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Review article

Mechanisms of quinolone resistance in *Salmonella*

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Abstract – As in other Gram-negative bacteria, mechanisms of resistance to quinolones in *Salmonella* include target gene mutations, active efflux, and decreased outer membrane permeability. However, the exact contribution of these individual mechanisms to resistance, which may nevertheless interplay to reach high-level resistance, has not yet clearly been defined as in other bacteria such as *Escherichia coli*. This paper reviews the current state of knowledge of quinolone resistance mechanisms in *Salmonella* by comparison with that of *E. coli* and future directions of research with particular attention to the recent development of efflux pump inhibitors as possible means of avoiding the emergence and spread of fluoroquinolone resistance.

***Salmonella* / quinolone / resistance / mechanism**

Résumé – Mécanismes de résistance aux quinolones chez *Salmonella*. Comme chez les autres bactéries Gram-négatives les mécanismes de résistance aux quinolones chez *Salmonella* comprennent les mutations dans les gènes cibles, l'efflux actif, et une diminution de la perméabilité de la membrane externe. Cependant la contribution exacte de ces mécanismes individuels, qui peuvent néanmoins interagir pour atteindre des niveaux de résistance élevés, n'a pas encore été clairement définie comme chez d'autres bactéries telle que *Escherichia coli*. Cette revue présente l'état actuel des connaissances des mécanismes de résistance chez *Salmonella* par comparaison avec *E. coli* et des perspectives futures de recherche avec une attention particulière au développement récent d'inhibiteurs de pompes d'efflux comme moyen possible pour éviter l'émergence et la diffusion de la résistance aux fluoroquinolones.

***Salmonella* / quinolone / résistance / mécanisme**

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1. INTRODUCTION

The evolution of resistance to fluoroquinolones is a problem in both veterinary and human medicine. Over the last years, we have faced the emergence of strains of *Escherichia coli* highly resistant to fluoroquinolones in humans as well as in different types of food animal production. The situation in *Salmonella* seems to be different. In humans and animals, a similar emergence of strains showing high-level resistance to fluoroquinolones is not frequently encountered. To date, only one example has been recorded with the emergence and probable clonal diffusion of the *S. enterica* Typhimurium variant Copenhagen in limited areas of Europe. These strains, which are highly resistant to fluoroquinolones, were mainly isolated between 1991 and 1995 from animals and humans [23, 25, 27]. Since then, at least in France, only certain strains with a decreased susceptibility to fluoroquinolones have been isolated [17].

As in other bacteria, mechanisms of resistance to quinolones in *Salmonella* include target gene mutations, active efflux, and decreased outer membrane permeability. However, the exact contribution of these individual mechanisms to resistance, which may nevertheless interplay to reach high level resistance, has not yet clearly been defined as in other bacteria such as *E. coli*. The following paragraphs will deal with the current state of knowledge of resistance mechanisms of *Salmonella*, points of discussion and future directions of research by comparison with the better studied individual quinolone resistance mechanisms in other bacteria such as *E. coli*.

2. TARGET GENE MUTATIONS

In *Salmonella*, quinolone resistance was firstly attributed to point mutations in the *gyrA* gene coding for the A subunit of gyrase, whose complex with DNA is the primary target of quinolones. Resistance mutations of *gyrA* have been clustered in a region of the gene product between amino acids 67 and 106, termed the quinolone resistance-determining region (QRDR). Amino acid changes at Ser-83 (to Phe, Tyr, or Ala) or at Asp-87 (to Gly, Asn, or Tyr) are the most frequently observed in nalidixic acid-resistant strains [6, 17, 20, 21, 26, 54, 57, 63–65, 70, 72]. Double mutations at both residues 83 and 87 have been identified in clinical isolates of *S. enterica* serovar Typhimurium showing high-level resistance to fluoroquinolones (e.g., MIC of ciprofloxacin: 32 $\mu g \cdot mL^{-1}$) [23]. This concerned a particular clone named Copenhagen variant encountered in Germany during the early 1990s. The double mutation was identical in several isolates (Ser-83 to Ala and Asp-87 to Asn) thereby suggesting the clonality of the strains [25]. These isolates additionally had an altered *gyrB* gene coding for the B subunit of gyrase [23]. Complementation of these strains with plasmids carrying either the *gyrA* or the *gyrB* wild-type genes of *E. coli* K-12 resulted in enhanced susceptibilities and provided evidence of a role for both altered *gyrA* and *gyrB* genes in

high-level fluoroquinolone resistance [23]. Probably the same clone described as phage-type DT204 showing high-level enrofloxacin resistance occurred in Belgium from 1991 to 1995 [27]. Mutations in the target genes were, however, not investigated in these cases. This clone has apparently disappeared and only clinical strains with a single mutation in the *gyrA* gene have since then been described to confer nalidixic acid resistance but decreased susceptibility to fluoroquinolones according to the clinical breakpoint admitted (MIC > 2 $\mu g \cdot mL^{-1}$). A *gyrB* gene mutation has also been identified in a quinolone-resistant post-therapy isolate of *S. enterica* serovar Typhimurium and consisted in a point mutation in codon 463 of *gyrB* giving rise to an amino acid substitution of Ser to Tyr [16]. In this case, complementing with a wild-type *gyrB* gene also resulted in increased susceptibility of the isolate to nalidixic acid and fluoroquinolones.

Less frequently, amino acid substitutions at other GyrA positions than Ser-83 and Asp-87 have been described in quinolone-resistant *Salmonella* strains such as those at Ala-67 (to Pro), Gly-81 (to Ser), and Ala-119 (to Glu) [17, 21, 63].

Unlike in *E. coli* [12, 14, 19, 24, 28, 32, 44, 51] no mutations have been described in the secondary target for quinolones, namely the *parC* gene coding for the ParC subunit of topoisomerase IV.

The role in *Salmonella* quinolone resistance of mutations in the target genes, in particular in *gyrA* and *gyrB*, has been confirmed by complementation studies using wild-type genes on plasmids [16, 20, 23, 54, 56, 57]. It nevertheless remains to be determined what the exact impact of these mutations is on quinolone resistance, as has been done for *E. coli* constructed isogenic mutants where mutations in *gyrA* and *parC* were experimentally introduced in the absence of quinolone selection [4]. Indeed, nalidixic acid and fluoroquinolones may select for mutations at other chromosomal loci, in particular those resulting in a multiple antibiotic resistance (Mar) phenotype due to the overproduction of broad-spectrum efflux pumps and decreased outer membrane permeability which may interplay with target gene mutation-mediated quinolone resistance (see below) [1, 2, 60].

3. ACTIVE EFFLUX

Quinolone resistance due to active efflux has been well studied in *E. coli*. A major efflux system involved in multidrug-comprising quinolone-resistance described in *E. coli* is the AcrAB-TolC system which is a three-component structure that crosses both the inner and the outer membrane. AcrB is the inner membrane component which acts as a proton-motive-force-dependent transporter and belongs to the class of resistance-nodulation-division (RND) efflux pumps [49, 50, 55, 60, 61, 76]. Its proposed structure consists of 12 transmembrane segments (TMS) or α-helices with two large hydrophilic extracytoplasmic domains between TMS 1 and 2 and TMS 7 and 8. These large periplasmic domains may play a critical role in the drug efflux. The AcrB transporter uses a wide variety of substrates extruding not only various classes of clinically important antibiotics such as quinolones, β-lactams, tetracycline, chloramphenicol, erythromycin, and rifampin but also disinfectants, dyes, detergents, bile salts, and organic solvents [29, 40, 43, 48–50, 53, 55, 60, 61, 68, 69, 71].

AcrA is a periplasmic lipoprotein which has an elongated shape thought to span the periplasmic space thus coordinating the concerted operation of inner and outer membrane components of the three-component structure [73, 74]. AcrA, in addition, forms oligomers, most probably trimers interacting specifically with the AcrB transporter independently of substrate and TolC [75]. AcrA might bring TolC and AcrB together by folding back on itself allowing the drug

efflux across the two membranes directly into the medium [73–76].

The functional outer membrane protein TolC is required for the operation of the AcrAB efflux system and for the expression of the Mar phenotype [15]. The crystal structure of TolC has recently been elucidated revealing a distinctive and previously unknown fold [30]. Three TolC protomers assemble to form a continuous, solvent accessible conduit – a "channel-tunnel" over 140 Å long that spans both the outer membrane and periplasmic space. Trimerisation results in an α-helical barrel, which forms a tunnel through the periplasm, anchored by a contiguous outer membrane β-barrel. The periplasmic end of the tunnel is sealed by sets of coiled helices and it is thought that these could be untwisted by an allosteric mechanism, mediated by protein-protein interactions, to open the tunnel.

In *E. coli* the level of production of the AcrAB-TolC efflux system is controlled by several regulatory genes at different chromosomal loci. The most important systems studied are the global *marRAB* and *soxRS* regulatory systems. *marA* and *soxS* code for homologous proteins that are positive regulators of the AcrAB-TolC efflux system [1, 2, 5, 40, 41, 46, 48, 53, 71]. The *marR* and *soxR* genes code for repressor proteins that locally downregulate the expression of the *marA* and *soxS* genes, respectively. Thus alteration of these repressor genes may lead to constitutive expression of *marA* and *soxS* resulting in the overproduction of the AcrAB-TolC efflux pump and resulting Mar phenotype. Upon selection with quinolones, several mutations have been identified in the repressor genes and also in the *marO* operator upstream of the *marRAB* operon. These mutations consisted of point mutations and deletions [1, 3, 10, 28, 37, 42, 51]. Such non-target gene mutations may thus lead to increased efflux-mediated quinolone resistance. Interestingly, MarA and SoxS also downregulate the synthesis of the major porin OmpF, through the increased production of an antisense RNA, *micF*, which results in decreased outer membrane permeability to quinolones and other antibiotics [8, 9]. Besides overproduction of the AcrAB-TolC efflux pump, it has recently been shown using macroarrays that *E. coli* strains constitutively expressing *marA* showed altered expression of more than 60 chromosomal genes [5].

Closely linked to the *acrAB* operon, is the *acrR* gene coding for a repressor of *acrAB*. Apart from the *marRAB* and *soxRS* regulons, AcrR would function as a specific secondary modulator to finely tune the level of *acrAB* transcription and to prevent the unwanted overexpression of *acrAB* [41].

In *Salmonella*, evidence for the participation of active efflux in quinolone resistance has recently been provided [18, 58]. Although no direct evidence has been provided for the role of the AcrAB-TolC efflux system in resistance, the production level of the AcrA protein in *in vitro* quinolone-selected mutants correlated well with the resistance levels to nalidixic acid and fluoroquinolones [18]. As in *E. coli*, quinolone-selection also resulted in a Mar phenotype with resistance to unrelated antibiotics such as β-lactams, chloramphenicol, and tetracycline. Interestingly, the *Salmonella acrB* gene has been shown to play an important role in resistance to bile salts, detergents and in mice intestinal colonisation [34]. A *S. enterica* serovar Typhimurium *acrB* mutant indeed exhibited a reduced capacity to colonise the intestinal tract. Similarly, the TolC protein of *S. enterica* serovar Enteritidis has been shown to be required for virulence in mice [66]. The *tolC* mutant was avirulent for mice when administered by the oral route. This mutant was also more sensitive than the wild-type strain to various detergents, antibiotics, and dyes suggesting that as in *E. coli* the TolC outer membrane channel of *Salmonella* is required for effective functioning of the AcrAB-TolC efflux system. These data suggest an important role of the AcrAB-TolC efflux system in the virulence of *Salmonella*.

The *marRAB* and *soxRS* operons also occur in *Salmonella* [11, 31, 33, 58, 59, 67]. As in *E. coli*, increased expression of *marA* and *soxS* results in the Mar phenotype [11, 31, 59]. Recently, Koutsolioutsou et al. [31] reported a *soxRS*-constitutive mutation in a clinical strain of *S. enterica* serovar Typhimurium that arose with the development of resistance to quinolones during treatment. The elevated quinolone resistance in this strain derived from a point mutation in the *soxR* gene and could be suppressed in trans by multicopy wild-type *soxRS* thus showing that constitutive expression of *soxRS* can contribute to antibiotic resistance in clinically relevant *S. enterica* [31]. In another recent study of Piddock et al. [58], evidence has been reported of a role of active efflux in the resistance phenotype of six isolates of *S. enterica* serovar Typhimurium recovered from a patient treated with ciprofloxacin. However, no mutations were found in the *marO*, *marR*, and *acrR* sequences and no increased expression of *marA*, *tolC*, or *soxS* was observed by Northern blotting although three of the six isolates showed increased expression of *acrB* [58]. To our knowledge these are the only two reports where mutations in the regulator genes of the AcrAB-TolC efflux system have been investigated. Thus much work remains to be done to study the role of efflux and its regulation in *Salmonella* quinolone resistance to reach the current state of knowledge of efflux in *E. coli*.

4. DECREASED OUTER MEMBRANE PERMEABILITY

A few studies have reported on alterations of outer membrane protein expression or of lipopolysaccharide in quinolone-resistant *Salmonella* [18, 20, 56, 57]. However, it does not appear clearly from these studies whether such alterations contributed significantly to decreased outer membrane permeability and consecutive quinolone resistance. The role of lipopolysaccharide composition on the accumulation of quinolones has been studied in several bacteria, but it remains unclear, as contradictory results have been obtained [7, 13, 22, 45, 47, 62]. It has been hypothesised in quinolone-selected *Pseudomonas aeruginosa* strains that increased amounts of lipopolysaccharide form a permeability barrier which acts preferentially against hydrophilic quinolones [45]. The lengthening of the O chains seen in in vitro quinolone-selected resistant *Salmonella* mutants could also result in a lower level of accessibility to the outer membrane [18].

It is thought that hydrophilic quinolones like ciprofloxacin preferentially use the porin pathway to penetrate the cells [7]. Lack of expression of the OmpF porin has been reported in some quinolone-resistant *Salmonella* strains [20, 56, 57]. However, it was not clear whether the lack of OmpF contributed to decreased levels of quinolone accumulation. As described for *E. coli* above, it has been shown for *Salmonella* that SoxS upregulates *micF* transcription [59] which may be responsible for the lack of expression of OmpF for these quinolone-resistant *Salmonella* isolates.

5. HOW CAN HIGH-LEVEL QUINOLONE RESISTANCE BE REACHED?

Interplay between several resistance mechanisms may lead to high-level resistance to quinolones and to other antibiotics when multidrug efflux pumps and decreased outer membrane permeability are involved [14, 28, 35, 36, 46]. For instance for in vitro selected quinolone-resistant *E. coli* mutants it has been shown that first-step quinolone-resistant mutants acquire a *gyrA* mutation [28]. Second-step mutants reproducibly acquire a Mar phenotype and show enhanced fluoroquinolone efflux. In some third-step mutants, fluoroquinolone efflux is further enhanced and additional

topoisomerase mutations are acquired. In clinical *E. coli* isolates from humans and animals the situation appears to be the same where high-level fluoroquinolone resistance is reached when mutations at several chromosomal loci are acquired [14, 51].

For *S. enterica* Typhimurium in vitro selected quinolone-resistant mutants, individual mechanisms of resistance also appear step by step and their accumulation also results in high-level fluoroquinolone resistance [18]. However, in contrast to *E. coli*, active efflux correlating with increased production of the AcrAB efflux pump appears prior to a mutation in *gyrA* [18]. In addition, no mutation in the *parC* topoisomerase IV gene occurs in late-step mutants but rather a gradual increase in the production of the AcrAB efflux pump. This suggests a probable step-by-step accumulation of mutations in regulator genes of the AcrAB-TolC efflux system of *Salmonella*.

High-level fluoroquinolone resistance in clinical isolates of *Salmonella* has only been reported for the Copenhagen variant clone of *S. enterica* serovar Typhimurium which appeared during the early 1990s in Germany [23, 25] and probably also in Belgium [27]. As described above only mutations in the *gyrA* and *gyrB* target genes have been investigated [23, 25]. It would therefore be worth further studying these isolates to determine whether accumulation of individual resistance mechanisms, in particular active efflux and target gene mutation, resulted in this high-level resistance phenotype.

Over the past few years no high-level fluoroquinolone-resistant clinical strains of *Salmonella* have been isolated which suggested a counterselection of highly fluoroquinolone-resistant strains in the field [17]. However, this situation might be temporary as the number of nalidixic acid-resistant isolates increases, in particular those of *S. enterica* serovar Typhimurium phagetype DT104 [70] and particular clones may always emerge showing high-level fluoroquinolone resistance as was the case in the early 1990s.

6. ESCAPING QUINOLONE RESISTANCE

It is particularly interesting to note that in *E. coli*, inactivation of the AcrAB efflux pump makes resistant strains, including those with target gene mutations, hypersusceptible to fluoroquinolones and certain other unrelated drugs [52]. Thus in the absence of the AcrAB efflux pump, gyrase mutations fail to produce clinically relevant levels of fluoroquinolone resistance [52]. The same observation has been made for *Pseudomonas aeruginosa* where deletion of the MexAB-OprM efflux pump, which is the homolog of the AcrAB-TolC efflux pump in this species, resulted in a significant decrease in resistance to fluoroquinolones even for strains carrying target gene mutations [38].

Therefore, multidrug efflux pumps such as AcrAB-TolC and MexAB-OprM appear as suitable targets for new therapeutics which, combined with fluoroquinolones, would decrease the level of intrinsic resistance, reverse acquired resistance, and result in a decreased frequency of emergence of fluoroquinolone-resistant strains [38].

One effective inhibitor of multidrug resistance efflux pumps has recently been discovered [39]. This molecule, termed MC-207,110, is active against all known Mex efflux pumps from *Pseudomonas aeruginosa* and the AcrAB-TolC efflux pump homolog of *E. coli* [39, 44]. The study of Lomovskaya et al. [39] showed that inhibition of efflux pumps by this molecule indeed (i) significantly decreased the level of intrinsic resistance, (ii) reversed acquired resistance, and (iii) resulted in a decreased frequency of emergence of *Pseudomonas aeruginosa* strains that are highly resistant to fluoroquinolones.

Thus the development and application of efflux pump inhibitors as new therapeutics combined with fluoroquinolones appear to be an attractive solution to avoid the emergence of pathogens highly resistant to fluoroquinolones, possibly including *Salmonella* which should be further investigated.

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