Decreased fluoroquinolone susceptibility in mutants of Salmonella serovars other than Typhimurium: detection of novel mutations involved in modulated expression of ramA and soxS

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Objectives: Mutants of five Salmonella enterica serovars were investigated for structural changes in regulatory regions known to be involved in the up-regulation of efflux pumps.

Methods: Five Salmonella field isolates and mutants, in which efflux pump inhibitor tests previously pointed towards an up-regulation of efflux, plus one negative control were included in the study. MIC values were determined of antibiotics that were indicative of AcrAB overexpression. The regulatory regions acrRA, soxRS, marORAB, acrSE and ramRA of original strains and mutants were sequenced and compared. The gene expression of acrA, tolC, ramA and soxS was assessed by quantitative real-time PCR. Conjugation experiments and tet gene PCRs were performed to explain unexpected variations in MIC values of tetracycline.

Results: In four mutant strains, changes in the ramRA regulatory region, causing up-regulation of ramA, were detected. These changes comprised point mutations and deletions of 10 or 15 bp within the ramR gene and a single bp exchange located in the binding site of the RamR protein in Salmonella Infantis, Paratyphi and Livingstone mutants. An insertion of 49 bp within the soxR gene was involved in soxS up-regulation and enhanced efflux activity in the fifth mutant from Salmonella Virchow. The loss of tetracycline resistance in one Salmonella Paratyphi mutant could be explained by the loss of a plasmid carrying a tet(A) gene.

Conclusions: Changes in the ramR-ramA region as well as in the soxR gene occur in mutants of Salmonella serovars other than Typhimurium and seem to be involved in the up-regulation of efflux activity.

Keywords: Salmonella enterica, efflux pumps, resistance

Introduction

Resistance to fluoroquinolones is believed to arise from an interplay of different resistance mechanisms, including mutations in topoisomerase genes, increased efflux via transporters, target site protection via Qnr proteins as well as enzymatic inactivation by Aac(6')-Ib-cr.1–3 Although initial studies focused on the target gene mutations as the major mechanism of resistance, it became obvious during the past decade that active efflux plays a highly relevant role in (fluoro)quinolone resistance. In Salmonella, decreased intracellular (fluoro)quinolone accumulation via active efflux has been studied most extensively in isolates of the serovar Typhimurium and was mainly attributed to the overexpression of the AcrAB-TolC multidrug efflux pump.4 Overproduction of this pump was considered to be responsible for a multiple antibiotic resistance phenotype, which also includes resistance to tetracyclines and phenicols.5,6 Composite efflux pumps of the resistance–nodulation–division (RND) family, such as AcrAB-TolC, are under precise transcriptional control and expression of the pumps is subject to...
multiple levels of regulation. Expression of AcrAB is known to be modulated by stress conditions as well as by global regulators such as MarA, SoxS or RamA. Furthermore, a local repressor, AcrR, has been identified to play a role in the regulation of acrAB genes. In Salmonella, a mutation in the acrR gene was reported to contribute to acrAB overexpression, as shown by quantitative real-time PCR (qRT–PCR). Recently, RamR, the local repressor of the gene ramA, was identified. Point mutations in ramR, which resulted in either amino acid exchanges or frame shifts, a deletion in the ramA promoter and also the inactivation of ramR by the insertion of an IS6 element have been proved to be involved in the up-regulation of efflux-mediated multidrug resistance in Salmonella Typhimurium. Although mutations in the global regulators have recently been investigated in Salmonella Enteritidis, very little is known about the role of ramR mutations or mutations in other regulatory genes, such as acrR, soxR, marR and acrS, that may influence AcrAB expression in Salmonella serovars other than Typhimurium.

In a previous study, we analysed nalidixic acid-resistant and -susceptible isolates of different Salmonella serovars for their mutant prevention concentrations of ciprofloxacin. Besides mutations in the target genes gyrA or gyrB, the results of studies with the efflux pump inhibitor Phe-Arg-b-naphthylamide pointed towards the involvement of up-regulated efflux in the observed higher (fluoro)quinolone MICs for the mutants. However, the molecular basis of this increased efflux was unknown. Therefore, it was the aim of this study to investigate the occurrence of structural changes in the aforementioned regulatory genes in selected mutants of Salmonella serovars Enteritidis, Infantis, Livingstone, Paratyphi B and Virchow.

Materials and methods

The origin of the three nalidixic acid-resistant and the two nalidixic acid-susceptible Salmonella field isolates, and the generation of the respective mutants during a study to determine the mutant prevention concentrations have been described previously. In brief, mutants were generated after 24 h of incubation on agar plates supplemented with 1–128× the MIC of ciprofloxacin for the original isolate. One mutant of each field isolate was chosen for further investigations. MIC values of nalidixic acid, ciprofloxacin, tetracycline, florenicol and chloramphenicol for the field isolates and the corresponding mutants were determined by broth microdilution. These antimicrobial agents were chosen because they belong to the substrate spectrum of AcrAB and their increased MICs pointed towards enhanced efflux via AcrAB. For MIC determination, Escherichia coli ATCC 25922 served as a reference strain for quality control purposes. All susceptibility tests were performed and evaluated according to the CLSI guidelines M7-A7 and M100-S17.

The preparation of whole-cell DNA followed a previously described protocol. Plasmid DNA from Salmonella enterica serovar Paratyphi B isolate 5 and the respective mutant 3 was extracted and purified following a modification of the alkaline lysis procedure. To confirm the origin of the mutant, macrorestriction analysis was conducted as described previously. To investigate the localization of a tetracycline resistance gene in isolate Paratyphi B 5, conjugation experiments were performed. For this, the rifampicin-resistant E. coli recipient strain HK225 was used and transconjugants were selected on agar plates containing 20 mg/L tetracycline and 100 mg/L rifampicin. The tet gene of isolate Paratyphi B 5 was identified by PCR assays as described previously.

To detect changes in the regions known to be involved in the regulation of the efflux pump AcrAB, we amplified the target regions acrR, soxRS, marORAB, acrSE and ramRA using primers and PCR conditions described previously. Subsequent sequencing of the PCR products was performed on both strands by primer walking (MWG-Biotech, Martinsried, Germany). The nucleotide and amino acid sequences of the field isolates and their ciprofloxacin-resistant mutants were compared using the DNAman software (Lynnon Biosoft, Quebec, Canada).

To assess the gene expression of ramA, acrA, tolC and soxS, qRT–PCR was performed. For that, overnight cultures were diluted 1:100 in Luria-Bertani (LB) broth and grown to mid-logarithmic phase. Total RNA was extracted by using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and contaminating genomic DNA was eliminated by DNase digestion (RNase-Free DNase Set, Qiagen). RNA yield and quality were measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany) and subsequently, cDNA was synthesized from 0.5 μg RNA templates using a QuantiTect Reverse Transcription Kit according to the manufacturer’s instructions (Qiagen). The absence of contaminating DNA was confirmed by including reverse-transcription-minus controls. qRT–PCR was carried out in a LightCycler 2.0 instrument (Roche, Mannheim, Germany) using a FastStart DNA MasterPLUS SYBR Green I Kit (Roche). Primers used for the qRT–PCRs were: ramA f, 5′-ttctcgcaggttagcagc-3′ and r, 5′-cgggcaatatcatcaatcg-3′ (82 bp); acrA f, 5′-tggtgagacagtatggcttcg-3′ and r, 5′-gaagcaggagctggcaaatg-3′ (252 bp, this study); tolC f, 5′-tgccgcaactgggtttagg-3′ and r, 5′-cagagaaggtgccttacttg-3′ (92 bp, this study); and soxS f, 5′-tatctgcataatgattg-3′ and r, 5′-aatctgcagctcaacag-3′ (71 bp). As a reference gene, gyrB was amplified with the primers gyrb f, 5′-tcctcagcaacaagataag-3′ and r, 5′-cagcatagctgtttcag-3′ (81 bp). PCR conditions were 10 s at 95°C, followed by 55 cycles of 95°C for 10 s, 54°C for 8 s and 72°C for 12 s. Salmonella Enteritidis 9 and its mutant 1, which did not exhibit changes in the MICs of phenicols and tetracycline (Table 1), were included in the qRT–PCR analysis as negative controls. The qRT–PCR experiments were performed three times independently, and the relative expression software tool (REST) was used for the calculation and analysis of qRT–PCR results.

Results and discussion

Antimicrobial susceptibility of field isolates and corresponding mutants

Previous investigations of the quinolone resistance determining regions (QRDRs) of these mutants have shown that only mutants from formerly nalidixic acid-susceptible isolates developed single mutations in the topoisomerase gene gyrA (Table 1), while mutants from formerly nalidixic acid-resistant isolates exhibited only the alteration in gyrB that was already present in the original isolate. The results of studies with efflux pump inhibitors suggested that enhanced efflux activity might play a role in the elevated MICs of ciprofloxacin for mutants derived from these latter isolates. To further investigate the role of enhanced efflux activity, the MIC values of tetracycline, florenicol and chloramphenicol were determined. These antimicrobial agents were reported to be substrates of the AcrAB efflux pump. A 2- to 16-fold increase in the MICs of these antimicrobial agents was detected in five mutants (Table 1). The observed changes in the MIC values between the original strain and its respective mutant were seen regardless of nalidixic acid resistance or susceptibility of the original strain, and this observation...
Table 1. Comparison of MIC values, mutations and changes in gene expression between *Salmonella* original isolates and their mutants

| Strains                        | MIC (mg/L) | x-Fold change in gene expression (based on group means) |
|-------------------------------|------------|--------------------------------------------------------|
|                               | NAL | CIP | TET | FLO | CHL | Target gene mutation | Regulatory gene mutation | ramA  | soxS  | acrA  | tolC   |
| *Salmonella* Virchow 2        | ≥128 | 0.25 | 64  | 4   | 256 | *gyrA*–S83F           | —                             | 1.1 ± 0.1 | 323.1 ± 14.2 | 8.7 ± 0.9 | 1.4 ± 0.1 |
| *Salmonella* Virchow 2 mutant 1 | ≥128 | 2   | 128 | 16  | 1024 | *gyrA*–S83F           | 49 bp insertion in *soxR*     |                     |                    |                    |        |
| *Salmonella* Paratyphi B 5    | ≥128 | 0.125 | 128 | 4   | 2   | *gyrA*–D87Y           | —                             | 10.0 ± 0.7 | 0.9 ± 0.2    | 10.1 ± 0.9 | 1.6 ± 0.1 |
| *Salmonella* Paratyphi B 5 mutant 3 | ≥128 | 1   | 2   | 16  | 16  | *gyrA*–D87Y           | 1 bp exchange in the RamR binding site |                     |                    |                    |        |
| *Salmonella* Paratyphi B 10   | ≥128 | 4   | 0.015 | 1  | 4   | 4   | —                             |                     | 94.8 ± 3.3 | 1.7 ± 0.4    | 7.2 ± 1.0 | 8.0 ± 0.4 |
| *Salmonella* Paratyphi B 10 mutant 2 | ≥128 | 4   | 0.015 | 1  | 2   | 8   | 8   | *gyrA*–D87Y           | 15 bp deletion in *ramR* + E160D exchange in RamR |                     |                    |                    |        |
| *Salmonella* Livingstone 3    | ≥128 | 0.5  | 256 | 16  | 64  | *gyrA*–S83Y           | R46P exchange in RamR        | 34.3 ± 5.2  | 1.2 ± 0.3     | 4.5 ± 0.1 | 2.2 ± 0.2 |
| *Salmonella* Livingstone 3 mutant 2 | ≥128 | 0.5  | 256 | 16  | 64  | *gyrA*–S83Y           | —                             |                     |                    |                    |        |
| *Salmonella* Infantis 1       | ≥128 | 0.25 | 128 | 4   | 4   | *gyrA*–S83Y           | —                             | 41.6 ± 7.4  | 1.5 ± 0.9     | 7.4 ± 2.0 | 2.0 ± 0.3 |
| *Salmonella* Infantis 1 mutant 2 | ≥128 | 1   | 256 | 32  | 16  | *gyrA*–S83Y           | 10 bp deletion in *ramR*      |                     |                    |                    |        |
| *Salmonella* Enteritidis 9    | ≥128 | 8   | 0.015 | 4  | 4   | 4   | —                             |                     | 0.9 ± 0.1  | 1.3 ± 0.2     | 0.7 ± 0.3 | 1.3 ± 0.1 |
| *Salmonella* Enteritidis 9 mutant 1 | ≥128 | 0.125 | 1  | 4   | 4   | *gyrA*–D87Y           | —                             |                     |                    |                    |        |

NAL, nalidixic acid; CIP, ciprofloxacin; TET, tetracycline; FLO, florfenicol; CHL, chloramphenicol.
suggested that enhanced efflux may occur independently of alterations in *gyrA* already present in the original isolates. Only for the mutant strain derived from isolate *Salmonella Paratyphi* B 5 was a striking decrease in the tetracycline MIC value detected (Table 1), and was the subject of further investigations.

**Tetracycline resistance gene in *Salmonella Paratyphi* B 5**

Since the original isolate *Salmonella Paratyphi* B 5 was tetracycline-resistant in contrast to its mutant 3 (Table 1), macro-restriction analysis was performed and confirmed the origin of the mutant strain. Plasmid profiling identified three plasmids of ~2.1, 5.8 and >54.0 kb in the original isolate, of which the largest plasmid was lost in the mutant strain. Conjugation experiments in combination with PCR assays confirmed that a tetracycline resistance gene of hybridization class A, *tet*(A), was located on the >54.0 kb plasmid.

**Characterization of mutations in *ramR-ramA* and other efflux pump regulatory regions, and expression analysis of *ramA*, *soxS*, *acrA* and *tolC* in *Salmonella* mutants**

Nucleotide sequence comparisons of the *acrRA*, *soxRS*, *marORAB*, *acrSE* and *ramRA* regions of original strains and

![Figure 1](https://academic.oup.com/jac/article-abstract/64/6/1175/741369)

**Figure 1.** Schematic presentation of the *ramR-ramA* (a) and *soxR-soxS* (b) region of *Salmonella Typhimurium* LT2 (GenBank accession no. NC_003197). The mutations identified in the respective region of *Salmonella* mutants selected during the determination of mutant prevention concentrations are indicated. The nucleotide sequence comprising the putative RamR binding site is shown in detail. Inverted repeated sequences located in that region according to Abouzeed *et al.* are indicated by arrows.
Non-target gene mutations in Salmonella mutants

their mutants detected changes in the ramRA and soxRS regions of five mutants.

Five sequence alterations were located in the ramA upstream region, four of which were detected in ramA and one in the non-coding region between ramA and its repressor gene (Figure 1a). In the mutant of Salmonella Livingston 3, a single bp exchange, leading to the amino acid exchange R46P in RamR, was present. Other changes in ramR comprised deletions of 10 or 15 nucleotides in the Salmonella Infantis 1 and the Salmonella Paratyphi B 10 mutant strains (Figure 1a). These deletions generated either an early stop codon (Salmonella Infantis 1 mutant 2) or caused the loss of five amino acids (Salmonella Paratyphi B 10 mutant 2), respectively. The aforementioned structural alterations differed from those reported in Salmonella Typhimurium or Enteritidis13–15 and suggested that sequence alterations may occur at various positions in the ramR gene. In Salmonella Paratyphi B 10 mutant 2, an additional alteration in the ramR gene was detected. This alteration comprised a single bp exchange, GAA→GAC, which resulted in the amino acid exchange E160D. A stop codon at position 160 has already been described in ramR in Salmonella Typhimurium.13 Both mutations detected in Salmonella Livingston 3 mutant 2 and Salmonella Infantis 1 mutant 2 increased the ramA expression 34.3- and 41.6-fold in comparison with the original strains (Table 1), whereas the two changes occurring in Salmonella Paratyphi B 10 mutant 2 led to a higher increase (94.8-fold) in the expression of the ramA gene. Minor increases in acrA and tolC expression were also observed in all three mutant strains and ranged from 4.5- to 7.4-fold (for acrA) and from 2.0- to 8.0-fold (for tolC).

Another point mutation, C→A, was located between the ramA gene and its repressor gene in mutant 3 of Salmonella Paratyphi B 5 (Figure 1a). This exchange was located exactly in one of the inverted repeated sequences formerly described as the RamR binding site13 and, thus, might result in impaired binding of RamR. This was confirmed by the relative expression analysis of ramA, whereby an increase of 10.0-fold (Table 1) could be detected. Although this up-regulation of ramA was considerably lower than the up-regulation due to mutations in ramR, decreases in antimicrobial susceptibility suggested that this point mutation is also able to enhance AcrAB-TolC efflux activity. A similar observation was made by Zheng et al.,14 who detected a 9 bp deletion in the ramA promoter region that led to activation of RamA in a single Salmonella Typhimurium strain.

Four-fold increases in the MICs of chloramphenicol and florfenicol suggested an overexpression of the AcrAB efflux pump in the Salmonella Virchow 2 mutant strain 1. In that mutant, an insertion of 49 bp was located after nucleotide position 149 within the soxR gene (Figure 1b). This insertion generated an early stop after codon 57. Analysis of the inserted region revealed that 33 of the 49 bp represented a duplication of the upstream nucleotide sequence. Although changes in the soxRS locus have been reported only rarely in Salmonella,12 exchanges in soxR have already been described to contribute to quinolone resistance in a clinical Salmonella Typhimurium isolate as well as in single in vitro mutants of Salmonella Typhimurium and Salmonella Enteritidis.9,14,15 Nevertheless, none of the soxRS mutations described before resulted in a similarly high up-regulation of soxS (by 323.1-fold) as observed in the Salmonella Virchow 2 mutant strain 1 (Table 1).

In the remaining Salmonella Enteritidis mutant, which was included as a negative control, no changes in the regulatory regions could be detected. This result was in good accordance with the comparison of MIC values. The mutant exhibited MICs of tetracycline, chloramphenicol and florfenicol that were indistinguishable from the MICs of the original isolate (Table 1) and, hence, an up-regulated efflux via AcrAB was not expected. This suggestion was confirmed by the relative expression analysis of acrA, ramA, tolC and soxS, where only slight differences in expression ratios compared with the original strain could be detected (Table 1).

In summary, the results of this study show that structurally different alterations in the ramR-ramA region as well as in the soxR gene lead to an AcrAB up-regulation and that they also occur in Salmonella serovars other than Typhimurium.

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Transparency declarations

None to declare.

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