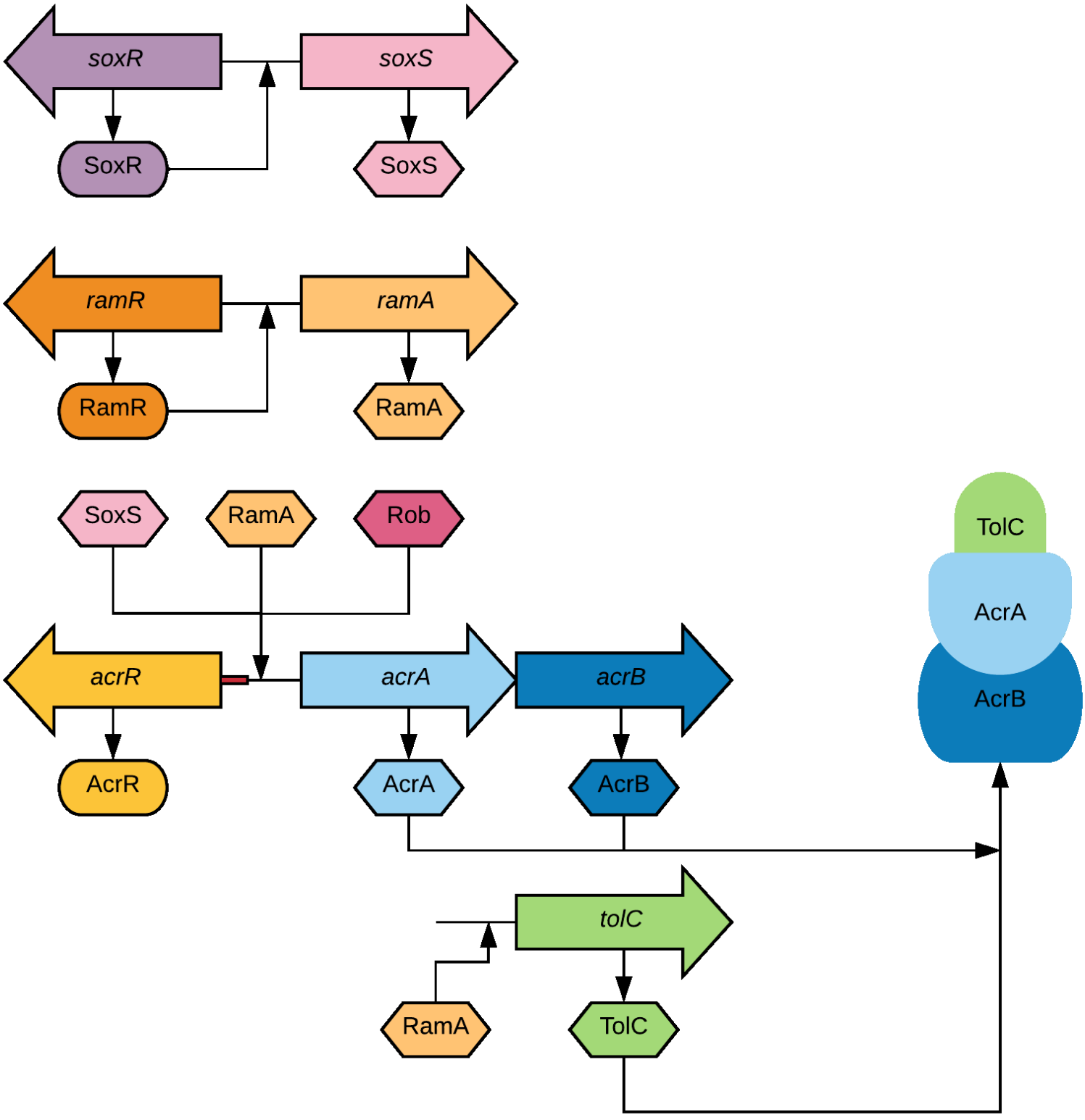
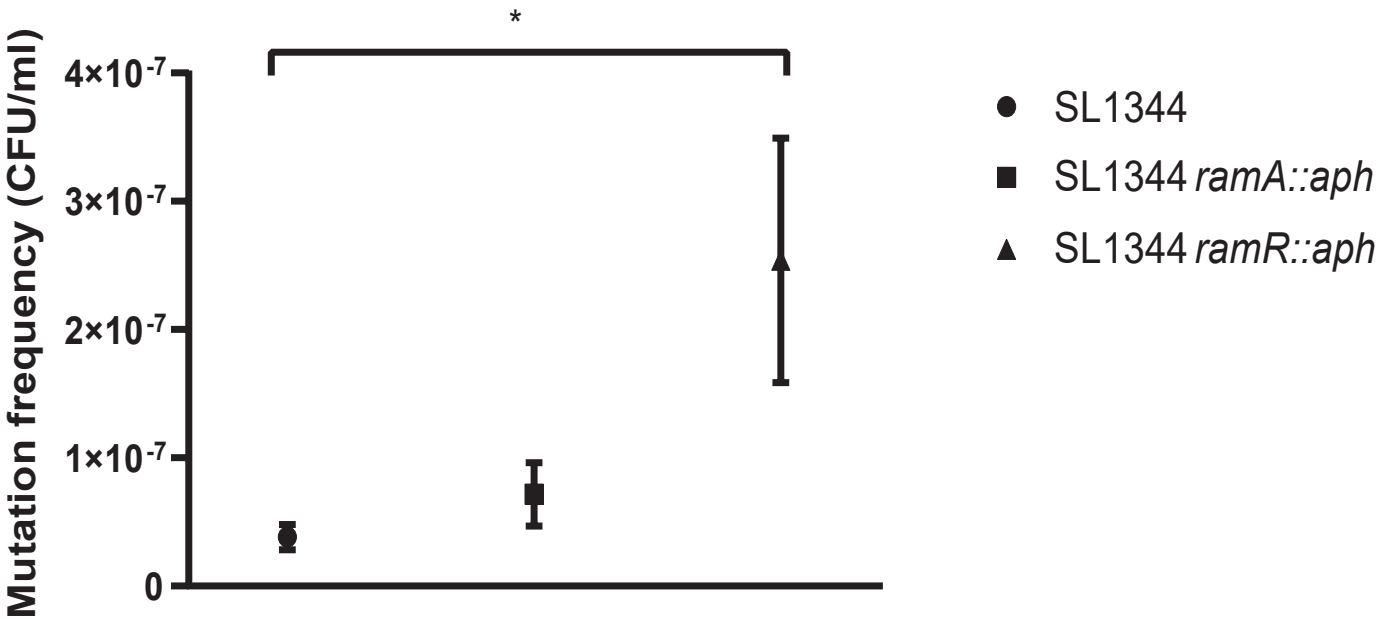
Table 3. MIC phenotype of the mutants obtained after exposure to ciprofloxacin. The genotype, determined by WGS of each mutant, is shown.

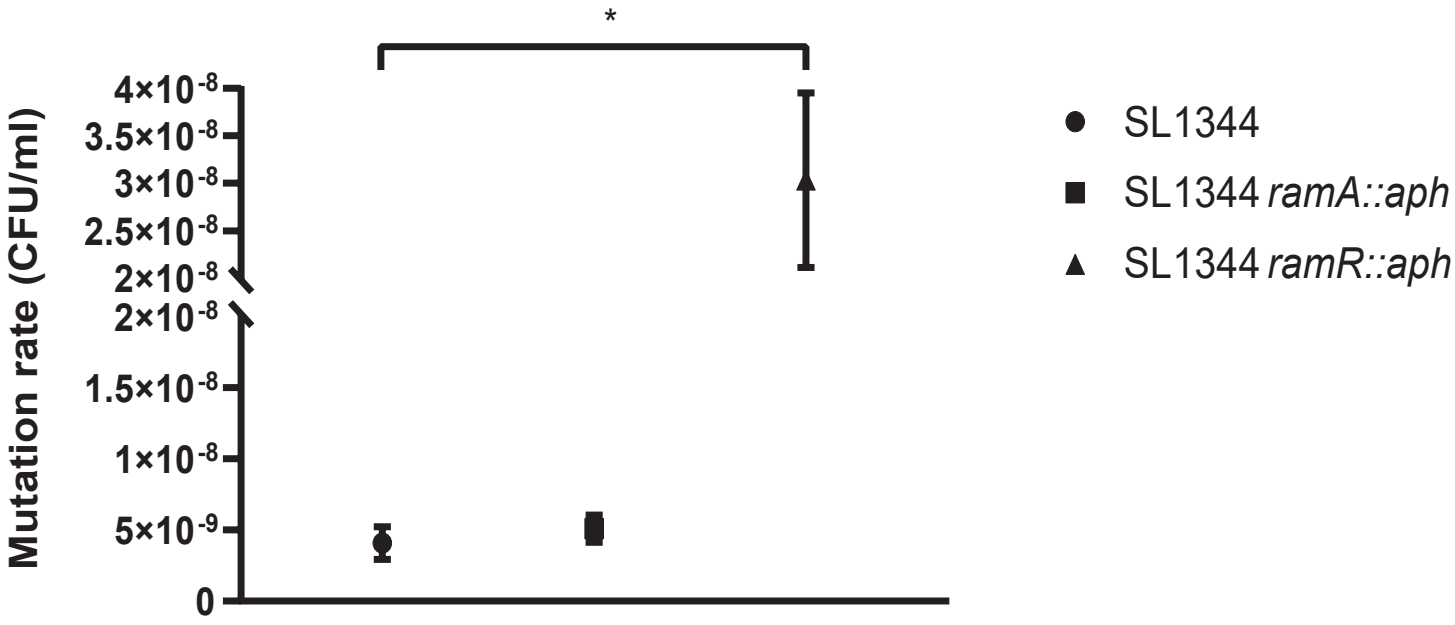
Strain	Genotype	MIC (µg/ml)					
		CIP	**NAL**	**CHL**	**TET**	**Et Br**	**AMP**
S. Typhimurium (SL1344)	WT	0.03	8	4	1	1024	1
L1881	**SoxR Asp137Asn**	***0.12***	**16**	***16***	**2**	***>2048***	***8***
S. Typhimurium (SL1344)	*ramA::aph*	0.03	8	4	1	1024	1
L1886	GyrA Ser83Phe	*0.5*	*>64*	4	1	2048	2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

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

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


soxR
soxS
SoxR
SoxS
ramR
ramA
RamR
RamA
SoxS
RamA
Rob
acrR
acrA
acrB
AcrR
AcrA
AcrB
TolC
AcrA
AcrB
tolC
RamA
TolC


*
Mutation frequency (CFU/ml)
4×10-7
3×10-7
2×10-7
1×10-7
0
SL1344
SL1344 ramA::aph
SL1344 ramR::aph


*
Mutation rate (CFU/ml)
4×10-8
3.5×10-8
3×10-8
2.5×10-8
2×10-8
2×10-8
1.5×10-8
1×10-8
5×10-9
0
SL1344
SL1344 ramA::aph
SL1344 ramR::aph