Introduction

The ever-increasing levels of resistance to antimicrobial compounds are of great concern, particularly for pathogens of clinical relevance. Salmonella enterica serovar Typhimurium is a pathogen distributed worldwide that typically causes gastroenteritis in humans. Fluoroquinolones and cephalosporins are the current first-line treatments; however, recent data have revealed that in particular geographical areas, such as China, high percentages of resistance to compounds such as nalidixic acid (61.9%) and cefepime, cefotaxime and ceftazidime (90%) have already been detected.

Quinolone resistance has been widely studied in Enterobacteriaceae, particularly in Escherichia coli and S. enterica. In E. coli, the mechanism that largely contributes to resistance and/or decreased susceptibility to quinolones is the acquisition of mutations located in the genes encoding the two quinolone targets: DNA gyrase (gyrA and gyrB) and topoisomerase IV (parC and parE). These mutations are usually acquired in the quinolone resistance-determining regions (QRDRs) detected in each of the target genes. On the other hand, increased drug extrusion by means of the overexpression of AcrAB-ToIC, the main efflux pump described in Enterobacteriaceae, is also of great concern since it confers cross-resistance to several unrelated compounds, including antimicrobial drugs. To a lesser extent, other efflux systems, such as AcrEF and EmrAB, have been reported to participate in the extrusion of antimicrobial compounds. In Salmonella, increased efflux has been described as the primary mechanism in quinolone resistance acquisition. Alternatively, decreased production of the OmpF porin has at times been related to the MDR phenotype, despite controversial data suggesting no clear role in S. enterica.

Several regulators have been reported to influence the expression of the acrAB operon in Salmonella. AcrR is the local repressor encoded upstream of the acrAB genes and mutations within its coding sequence have been associated with increased expression of the pump. In addition, three homologous transcriptional activators, RamA, SoxS and MarA, have been associated with increased acrB and tolC expression levels. While clear associations have been reported for enhanced production of SoxS and RamA and overexpression of acrAB, only indirect results have suggested greater production of MarA with increased levels of

Differential impact of ramRA mutations on both ramA transcription and decreased antimicrobial susceptibility in Salmonella Typhimurium

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Objectives: This study was focused on analysing the heterogeneity of mutations occurring in the regulators of efflux-mediated MDR in Salmonella Typhimurium. Moreover, the impact of such mutations on impairing the transcription of ramA, acrB, tolC and acrF was also assessed as was the impact on the resistance or decreased susceptibility phenotype.

Methods: Strains were selected in vitro under increasing ciprofloxacin concentrations. Etest and broth micro-dilution tests were used to determine the MICs of several unrelated compounds. Screening of mutations in the quinolone target genes and MDR regulators was performed. RT–PCR analysis was used to detect the levels of expression of acrB, tolC, ompF, acrF, emrB, acrR, ramA, soxS and marA.

Results: All mutant strains showed increased MICs of most of the antimicrobials tested, with the exception of kanamycin. Mutations in the quinolone target genes did not occur in all the mutants, which all harboured mutations in the ramRA regulatory region. All the mutants overexpressed ramA, tolC and acrB (only tested in 60-wt derivatives), whereas differential results were seen for the remaining genes.

Conclusions: Mutations in the ramRA region related to resistance and/or decreased susceptibility to antimicrobials predominate in Salmonella. There is heterogeneity in the types of mutations, with deletions affecting RamR-binding sites having a greater impact on ramA expression and the MDR phenotype.
resistance, supposedly mediated by higher levels of AcrAB,\textsuperscript{11,17} In terms of regulation, each of these three activators has its own regulator: RamR, SoxR and MarR, respectively.\textsuperscript{3} In terms of the MDR phenotype, the clinical relevance of mutations located in the genes encoding these latter regulators has been clearly shown for RamR,\textsuperscript{18,19} while there have been few reports for mutations located in the soxRS region.\textsuperscript{11,15} Concerning MarA, even though its overexpression has been detected in MDR \textit{S. enterica} strains,\textsuperscript{8,20} the putative responsible mutations in the \textit{marRAB} region have not been mapped. Naturally occurring mutations in this region have been widely reported in \textit{E. coli},\textsuperscript{21,22} whereas, to our knowledge, such mutations in \textit{S. enterica} have only been reported in a single study, associating it with high MarA overexpression and an MDR phenotype.\textsuperscript{12}

The aim of this study was to determine the mechanisms involved in increasing the MICs of different antimicrobial agents for a collection of \textit{Salmonella} Typhimurium mutants selected in \textit{vivo}, particularly when studying strains with low MICs of ciprofloxacin and their derivative mutants selected at the initial steps of drug exposure following a stepwise procedure. The mechanisms studied included target gene mutations and the expression of several genes involved in decreasing the intracellular concentration of the drug. Moreover, and as a novel approach, we also assessed the role and heterogeneity of ramRA mutations and their impact on increasing the expression of ramA and the phenotype of decreased susceptibility to multiple antibiotics or MDR.

**Materials and methods**

**Bacterial strains and selection of resistant mutants**

Two \textit{Salmonella} Typhimurium clinical isolates, strains 59-wt and 60-wt, were recovered from independent stool samples in the Department of Clinical Microbiology at the Hospital Clinic of Barcelona, Spain. Strain 59-wt has previously been characterized, as have its derivative mutants displaying increasing ciprofloxacin MICs, including the highly resistant mutant 59-64.\textsuperscript{23} As indicated, the clinical isolate 59-wt was grown at 37°C on MacConkey agar plates in the presence of ciprofloxacin (Fluox) in a multistep selection process with doubling concentrations of the drug.\textsuperscript{23} Single colonies were randomly selected at different steps and previously characterized. In the present study, we characterized additional randomly selected colonies during the process (59-mut1, 59-mut2 and 59-mut3) to assess the occurrence of heterogeneity in the mechanisms of resistance. Likewise, strain 60-wt was similarly treated and exposed to increasing ciprofloxacin concentrations and two different mutants were randomly selected (60-mut1 and 60-mut2).

**Susceptibility testing**

The MICs of several quinolones and unrelated antimicrobial compounds were determined by Etest (AB Biodisk) according to the manufacturer’s recommendations and interpreted according to CLSI guidelines.\textsuperscript{24} The broth microdilution method was used to evaluate the MICs of ciprofloxacin, norfloxacin and nalidixic acid when maximum Etest values were reached. The compounds tested method was used to evaluate the MICs of ciprofloxacin, norfloxacin and nalidixic acid when maximum Etest values were reached. The compounds tested included target gene mutations and the expression of several genes involved in decreasing the intracellular concentration of the drug. Moreover, and as a novel approach, we also assessed the role and heterogeneity of ramRA mutations and their impact on increasing the expression of ramA and the phenotype of decreased susceptibility to multiple antibiotics or MDR.

**Detection of mutations within the QRDRs and regulatory loci**

Mutations acquired in the QRDRs of the \textit{gyrA}, \textit{gyrB}, \textit{parC} and \textit{parE} genes as well as in the MDR regulatory loci \textit{soxRS}, \textit{marRAB}, \textit{acrR} and \textit{ramR} were screened by PCR amplification as described previously.\textsuperscript{25} Amplicons were purified and sent to Beckman Coulter Genomics (Essex, UK) for sequencing reactions. Detection of mutations was carried out using BioEdit\textsuperscript{8} software (Ibis Biosciences, Carlsbad, CA, USA) by comparison with the genome of \textit{Salmonella} Typhimurium LT2 as the reference strain (RefSeq: NC_003197.1).

**RNA extraction and real-time PCR**

Bacterial pellets were obtained as described previously.\textsuperscript{25} Briefly, strains were grown in LB broth at 37°C with shaking to reach exponential phase (OD\textsubscript{600} = 0.6). Four millilitres of bacterial cells was treated with 8 mL of RNA Protect Bacteria Reagent (Qiagen) and subsequently incubated with Tris-EDTA buffer supplemented with lysozyme. RNA extractions were obtained using the Maxwell\textsuperscript{18} 16 Research Instrument (Promega) and the Maxwell\textsuperscript{18} 16 LEV simplyRNA Blood Kit (Promega) following the manufacturer’s recommendations. Five independent RNA extractions were made. The acrR, tolC, ompF, acrF, emrB, ramA, marA, soxS and acrR genes were analysed by RT–PCR following previously described conditions.\textsuperscript{25} The 16S rRNA gene was used as an internal control for normalization and susceptible strains 59-wt and 60-wt were the reference strains for their respective derived mutants. The 2−ΔΔCT method was used for relative gene expression calculations.\textsuperscript{27} Five independent assays were performed and each RNA sample was tested in triplicate. The primers used are reported in Table 1. Mean (+SD) values are detailed in Table 2.

**Results and discussion**

**Quinolone resistance and the MDR phenotype**

Three and two derivative mutants were selected from the quinolone-susceptible clinical isolates 59-wt and 60-wt, respectively. Susceptibility testing to several unrelated compounds was
used to determine the acquisition of the quinolone resistance and MDR phenotypes (Table 3). The term MDR has been defined as resistance to one agent in three or more antimicrobial categories28 or to four or more antimicrobials in the particular case of non-typhoidal Salmonella.29 In the present study, we used instead the term decreased susceptibility to multiple antibiotics when increased MICs of more than four antimicrobial compounds were seen even though the resistance breakpoints were not reached. Strain 59-64, already characterized in a previous study,23 was also included in the present work for comparison with the mutants.

The results showed that in comparison with their WT strain, all selected mutants had increased MICs (1.5- to >8-fold) of all the drugs tested, except for kanamycin, for which no increase was recorded. Only 59-wt derivative mutants showed the acquisition of QRDR mutations (Table 4). Strains 59-mut1 and 59-mut2 showed a similar genetic background in terms of target gene mutations. However, higher MIC values were seen for 59-mut2 concerning all the drugs (except for amoxicillin and chloramphenicol, which had already shown maximum Etest values for 59-wt, and tetracycline). Likewise, on comparing strains 60-mut1 and 60-mut2 a similar conclusion was obtained, with higher MIC results seen for 60-mut2 despite having background similarity.

In accordance with the fact that strains 59-mut3 and 59-64 were selected at higher ciprofloxacin concentrations, these strains showed the highest MICs, mostly concerning quinolones, being maximal for strain 59-64.

Taking into account the increased MICs of most of these compounds for all the mutants, and the fact that increased efflux confers a cross-resistance phenotype by means of increased AcrAB or even a hitherto uncharacterized efflux pump,6,23 enhanced exclusion activity was the most likely mechanism underlying this phenotype. Moreover, the results obtained from 60-wt and its derivative mutants strengthen the idea that efflux is selected at primary stages of the process of quinolone resistance acquisition as suggested previously10,25 and this mechanism is selected even before target gene mutations. On the contrary, it should be noted that mutants selected in a single step-selection process, usually performed at concentrations higher than the initial MIC, may follow a different pattern of acquisition of resistance mechanisms.

### Expression of structural genes involved in MDR

Gene expression analysis was performed to determine the expression patterns of genes related to bacterial efflux and permeability. The results were interpreted after comparison of the expression

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### Table 2. Mean (±SD) values of RT–PCR analysis obtained in five independent experiments

| Strain   | acrB | tolC | ompF | acrF | emrB | acrR | ramA | soxS | marA |
|----------|------|------|------|------|------|------|------|------|------|
| 59-wt    | ND   | 1    | 1    | 1    | 1    | 1    | 1    | 1    | 1    |
| 59-mut1  | ND   | 2.7  (0.59) | −1.2 (0.23) | 1.7 (0.27) | −1.5 (0.13) | 1.0 (0.25) | 19.6 (8.05) | 1.2 (0.57) | 1.3 (0.23) |
| 59-mut2  | ND   | 6.2  (0.36) | −1.8 (0.12) | 6.0 (1.43) | −1.8 (0.22) | −1.9 (0.16) | 66.0 (12.68) | 1.8 (1.77) | 4.3 (4.13) |
| 59-mut3  | ND   | 2.5  (0.49) | −1.8 (0.05) | 1.7 (0.27) | −1.4 (0.35) | 1.0 (0.41) | 17.3 (9.44) | 1.7 (0.85) | 2.0 (0.51) |
| 59-64    | ND   | 3.2  (1.11) | −3.3 (0.10) | 1.4 (0.33) | −1.9 (0.27) | 2.1 (1.57) | 13.4 (4.71) | 4.6 (2.06) | 3.7 (0.48) |
| 60-wt    | 1    | 1    | 1    | 1    | 1    | 1    | 1    | 1    | 1    |
| 60-mut1  | 5.2  (1.33) | 2.3  (1.02) | −1.4 (0.45) | 1.6 (0.86) | −1.2 (0.57) | −1.3 (0.54) | 15.4 (7.78) | 1.1 (0.46) | 2.0 (1.10) |
| 60-mut2  | 9.5  (4.85) | 5.4  (2.69) | −2.2 (0.27) | 4.9 (4.77) | −1.6 (0.37) | −1.2 (0.49) | 74.2 (30.19) | 4.3 (4.28) | 3.6 (2.35) |

ND, not determined.

### Table 3. Susceptibility testing of all the strains and ciprofloxacin concentrations used for the selection of mutants

| Strain   | CIP concentration (mg/L) at selection | CIP | NOR | NAL | AMX | CRO | FOX | TET | CHL | ERY | KAN |
|----------|-------------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 59-wt    | a                                   | 0.012 | 0.094 | 4 | >256 | 0.094 | 2 | 64 | >256 | 32 | 1.5 |
| 59-mut1  | 0.06                                | 0.125 | 2 | 32 | >256 | 0.190 | 6 | 128 | >256 | 128 | 1.5 |
| 59-mut2  | 0.25                                | 0.38 | 6 | 96 | >256 | 0.5 | 12 | 128 | >256 | 256 | 1.5 |
| 59-mut3  | 2                                   | 8 | 16 | 8128 | >256 | 0.25 | 4 | 96 | >256 | 128 | 1.5 |
| 59-64    | 64                                  | 256 | 512 | 8128 | >256 | 1 | 96 | 256 | >256 | >256 | 1.5 |
| 60-wt    | a                                   | 0.016 | 0.094 | 3 | 1 | 0.032 | 3 | 3 | 32 | 1 | 1 |
| 60-mut1  | 0.015                               | 0.047 | 0.19 | 6 | 1.5 | 0.064 | 8 | 8 | 192 | 1 |
| 60-mut2  | 0.015                               | 0.094 | 0.38 | 24 | 3 | 0.125 | 12 | 12 | 24 | >256 | 1 |

CIP, ciprofloxacin; NOR, norfloxacin; NAL, nalidixic acid; AMX, amoxicillin; CRO, ceftriaxone; FOX, cefoxitin; CHL, chloramphenicol; TET, tetracycline; ERY, erythromycin; KAN, kanamycin.

*aClinical isolate not exposed to ciprofloxacin in vitro*.
levels of each clinical isolate with their respective mutant derivatives. The genes studied were acrB, tolC, ompF, acrF and emrB (Figure 1 and Table 2). Overexpression of the AcrAB-TolC efflux pump has been reported as the most relevant mechanism in terms of efflux.\(^3\) In the present study, acrB was only analysed in 60-wt and its derivatives, which all overexpressed this gene (5.2- to 9.5-fold), since it was reported that 59-wt has a mutation inactivating the acrB operon.\(^2\) The tolC gene was found to be consistently overexpressed in all the mutants (≥2.3-fold), particularly for strains 59-mut2 and 60-mut2 (6.2- and 5.4-fold, respectively). In contrast, ompF always showed decreased expression with the strongest results being seen in strains 59-wt derivatives, whereas emrB showed a slightly decreased expression in the all the mutants (−1.2- to −1.9-fold). Thus, we can only suggest a role in increasing the MICs mentioned for the AcrEF efflux system in these two particular mutants, one of which is also an AcrAB overproducer (60-mut2).

Expression of the MDR regulators: the key role of ramA

In addition to the analysis of these structural genes, we also studied the levels of expression of the AcrAB regulators: acrR, ramA, soxS and marA (Figure 1 and Table 2). We could not find a clear interpretation for acrR expression. In contrast, ramA was overexpressed in all the mutants, thereby suggesting this regulator as the cause of the increased MICs for both the mutants over-expressing acrB and those over-expressing an unknown efflux system. Similar results have also highlighted the greater importance and prevalence of increased RamA over that of the other regulators.\(^{16,30}\) Maximal ramA expression levels were seen for 59-mut2 and 60-mut2 (66- and 74.2-fold, respectively) above the levels detected for the remaining mutants (13.4- to 19.6-fold). In line with these results, these two strains also showed higher MICs and acrB (only reported for 60-wt derivatives) and tolC expression values in comparison with their closely related mutants 59-mut1 and 60-mut1, respectively. In addition, as mentioned above, 59-mut2 and 60-mut2 were also reported to clearly overexpress

\(\text{Table 4. Mutations acquired in the quinolone target genes and the ramRA regulatory region; comparison of ramA transcriptional levels and regulatory mutations with previously reported mutants}\)

| Strain    | GyRA | GyRB | ParC | ParE | ramR/RamR \(^d\) | ramA promoter \(^a\) | Salmonella serovar | Reference     |
|-----------|------|------|------|------|----------------|-------------------|-------------------|--------------|
| 59-wt     | —    | —    | —    | —    | —             | —                | —                 |              |
| 59-mut1   | —    | —    | —    | —    | Del C\(_{514}\)–G\(_{557}\) | —                | Typhimurium       | this study    |
| 59-mut2   | —    | —    | —    | —    | —             | —                | Typhimurium       | this study    |
| 59-mut3   | S83Y | —    | —    | —    | Del A\(_{346}\)–G\(_{352}\) | —                | Typhimurium       | this study    |
| 59-64     | S83Y | D87G | E466D| S80R | —             | —                | Typhimurium       | this study    |
| 60-wt     | —    | —    | —    | —    | —             | —                | Typhimurium       | this study    |
| 60-mut1   | —    | —    | —    | —    | Q19P           | —                | Typhimurium       | this study    |
| 60-mut2   | —    | —    | —    | —    | Del A\(_{174}\)–C\(_{159}\) | —                | Typhimurium       | this study    |

| Previously reported mutant | GyRA | GyRB | ParC | ParE | ramR/RamR \(^d\) | ramA promoter \(^a\) | Salmonella serovar | Reference     |
|----------------------------|------|------|------|------|----------------|-------------------|-------------------|--------------|
| LTL                        | S83F | —    | —    | —    | —             | —                | Typhimurium       | 36            |
| BN10055                    | S83Y | —    | —    | —    | —             | —                | Typhimurium       | 16,37         |
| 5408-Cip                   | D87Y | —    | —    | —    | V461G         | —                | Enteritis         | 11            |
| 05-8560                    | S83F | D87N | —    | —    | G25A           | —                | Kentucky          | 38            |
| 02-8141                    | S83F | —    | —    | —    | Del G\(_{332}\)–G\(_{333}\) | —                | Kentucky          | 38            |
| 5 mutant 3                 | D87Y | —    | —    | —    | Dup (4 nt) C\(_{508}\) | —                | Paratyphi B       | 30            |
| 10 mutant 2                | D87Y | —    | —    | —    | —             | —                | Paratyphi B       | 30            |
| 3 mutant 2                 | S83Y | —    | —    | —    | R46P           | —                | Infantis          | 30            |
| 1 mutant 2                 | S83Y | —    | —    | —    | —             | —                | Infantis          | 30            |

\(^{a}\)Del, deletion; Ins, insertion; Dup, duplication.
\(^{b}\)Mutations leading to the maximum ramA expression values are represented in bold.
\(^{c}\)Maximum ramA expression values are represented in bold.
\(^{d}\)Mutations are indicated by either the nucleotide positions deleted relative to the translation start site or the amino acid substitution.
\(^{e}\)Numbers indicate the upstream positions relative to the translation start site.
\(^{f}\)This is a 2 nt deletion although only 1 nt affects a RamR-binding site.
ramA expression (>60-fold in the present study) and tolC and acrF overexpression agrees with a previously reported study.31

The soxS expression values detected in the present study were <2-fold higher in most of the mutants versus the expression levels seen in the two clinical isolates (Figure 1). Only two mutants, strains 59-64 and 60-mut2, showed overexpression of >4-fold. However, it was not possible to consistently associate this trait with higher expression values of ramA or acrF in both mutants. In contrast, these two strains did show the minimum levels of ompF expression (2.3.3- and 2.2-fold, respectively). Similarly, marA transcription also showed ≤2-fold increased expression in three mutant strains: 59-mut1, 59-mut3 and 60-mut1. In contrast, the highest levels were seen in 59-mut2 (4.3-fold), 59-64 (3.7-fold) and 60-mut2 (3.6-fold).

To understand our results, it is worth mentioning that RamA-binding sites have already been reported in Salmonella concerning the acrAB and tolC promoters.32 The 20 bp sequences recognized by this regulator resemble those initially reported to be present in all members of the marA/soxS/rob regulon in E. coli.13 It has been described that most of the residues of the two helix-turn-helix motifs (important for DNA sequence recognition) of MarA from E. coli are conserved in RamA from S. enterica serovar Paratyphi B.8,34 Moreover, it has previously been reported that the marRAB promoter contains its own marbox sequence.13 In agreement with this, RamA from Salmonella Paratyphi B has been shown to bind the MarA operator of E. coli.34 Thus, the binding sites characterized for MarA and SoxS in E. coli, equally termed marbox or soxbox, are similar to the already-mentioned rambox in Salmonella.31,32 Therefore, increased levels of RamA (>60-fold) and/or SoxS (>4-fold) could bind to the rambox/marbox located in the marRAB promoter and activate marA transcription, hence explaining the increased levels of marA expression observed for strains 59-mut2 [RamA overproducer (>60-fold)], 59-64 [SoxS overproducer (>4-fold)] and 60-mut2 [RamA overproducer (>60-fold) and SoxS overproducer (>4-fold)]. Nonetheless, lower ramA overexpression values (13- to 20-fold) would not have the same effect, thereby reinforcing the idea of an activator concentration-dependent response.31,35

Unravelling the mutations leading to the phenotype of decreased susceptibility to multiple antibiotics

In order to determine the mutations underlying the resistance phenotypes, sequencing and detection of mutations was performed in all the strains for all known regulators of MDR (acrRA, ramRA, soxRS, marRAB and acrSE). The results revealed the acquisition of mutations in the ramRA loci for all the mutants (Table 4). Mutations were located within the ramR coding sequence, either leading to a single amino acid substitution (Gln-19 → Pro, strain 60-mut1) or even deletions of 44 and 6 nt (strains 59-mut1 and 59-mut3, respectively). Surprisingly, the two strains (59-mut2 and 60-mut2) with the highest ramA overexpression values harboured a similar genotype: 6 nt deletion and 16 nt deletion, respectively, in the ramA promoter. Lastly, and as previously reported,23 strain 59-64 showed a single nucleotide change also located in the ramA promoter.

Previous reports have revealed that mutations or gene interruptions can be either acquired within ramR or in the ramA promoter.11,16,30 However, no association has ever been made between the type of mutation and transcription levels of ramA. The results observed in the present study point out that severe...
nucleotide deletions located in the \textit{ramA} promoter have a higher impact on increasing the expression of this regulator, whereas mutations within \textit{ramR} or single nucleotide changes in the \textit{ramA} promoter have a lesser effect. We performed an exhaustive analysis of the literature looking for studies that determined both \textit{ramA} transcription levels and \textit{ramRA} mutations in strains with resistance or decreased susceptibility to fluoroquinolones. Studies conducted in serovars Typhimurium,\textsuperscript{16,17} Enteritidis,\textsuperscript{51} Kentucky\textsuperscript{18} and other serovars\textsuperscript{30} were found to report similar results (Table 4). In order to understand this situation, it is necessary to note that RamR has been reported to bind as a homodimer to two RamR-binding sites located in the \textit{ramA} promoter (Figure 2).\textsuperscript{37} Thus, taking into account all this information, we hypothesize that important deletions occurring in these binding sites seriously impair the RamR repressive activity by preventing RamR binding and lead to high levels of \textit{ramA} expression (>60-fold). In contrast, mutations or deletions occurring in RamR or single nucleotide modifications affecting one binding site do not seem to abolish repression to the same extent and lead to moderate levels of \textit{ramA} transcription (less than ~40-fold). This latter situation would be supported by the capacity of the mutated form of RamR to partially preserve its repressive activity or by the existence of other regulators capable of binding to the \textit{ramA} promoter even in the absence of a functional RamR protein. Nonetheless, to our knowledge, two exceptions have been reported: one Salmonella Kentucky strain\textsuperscript{18} and one Salmonella Paratyphi B mutant (Table 4).\textsuperscript{30} The former situation might be explained by a large deletion detected at the very beginning of the repressor (affecting the protein sequence from the amino acid at position 14), whereas no clear explanation could justify the latter situation. Therefore, in order to elucidate the role of these mutations and strengthen or not our hypothesis, a larger number of strains need to be analysed in further studies.

In no strain did we find any mutation in any of the other regulatory sequences analysed in the present study. Consequently, we are unable to explain the increased \textit{soxS} transcription reported in 59-64 and 60-mut2. Concerning \textit{acrF} overexpression, previous results have associated it with mutations within the \textit{acrS} gene or in the \textit{acrEF} promoter.\textsuperscript{14} However, in the present study, no mutation in the \textit{acrSE} regulatory region could explain our findings. Instead, and as previously mentioned and reinforced by our results, overexpression of this efflux component is related to the levels of \textit{ramA} transcription.\textsuperscript{51} High levels of \textit{ramA} expression trigger \textit{acrF} overexpression, whereas intermediate levels do not. In line with these results, a previous study has also associated nucleotide deletions in the \textit{ramA} promoter with \textit{acrEF} overexpression.\textsuperscript{19} In view of these findings, the regulatory network that controls the expression of genes involved in the phenotype of decreased susceptibility to multiple antibiotics or MDR still needs further research to completely understand the bacterial response for survival under antimicrobial exposure. Nonetheless, we must keep in mind that our observations have arisen from mutants selected in a stepwise process, which may harbour additional mutations with unknown influence. Additional experiments are required in order to validate these results.

Conclusions

The results of our study indicate that RamA overexpression leads to the phenotype of decreased susceptibility to multiple antibiotics by using two different efflux-related strategies: overexpression of \textit{AcrAB} and overexpression of a hitherto uncharacterized efflux pump. Moreover, we provide further evidence of the prevalence of \textit{ramRA} mutations versus other \textit{acrB} regulators in the acquisition of MDR. However, heterogeneity was observed in the types of mutations acquired, which may be associated with different levels of \textit{ramA} transcription. Large deletions affecting the RamR-binding sites in the \textit{ramA} promoter were observed in strains with higher \textit{ramA} transcription levels, a trait that may account for the highest expression levels of \textit{acrB}, \textit{tolC}, \textit{marA} and \textit{acrF}, hence related to a major contribution to the phenotype of decreased susceptibility to multiple antibiotics.

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Transparency declarations

None to declare.
References

1. Fábregas A, Vila J. Salmonella enterica serovar Typhimurium skills to succeed in the host: virulence and regulation. Clin Microbiol Rev 2013; 26: 308–41.

2. Liang Z, Ke B, Deng X et al. Serotypes, seasonal trends, and antibiotic resistance of non-typhoidal Salmonella from human patients in Guangdong Province, China, 2009–2012. BMC Infect Dis 2015; 15: 53.

3. Fábregas A, Madurga S, Giralt E et al. Mechanism of action of and resistance to quinolones. Microbiol Biotechnol 2009; 2: 40–61.

4. Heisig P. Genetic evidence for a role of parC mutations in development of high-level fluoroquinolone resistance in Escherichia coli. Antimicrob Agents Chemother 1996; 40: 879–85.

5. Vila J, Ruiz J, Goni P et al. Detection of mutations in parC in quinolone-resistant clinical isolates of Escherichia coli. Antimicrob Agents Chemother 1996; 40: 491–3.

6. Cohen SP, McMurry LM, Hooper DC et al. Cross-resistance to fluoroquinolones in multiple-antibiotic-resistant (Mar) Escherichia coli selected by tetracycline or chloramphenicol: decreased drug accumulation associated with membrane changes in addition to OmpF reduction. Antimicrob Agents Chemother 1989; 33: 1318–25.

7. Nishino K, Yamauchi A. Analysis of a complete library of putative drug transporter genes in Escherichia coli. J Bacteriol 2001; 183: 5803–12.

8. Chen S, Cui S, McDermott PF et al. Contribution of target gene mutations and efflux to decreased susceptibility of Salmonella enterica serovar Typhimurium to fluoroquinolones and other antimicrobials. Antimicrob Agents Chemother 2007; 51: 535–42.

9. Olliver A, Vallee M, Chaslus-Danchel E et al. Overexpression of the multi-drug efflux operon acrEF by insertional activation with IS1 or IS10 elements in Salmonella enterica serovar Typhimurium DT204 acrB mutants selected with fluoroquinolones. Antimicrob Agents Chemother 2005; 49: 289–301.

10. Giraud E, Cloeckaert A, Kerboeuf D et al. Evidence for active efflux as the primary mechanism of resistance to ciprofloxacin in Salmonella enterica serovar Typhimurium. Antimicrob Agents Chemother 2000; 44: 1223–8.

11. O'Regan E, Quinn T, Pages JM et al. 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 2009; 53: 1080–7.

12. Ballesté-Delpierre C, Fábregas A, Ferrer-Navarro M et al. Attenuation of in vitro host–pathogen interactions in quinolone-resistant Salmonella Typhi mutants. J Antimicrob Chemother 2016; 71: 111–22.

13. Piddock LJV, Griigs DJ, Hall MC et al. Ciprofloxacin resistance in clinical isolates of Salmonella typhimurium obtained from two patients. Antimicrob Agents Chemother 1993; 37: 662–6.

14. Olliver A, Vallee M, Chaslus-Danchel E et al. Role of an acrR mutation in multidrug resistance of in vitro-selected fluoroquinolone-resistant mutants of Salmonella enterica serovar Typhimurium. FEMS Microbiol Lett 2004; 238: 267–72.

15. Koutsosiloutou A, Martins EA, White DG et al. A soxRS-constitutive mutation contributing to antibiotic resistance in a clinical isolate of Salmonella enterica (serovar Typhimurium). Antimicrob Agents Chemother 2001; 45: 38–43.

16. Abouzeed YM, Baucheron S, Cloeckaert A. ramRA mutations involved in efflux-mediated multidrug resistance in Salmonella enterica serovar Typhimurium. Antimicrob Agents Chemother 2008; 52: 2428–34.

17. Sulavik MC, Dazer M, Miller PF. The Salmonella typhimurium mar locus: molecular and genetic analyses and assessment of its role in virulence. J Bacteriol 1997; 179: 1857–66.

18. Giraud E, Baucheron S, Virlogeux-Payant I et al. Effects of natural mutations in the ramRA locus on invasiveness of epidemic fluoroquinolone-resistant Salmonella enterica serovar Typhimurium isolates. J Infect Dis 2013; 207: 794–802.

19. Ricci V, Tzakas P, Buckley A et al. Ciprofloxacin-resistant Salmonella enterica serovar Typhimurium strains are difficult to select in the absence of AcrB and TolC. Antimicrob Agents Chemother 2006; 50: 38–42.

20. Tibbetts RJ, Lin TL, Wu CC. Phenotypic evidence for inducible multidrug resistance in Salmonella choleraesuis. FEMS Microbiol Lett 2006; 218: 333–8.

21. Gethinger M, Podgljen I, Kern WV et al. Overexpression of the marK or soxS regulatory gene in clinical topoisomerase mutants of Escherichia coli. Antimicrob Agents Chemother 1998; 42: 2089–94.

22. Alekshun MN, Levy SB. Regulation of chromosomally mediated multiple antibiotic resistance: the mar regulon. Antimicrob Agents Chemother 1997; 41: 2067–75.

23. Fábregas A, Soto SM, Ballesté-Delpierre C et al. Impact of quinolone-resistance acquisition on biofilm production and fitness in Salmonella enterica. J Antimicrob Chemother 2014; 69: 1815–24.

24. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-third Informational Supplement M100-S23. CLSI, Wayne, PA, USA, 2013.

25. Fábregas A, du Merle L, Le Bouguenec C et al. Repression of invasion genes and decreased invasion in a high-level fluoroquinolone-resistant Salmonella Typhimurium mutant. PLoS One 2009; 4: e8029.

26. Ballesté-Delpierre C, Sole M, Domenech O et al. Molecular study of quinolone resistance mechanisms and clonal relationship of Salmonella enterica clinical isolates. Int J Antimicrob Agents 2014; 43: 121–5.

27. Livkj KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 2001; 25: 402–8.

28. Magiorakos AP, Srinivasan A, Carey RB et al. Multidrug-resistant, extensively drug-resistant, and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 2012; 18: 268–81.

29. Parry CM, Threlfall EJ. Antimicrobial resistance in typhoidal and nontyphoidal salmonellae. Curr Opin Infect Dis 2008; 21: 531–8.

30. Kehrenberg C, Cloeckaert A, Klein G et al. Decreased fluoroquinolone susceptibility in mutants of Salmonella serovars other than Typhimurium: detection of novel mutations involved in modulated expression of ramA and soxS. J Antimicrob Chemother 2009; 64: 1175–80.

31. Bailey AM, Ivens A, Kingsley R et al. RamA, a member of the AraC/XylS family, influences both virulence and efflux in Salmonella enterica serovar Typhimurium. J Bacteriol 2010; 192: 1607–16.

32. Nikaido E, Yamauchi A, Nishino K. AcrAB multidrug efflux pump regulation in Salmonella enterica serovar Typhimurium by RamA in response to environmental signals. J Biol Chem 2008; 283: 24245–53.

33. Martin RG, Rosner JL. Genomics of the marK/soxS/rob regulon of Escherichia coli: identification of directly activated promoters by application of molecular genetics and informatics to microarray data. Mol Microbiol 2002; 44: 1611–24.

34. Yassini MA, Ewis HE, Lu CD et al. Molecular cloning and characterization of the Salmonella enterica serovar Paratyphi B rmp gene, which confers multiple drug resistance in Escherichia coli. Antimicrob Agents Chemother 2002; 46: 360–6.

35. Martin RG, Bartlett ES, Rosner JL et al. Activation of the Escherichia coli marK/soxS/rob regulon in response to transcriptional activator concentration. J Mol Biol 2008; 380: 278–84.

36. Zheng J, Cui S, Meng J. Effect of transcriptional activators RamA and SoxS on expression of multidrug efflux pumps AcrAB and AcrEF in Salmonella enterica serovar Typhimurium DT204.
fluoroquinolone-resistant Salmonella Typhimurium. J Antimicrob Chemother 2009; 63: 95–102.

37 Baucheron S, Coste F, Canepa S et al. Binding of the RamR repressor to wild-type and mutated promoters of the RamA gene involved in efflux-mediated multidrug resistance in Salmonella enterica serovar Typhimurium. Antimicrob Agents Chemother 2012; 56: 942–8.

38 Baucheron S, Le HS, Doublet B et al. ramR mutations affecting fluoroquinolone susceptibility in epidemic multidrug-resistant Salmonella enterica serovar Kentucky ST198. Front Microbiol 2013; 4: 213.

39 Spengler G, Rodrigues L, Martins A et al. Genetic response of Salmonella enterica serotype Enteritidis to thioridazine rendering the organism resistant to the agent. Int J Antimicrob Agents 2012; 39: 16–21.