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Review

Mechanism of action of and resistance to quinolones

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Summary

Fluoroquinolones are an important class of wide-spectrum antibacterial agents. The first quinolone described was nalidixic acid, which showed a narrow spectrum of activity. The evolution of quinolones to more potent molecules was based on changes at positions 1, 6, 7 and 8 of the chemical structure of nalidixic acid. Quinolones inhibit DNA gyrase and topoisomerase IV activities, two enzymes essential for bacteria viability. The acquisition of quinolone resistance is frequently related to (i) chromosomal mutations such as those in the genes encoding the A and B subunits of the protein targets (*gyrA*, *gyrB*, *parC* and *parE*), or mutations causing reduced drug accumulation, either by a decreased uptake or by an increased efflux, and (ii) quinolone resistance genes associated with plasmids have been also described, i.e. the *qnr* gene that encodes a pentapeptide, which blocks the action of quinolones on the DNA gyrase and topoisomerase IV; the *aac(6′)-Ib-cr* gene that encodes an acetylase that modifies the amino group of the piperazin ring of the fluoroquinolones and efflux pump encoded by the *qepA* gene that decreases intracellular drug levels. These plasmid-mediated mechanisms of resistance confer low levels of resistance but provide a favourable background in which selection of additional chromosomally encoded quinolone resistance mechanisms can occur.

Fluoroquinolones are an important class of broad-spectrum antibacterial agents, whose spectra of activity has been parallel to modifications in the structure of the first quinolone, nalidixic acid. Nalidixic acid, which can be considered as the first generation of quinolones, was introduced for clinical use in 1962 (Lesher *et al*., 1962) and was initially administered to treat Gram-negative urinary tract infections in humans and animals (Suh and Lorber, 1995). Subsequently, the molecular structures of quinolones were modified to improve their antimicrobial properties and pharmacokinetic profiles (Ball, 1998; 2000; Kim *et al*., 2001). On the basis mainly of their antibacterial spectra, quinolone drugs are classified into generations. The second generation of quinolones started with fluoroquinolones obtained by fluoridation of the quinolone molecule at position C6. The first fluoroquinolone, norfloxacin, was synthesized in 1978 and became available for clinical use in 1986 (Paton and Reeves, 1988). Ciprofloxacin, one of the most used fluoroquinolones, was introduced into the clinical market in 1987. Fluoroquinolone drugs are active against a wide range of Gram-negative and Gram-positive pathogens and show improved oral absorption and systemic distribution. Thus, the clinical applications of these compounds have been extended to the treatment of lower respiratory tract infections, skin and soft tissue infections, sexually transmitted diseases and urinary tract infections (Chu, 1996). However, this second generation of quinolones has limited activity against a number of clinically relevant Gram-positive bacteria and anaerobes (Ball, 1998; Ball *et al*., 1998; Zhanel *et al*., 2002). Since 1987, structural variations of fluoroquinolones have provided numerous new agents suitable for the treatment of a variety of bacterial infections. In the third generation of quinolones, more potent fluoroquinolones were developed, such as levofloxacin, gatifloxacin (Perry *et al*., 1999) and moxifloxacin (Barrett, 2000), which exhibit improved bactericidal activity against Gram-positive bacteria. The fourth generation of quinolone drugs, such as gemifloxacin (Lowe and Lamb, 2000), shows good activity against Gram-positive cocci and significant activity against anaerobes (Bhavnani and Ballow, 2000; Kim *et al*., 2001).

Structure of the quinolones

From the structural perspective, quinolones are heterocycles with a bicyclic core structure (Fig. 1). The carboxylic acid group at position 3 and the carbonyl at position 4

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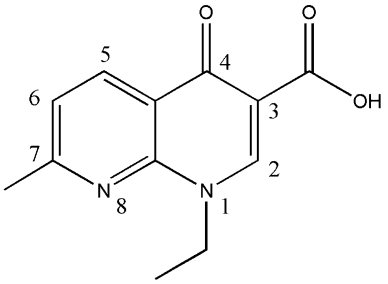
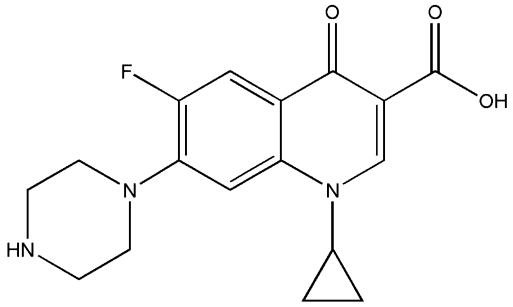
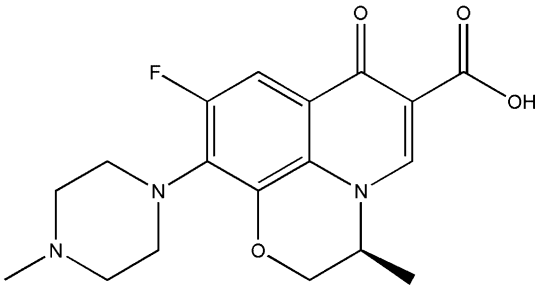


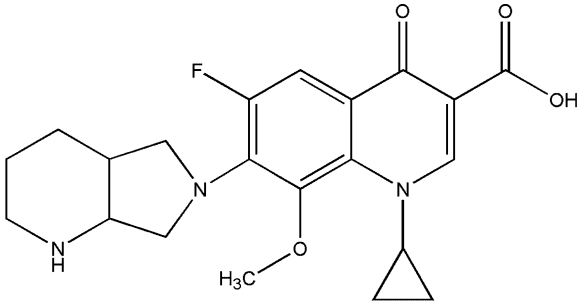
Nalidixic acid



Ciprofloxacin



Levofloxacin



Moxifloxacin

Fig. 1. Structure of representative quinolones.

seem to be essential for the activity of the quinolones. In addition, bulky substituents on one face of the bicyclic core, namely at positions 1 and 7 and/or 8, are permissible and they seem to play a relevant role to determine the quinolone antibiotic spectrum. With respect to these substituents, most quinolones can be arranged into three main categories: piperazinyl-, pyrrolidinyl- and piperidinyl-type side-chains (Hu *et al*., 2003). Piperazinyl-based quinolones usually have a wide Gram-negative coverage but a limited Gram-positive spectrum (e.g. ciprofloxacin and levofloxacin). On the other hand, piperidinyl- and pyrrolidinyl-based quinolones have a more balanced spectrum (e.g. gemifloxacin).

Mechanism of action

Quinolone antibiotics inhibit DNA synthesis by targeting two essential type II topoisomerases, DNA gyrase and topoisomerase IV (Topo IV). Both targets allow one double-stranded DNA molecule to pass through another, followed by religation of the original strand, thereby changing the linking number of DNA by two in each enzymatic step. Although both enzymes show a high degree of similarity in their structures and functions, their specific function during DNA replication differs (Levine *et al*., 1998).

DNA gyrase is an enzyme found only in bacteria. This enzyme uses the energy of ATP hydrolysis to introduce negative supercoils into DNA (Gellert *et al*., 1976; Champoux, 2001; Corbett and Berger, 2004). This unidirectional supercoiling activity is caused by chiral wrapping of the DNA (Liu and Wang, 1978a,b) around a specialized domain of the enzyme before strand passage (Reece and Maxwell, 1991; Kampranis and Maxwell, 1996; Corbett *et al*., 2004; Ruthenburg *et al*., 2005). Negative DNA supercoiling is essential for chromosome condensation, relieving torsional strain during replication, and promoting local melting for vital processes such as transcript initiation by RNA polymerase (Levine *et al*., 1998; Wang, 2002). DNA gyrase is an excellent target for quinolones because it is not present in eukaryotic cells and is essential for bacterial growth. This enzyme comprises two subunits, A (97 kDa) and B (90 kDa), which form an A_2B_2 tetramer (Higgins *et al*., 1978; Liu and Wang, 1978b; Klevan and Wang, 1980). The A subunit is encoded by the *gyrA* gene and is involved mainly in DNA breakage and reunion, while the B subunit is encoded by the *gyrB* gene and exhibits ATPase activity (Ali *et al*., 1993; 1995). To develop supercoiling activity, the DNA gyrase generates a pair of single-stranded breaks of a first (G or gate) DNA segment in which the broken ends are 4 bp apart (Morrison and Cozzarelli, 1979; Wang, 1998). These two DNA ends are separated, thereby forming a transient gate, through which the second (T or transported strand) DNA segment, wrapped around the DNA gyrase, is then

passed. In this process, the C-terminus of the GyrA subunit is responsible for the unique negative supercoiling activity of the DNA gyrase. This conclusion has been made on the basis of observations that mutants lacking the C-terminus lose their capacity to form negative supercoils (Kampranis and Maxwell, 1996; Kampranis *et al*., 1999).

In addition to quinolones, naturally occurring bacterial DNA gyrase inhibitors, such as coumarins, which include novobiocin, are also antibacterial agents (Maxwell, 1993; Kim and Ohemeng, 1998). Coumarins inhibit the ATPase activity of the DNA gyrase by competing with ATP for binding to the GyrB subunit. However, because of side-effects, to date few pharmaceutically useful drugs have been derived from coumarins. Topoisomerase IV has two functions in the cell. First, it serves as a decatenating enzyme that resolves interlinked daughter chromosomes after DNA replication. Topoisomerase IV is required at the terminal stages of DNA replication for unlinking newly replicated daughter chromosomes (Drlica and Zhao, 1997). These links must be removed in order to segregate chromosomes (and plasmids) into daughter cells so that cell division can be completed. The second function, shared with the DNA gyrase, of Topo IV is to relax positive supercoils. Like the DNA gyrase, Topo IV uses a double-strand passage mode; however, the mechanism of this passage differs. The gyrase wraps the DNA around itself, while Topo IV does not. Topoisomerase IV is also a heterotetramer made of two A subunits (ParC) and two B subunits (ParE) (Kato *et al*., 1990). ParC is encoded in the *parC* gene (also called *grlA* gene in *Staphylococcus aureus*) and ParE is encoded in the *parE* gene. These subunits share about 35% identity with GryA and GyrB of the DNA gyrase. During the catalytic cycle, Topo IV binds the gate (G) segment of the DNA. Upon binding of a second DNA segment, the transport (T) segment, the ParE subunits dimerize around the T segment DNA. The enzyme then cleaves the G segment, passes the T segment through the break and reseals the broken duplex. ParC are the subunits responsible for DNA binding and the cleavage and religation reaction, while ParE are responsible for ATP binding and hydrolysis (Levine *et al*., 1998).

However, some microorganisms such as *Mycobacterium* spp., *Campylobacter* spp., *Corynebacterium* spp. and *Helicobacter pylori* do not possess Topo IV and it has been shown that the DNA gyrase of *Mycobacterium smegmatis* presents an enhanced decatenating activity and, hence, likely assumes the role of Topo IV in these microorganisms (Manjunatha *et al*., 2002). The main physiological role, of both DNA gyrase and Topo IV , is the replication and transcription of the DNA and Topo IV in addition to the decatenation of daughter replicons following DNA replication. The DNA gyrase may also play a role in the organization of the chromosome as it has been suggested that it is organized in negative supercoiled domains.

As mentioned above, quinolone drugs are active against type II topoisomerases and act by blocking DNA replication and inhibiting synthesis and cell division (Vila, 2005). The mechanism of quinolone inhibition occurs via formation of a ternary cleavage complex with the topoisomerase enzyme and DNA Figure 2 (Hiasa and Shea, 2000). However, the molecular details of the mode of action of these drugs remain unclear.

It is accepted that for quinolones to inhibit DNA gyrase activity, they must form a stable interaction with the DNA gyrase–DNA complex. To overcome the lack of crystallographic data for the ternary complex, computational tools,

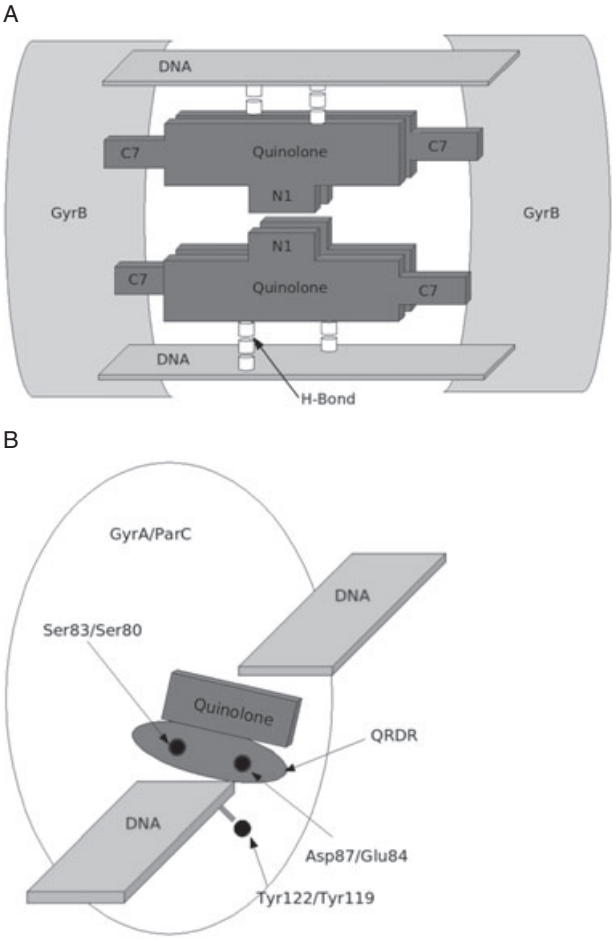


Fig. 2. A. Cooperative quinolone–DNA binding model of Shen *et al.* for the inhibition of the DNA gyrase. Four molecules of quinolones are self-associated. Quinolones bind to DNA via hydrogen bonds to the unpaired bases.
B. Model of Maxwell *et al.* for quinolone binding to DNA and GyrA (DNA gyrase) or ParC (Topo IV). Mutations in DNA gyrase or Topo IV that confer quinolone resistance are clustered principally within a small region (QRDR). The most common mutations of the QRDR include Ser-83 and Asp-87 for GyrA, or Ser-80 and Glu-84 for ParC.

such as molecular docking, are useful for predicting the structures of protein–ligand complexes and providing information on the modes of interaction between ligands and receptors. Several docking studies have been performed with the ATP binding site of the GyrB subunit (Boehm *et al*., 2000; Schulz-Gasch and Stahl, 2003) or outside the QRDR region of GyrA (Ostrov *et al*., 2007). A docking study of fluoroquinolones to the QRDR region of the DNA gyrase recently put forward a structural hypothesis of their binding mode (Madurga *et al*., 2008). It was found that Asp-87 is critical in the binding of quinolone drugs because it interacts with the positively charged nitrogen of the fluoroquinolones. In addition, Arg-121, located next to the active-site tyrosine, was postulated to be another relevant point of binding (Madurga *et al*., 2008).

Mechanisms of resistance

The acquisition of quinolone resistance may be related to: (i) chromosomal mutations in genes encoding the protein targets, or mutations causing reduced drug accumulation, either by a decreased uptake or by an increased efflux, and (ii) plasmid-located genes associated with quinolone resistance (Vila, 2005).

Chromosome-mediated quinolone resistance

Enterobacteriaceae. The process by which susceptible strains become highly fluoroquinolone-resistant is thought to be a result of a series of sequential steps. Overall, in *Enterobacteriaceae* the first step is often a single mutation in the *gyrA* gene, which confers low-level quinolone resistance [minimal inhibitory concentration (MIC) of ciprofloxacin of 0.125–0.25 mg l^{-1}]. The acquisition of a second mutation either in the amino acid codon Ser-80 or in the amino acid codon Glu-84 of the *parC* gene is associated with a moderate level of ciprofloxacin resistance (1–4 mg l^{-1}). A third mutation, the second in *gyrA*, is associated with a high level of ciprofloxacin resistance (8–64 mg l^{-1}), and a fourth mutation, the second in *parC*, is associated with the highest level of resistance (128 mg l^{-1}) (Table 1) (Vila *et al*., 1996). Therefore, several mutations are needed to produce a high level of quinolone resistance. The most important mutations leading to a quinolone-resistant phenotype in *Escherichia coli* are in the *gyrA* gene, mainly amino acids Ser-83–Leu and Asp-87–Asn (this position can occasionally be changed to Val, Tyr and Gly), and in the *parC* gene changing Ser-80–Arg (Ile can also be found) and Glu-84–Val (Gly can also be found) (Nakamura *et al*., 1989; Vila *et al*., 1994; Hiasa, 2002). Nakamura and colleageus (1989) found that mutations in the *gyrB* gene also contribute to low-level quinolone resistance. Yoshida and colleageus (1991) evaluated mutations in *gyrB* and found two mutations: Asp-426–Asn (associated with a higher level of quinolone and fluoroquinolone resistance) and Lys-447–Glu (associated with hypersusceptibility to fluoroquinolones but nalidixic acid resistance). However, in *E. coli* clinical isolates this does not appear to be a common phenomenon, since an Asp-426–Asn change was only found in one out of 27 *E. coli* clinical isolates investigated (Vila *et al*., 1994). No mutations were found in the *parE* gene in 27 *E. coli* clinical isolates (Ruiz *et al*., 1997). However, Sorlozano and colleageus (2007) have recently described the acquisition of a new previously undetected mutation within the QRDR of the *parE* gene at position 458 (Ser→Ala). The above mentioned mutations can be extrapolated to other *Enterobacteriaceae.* In addition to point mutations in the *gyrA* gene, the decreased susceptibility to fluoroquinolones may be due to the decreased accumulation of the quinolone or to the presence of some plasmid mediated quinolone resistance mechanism (see specific section). Moreover, the overepression of efflux

Table 1. The most frequent amino acid substitutions found in GyrA and ParC of different *Enterobacteriaceae*.

Microorganism	Amino acid change				MIC (mg l^{-1}) CIP
	GyrA		ParC		
Escherichia coli wt	Ser-83	Asp-87	Ser-80	Glu-84	
	Leu				0.25–4
	Leu		Arg		1–4
	Leu			Lys	4
	Leu	Asn/Tyr			8–128
	Leu	Asn	Arg/Ile		4–128
	Leu	Asn/Tyr	Ile	Val/Lys	64–128
Salmonella spp. wt	Ser-83	Asp-87	Ser-80		
	Phe				0.25–2
		Gly/Tyr			0.12–0.5
	Phe		Ile		4

MIC, minimum inhibitory concentration; CIP, ciprofloxacin.

pumps may also play a role in the high level of resistance in strains with two or three mutations.

The decrease of drug accumulation can be associated with: (i) an upregulation of certain cell envelope proteins, which can facilitate extrusion of these agents – these proteins are energy-dependent efflux systems that can be specific to a drug or can have broad specificity, then called multidrug transporters, and (ii) decrease of permeability often related to decreased expression of porins, which are outer membrane proteins that form channels for passive diffusion and are only present in Gram-negative bacteria (Markham and Neyfakh, 2001; Jacoby, 2005).

Active efflux transporters have been classified into five superfamilies: (i) the major facilitator superfamily (MFS), (ii) the ATP-binding cassette (ABC) family, (iii) the resistance/nodulation/division (RND) family, (iv) the small multidrug resistance (SMR) family and (v) the multidrug and toxic compound extrusion (MATE) family. These antibiotic efflux pumps utilize the energy of the proton-motive force to expel antibiotics, with the exception of the ABC family that utilizes the energy generated from the hydrolysis of ATP. A remarkable feature of some of these transporters is wide range of substrates that are recognized by a single pump protein (Poole, 2000a; Fàbrega *et al*., 2008).

The *Enterobacteriaceae*, as most Gram-negative bacteria, are protected by the action of multidrug efflux transporters, which usually belong to the RND family followed by members of the MFS family and are expressed in a constitutive way leading to their intrinsic resistance phenotype and providing immediate response to structurally diverse antimicrobial agents by means of their overexpression (Nikaido, 1996; Zgurskaya and Nikaido, 2000).

There are many genes that are assumed to encode a drug transporter protein in *Enterobacteriaceae* because of sequence similarities in their open reading frames (ORFs). However, only AcrAB/TolC overexpression has been shown to play a major role as a main efflux pump implicated in extruding quinolones (Oethinger *et al*., 2000; Webber and Piddock, 2001; Schneiders *et al*., 2003; Baucheron *et al*., 2004; Hasdemir *et al*., 2004; Chen *et al*. 2007). This efflux pump, which belongs to the RND superfamily, is a three-component system: *acrA* and *acrB* genes are co-transcribed from the same operon and the resulting proteins are AcrA, the membrane fusion protein (MFP), and AcrB, the energy-dependent transport protein anchored in the inner membrane respectively. The third component is TolC, the outer membrane protein (Okusu *et al*., 1996; Fàbrega *et al*., 2008). The inactivation of the *acrB* or *tolC* genes in fluoroquinolone-susceptible strains shows its contribution to the intrinsic resistance levels to fluoroquinolones and other antibiotics (tetracyclines, chloramphenicol, β-lactams, trimethoprim, rifampin, aminoglycosides and toxic compounds) due to a constitutive expression (Okusu *et al*., 1996; Oethinger *et al*., 2000; Sulavik *et al*., 2001; Yang *et al*., 2003; Baucheron *et al*., 2004; Chen *et al*., 2007).

In addition to AcrAB overexpression there are particular situations described in *E. coli* and *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium) when fluoroquinolone-resistant mutants have been obtained '*in vitro*' after *acrAB* inactivation. These mutants have reached this phenotype by overexpressing another efflux pump, AcrEF (also an RND family member), which may be a compensatory mechanism and whose substrate specificity is very similar to that of AcrAB (Jellen-Ritter and Kern, 2001; Olliver *et al*., 2005).

The mechanisms of resistance by which AcrAB can be overexpressed are those that affect the regulatory genes that determine the protein levels. *acrAB* genes are regulated by four known transcriptional factors. Rob, MarA and SoxS are transcriptional activators that belong to the Xlys/AraC family (Gallegos *et al*., 1997) and promote *acrAB* expression by binding to the marbox found upstream from the *acrAB* operon; whereas AcrR is the local repressor for this pump, localized upstream of *acrA* gene but transcribed into the opposite direction (Fig. 3) (Gallegos *et al*., 1997; Martin *et al*., 1999; Martin and Rosner, 2002).

The SoxS protein belongs to the SoxRS regulon. In this system, the *soxS* gene is only transcribed in the presence of an oxidized form of the SoxR protein (Fig. 3) (Amabile and Demple, 1991; Pomposiello and Demple, 2000). Among *E. coli* clinical isolates that show a MAR phenotype, it is frequent to find overexpression of SoxS (Oethinger *et al*., 1998; Kern *et al*., 2000; Webber and Piddock, 2001). Constitutive *soxS* expression can be triggered, in principle, by mutations within the *soxR* gene that render the protein constitutively active, by mutations in the *soxS* promoter that turn on its own transcription constitutively or by mutations in other genes that regulate the redox status of SoxR (Amabile and Demple, 1991). To date, only mutations randomly distributed within the *soxR* gene have been found as a factor responsible for increased *soxS* expression in *E. coli* and *S.* Typhimurium clinical isolates, as these mutations lead SoxR to be in a permanent activated state (Nunoshiba and Demple, 1994; Koutsolioutsou *et al*., 2001; 2005; Webber and Piddock, 2001).

The MarA protein belongs to the *marORAB* operon, where MarR is a transcriptional repressor (Fig. 3). Once MarA is transcribed it can autoactivate the operon itself by binding to the marbox upstream from the *marRAB* promoter (Sulavik *et al*., 1997; Martin *et al*., 1999; Martin and Rosner, 2002). To date, mutations that trigger overexpression of MarA have only been found in *E. coli*, usually within the codifying sequence of MarR, and focus their

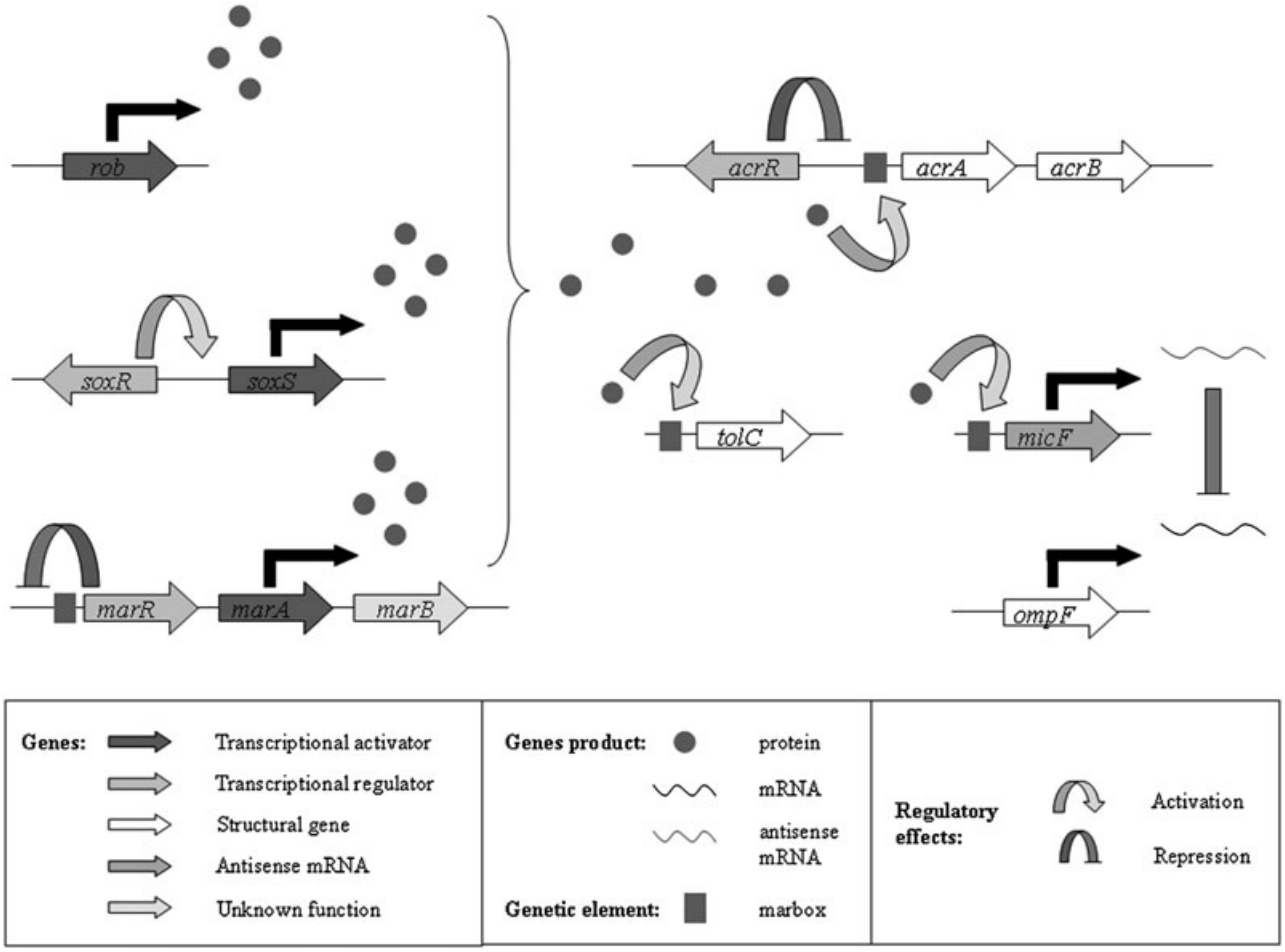


Fig. 3. Regulation of *acrAB*, *tolC* and *ompF* genes involved in decreasing the internal accumulation of quinolones. MarA, SoxS and Rob are the transcriptional activators which turn on these genes. AcrR is the local repressor and only affects AcrAB expression.

effects on AcrAB, because when this pump is inactivated, overproduction of MarA becomes useless at increasing fluoroquinolone resistance (Okusu *et al*., 1996; Oethinger *et al*., 1998).

The Rob protein also belongs to the same family of activators but differs in size, as only its N-terminal domain, which is the DNA-binding domain, shows homology with both MarA and SoxS proteins. It has been shown that Rob activates many regulatory genes leading to a global effect, although the magnitude of its effects is modest (Fig. 3). No clinical data have been reported to date linking fluoroquinolone resistance with Rob overexpression.

The last of the four regulators for AcrAB is AcrR, the repressor that controls *acrAB* expression; it only affects the level of these two structural proteins (Fig. 3) (Okusu *et al*., 1996; Webber and Piddock, 2001). *Escherichia coli*, *S. enterica* and *Klebsiella pneumoniae* clinical isolates as well as MAR mutants selected '*in vitro*' can overexpress AcrAB by acquiring mutations within the transcriptional DNA that inactivate the *acrR* gene (Jellen-Ritter and Kern, 2001; Webber and Piddock, 2001; Schneiders *et al*., 2003; Olliver *et al*., 2004).

In addition to these regulatory loci generally found in *Enterobacteriaceae*, it has been reported that some bacterial species have a homologue of MarA, dubbed RamA, which belongs to the same family of transcriptional activators. This gene was first described in *K. pneumoniae* (George *et al*., 1995), but it is also present in *Salmonella* spp., *Enterobacter aerogenes* and *Enterobacter cloacae*. However, it is absent in *E. coli*. The resulting protein, which has also been shown to bind to the marbox, when overexpressed in a susceptible *E. coli* strain, allowed this microorganism to display a MAR phenotype related to both increased efflux and loss of the OmpF porin (see below for porin regulation). This can be explained by assuming that RamA may trigger the same effect as MarA or SoxS (George *et al*., 1995; Schneiders *et al*., 2003; van der Straaten *et al*., 2004). Overexpression of the *ramA* gene has been detected in some

fluoroquinolone-resistant clinical *K. pneumoniae* strains in concordance with elevated levels of AcrAB when neither MarA nor SoxS are overexpressed. In addition, RamA overexpression in MAR strains overexpressing AcrAB has been justified by the presence of mutations within the encoding region of the repressor leading to its inactivation. In addition, a deletion in the putative RamR binding site upstream *ramA* prevents RamR binding and thus, its repressor effect over RamA. The regulatory effects of RamA in fluoroquinolone resistance acquisition may play a key role in strains lacking an altered level of any of the other regulators, such as MarA, SoxS or AcrR, suggesting that the role of these regulators is not as significant as in *E. coli* (Abouzeed *et al*., 2008). However, other studies compromise these conclusions as they reveal that *ramA* inactivation in some *S.* Typhimurium clinical isolates showing a MAR phenotype does not result in any change in ciprofloxacin susceptibility (van der Straaten *et al*., 2004).

These mutations in the regulatory loci are acquired individually as only one of these genes is completely affected (Oethinger *et al*., 1998; Kern *et al*., 2000; Webber and Piddock, 2001). However, an exception has been reported in *K. pneumoniae* when both genetic mechanisms, increased RamA expression and *acrR* inactivation, have sometimes been found at the same time (Schneiders *et al*., 2003). A reasonable explanation may be that some of these transcriptional factors (MarA, SoxS or Rob) show overlapping effects as many of the genes of their regulons are the same (Martin *et al*., 1999; Martin and Rosner, 2002).

Despite the proposal of all these mechanisms further investigation is needed for a complete explanation, for example, when: (i) fluoroquinolone resistance has at times been reported not to be linked to *marOR*, *soxRS* or *acrR* mutations, even when AcrAB is overproduced in *E. coli* and *Salmonella* strains, suggesting that mutations in unidentified chromosomal loci may turn on other regulatory mechanisms that increase efflux via AcrAB (Oethinger *et al*., 2000; Piddock *et al*., 2000; Webber and Piddock, 2001; Chu *et al*., 2005; Koutsolioutsou *et al*., 2005), (ii) an increasing level of SoxS linked with fluoroquinolone resistance has been reported with an absence of an AcrAB-inducing effect, suggesting that alternative ways may be implicated (Oethinger *et al*., 1998; Oethinger *et al*., 2000; Webber and Piddock, 2001; Koutsolioutsou *et al*., 2005), and (iii) inactivation of the *acrB* gene is performed and there is an important decrease in resistance in quinolone-resistant mutants, but in contrast, the wild-type conditions are not reached, suggesting that another mechanism may contribute (Baucheron *et al*., 2004).

Furthermore, the outer membrane protein profile has also been studied in strains with a high level of fluoroquinolone resistance. It has been found that the major outer membrane proteins of *E. coli*, OmpF and OmpC [as their analogous proteins in other bacterial species, like OmpK35 and OmpK36, respectively, found in *K. pneumoniae* (Hernández-Allés *et al*., 2000)], when downregulated play a role in decreasing the outer membrane permeability and thereby reducing the internal accumulation of the antibiotic leading to a two- to fourfold increase in the MIC of fluoroquinolones (Mizuno *et al*., 1984; Hirai *et al*., 1986; Martínez-Martínez *et al*., 2002). These two genes, *ompF* and *ompC*, are transcriptionally regulated, depending on the temperature and the osmolarity of the media, by the two-component regulatory system OmpR-EnvZ that mediates both positive and negative control. There is also a post-transcriptional control by the small regulatory RNA molecules *micC* and *micF* which downregulate OmpC and OmpF expression respectively. MicC is complementary to the leader sequence of the *ompC* mRNA (Chen *et al*., 2004), whereas *micF* is partially complementary to the 5′ end of the *ompF* mRNA (Mizuno *et al*., 1984). The *micF* promoter contains a marbox so that it is turned on by MarA (Cohen *et al*., 1988), SoxS (Chou *et al*., 1993), Rob (Ariza *et al*., 1995) and RamA (George *et al*., 1995) what can downregulate OmpF expression independently of OmpC production (Fig. 3). This explains why it is more frequent to find only an OmpF loss or decrease in fluoroquinolone-resistant strains, whereas few cases have reported only an OmpC reduction, or both proteins at the same time (Hirai *et al*., 1986; Cohen *et al*., 1989; Tavío *et al*., 1999; Hernández-Allés *et al*., 2000; Hasdemir *et al*., 2004; Chenia *et al*., 2006). This deficiency in porins has been reported to achieve a significant effect only when mutations occur within the QRDR or efflux mechanisms appear simultaneously. Furthermore, an altered protein profile seems to be linked with AcrA overexpression (Deguchi *et al*., 1997; Hernández-Allés *et al*., 2000; Martínez-Martínez *et al*., 2002; Hasdemir *et al*., 2004).

Despite this general information, other efflux systems as well as particular situations affect bacteria individually:

(i) *Escherichia coli.* Concerning quinolone resistance, only four efflux pumps have been able to show a clear implication in quinolone efflux by overexpression from a plasmid: (i) AcrAB and (ii) AcrEF which confer an eight- and a fourfold increase in norfloxacin and nalidixic acid resistance respectively, (iii) MdfA, which belongs to the MFS superfamily and (iv) YdhE (also called NorE), belonging to the MATE superfamily. These two latter proteins, MdfA and YdhE, confer an eightfold increase in norfloxacin resistance and do not affect nalidixic acid susceptibility (Table 5) (Edgar and Bibi, 1997; Nishino and Yamaguchi, 2001; Yang *et al*., 2003). However, another study has shown that the inactivation in *E. coli* strain W3110 of either the

mdfA or *acrEF* genes does not trigger any change in fluoroquinolone susceptibility (Sulavik *et al*., 2001).

When AcrAB overexpression is in combination with other multidrug resistance pumps, such as MdfA or YdhE, it has been shown to confer a synergistic effect (7- and 11-fold increased in ciprofloxacin and norfloxacin resistance respectively) (Yang *et al*., 2003). Despite this effect, it has been suggested that resistance levels mediated by individual or simultaneous overexpression of pumps may have an upper limit, approximately a 10-fold increase in drug resistance, because when a determined high level of expression for these proteins is reached, the correlation with high efflux of the antibiotic and its MIC is no longer provided (Webber and Piddock, 2001; Yang *et al*., 2003).

(ii) *Salmonella enterica.* Fluoroquinolone-resistant strains have been able to show a substantial increased expression of AcrF, EmrD or MdlB (a part from AcrB), whereas overexpression of TolC, MdtB, MdtC or EmrA is also achieved but to a lesser extent (Chen *et al*., 2007). However, the individual inactivation of AcrEF, MdtABC, EmrAB, MdlAB or even AcrD (but maintaining an active AcrAB) does not lead to any significant change in the MIC of any fluoroquinolone, suggesting that limited or no role is played (Olliver *et al*., 2005; Chen *et al*., 2007).

(iii) *Klebsiella pneumoniae.* Other ORFs have been found to extrude quinolones. An example of these pumps are two MFS members: KmrA which has been reported to be overexpressed in a *K. pneumoniae* clinical isolate showing the MAR phenotype (Ogawa *et al*., 2006) and KdeA, a homologue of MdfA of *E. coli*, whose expression level is similar in the same clinical isolate and in the ATCC strain, suggesting a possible role in the intrinsic resistance in *Klebsiella* (Table 5) (Ping *et al*., 2007).

Non-fermenting Gram-negative bacteria. In microorganisms such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, a single mutation in the *gyrA* gene is sufficient to cause clinically important levels of resistance to fluoroquinolones as these bacteria already show an intrinsic resistance to these antibacterial agents, likely due to low permeability or constitutive expression of some efflux pump(s) or the interplay between both. Therefore, this decreased susceptibility (low level of resistance) can favour the acquisition of a mutation and increase the MIC of fluoroquinolones.

• *Pseudomonas aeruginosa.* Beside mutations in target genes, DNA modifications increasing the efflux of the antibiotics by overexpression of the efflux pump systems play an important role in triggering resistance. The most important group of efflux pumps found in *P. aeruginosa* is the RND family (Vila and Martínez, 2008). Nine different RND efflux pumps have been characterized (Table 5): (i) *mexAB-oprM* was the first operon found in 1993 with substantial homology to *acrAB/TolC* of *E. coli* conferring multiple antibiotic resistance, including quinolones such as nalidixic acid and ciprofloxacin in *nalB* mutants (Poole *et al*., 1993). MexAB-OprM has been reported to play a main role in conferring intrinsic resistance to several antibiotics, including quinolones, due to its constitutive expression (Li *et al*., 1995). It extrudes a wide range of diverse unrelated antibiotics: quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin and β-lactams (except imipenem) are susceptible to be pumped out (Vila and Martínez, 2008), (ii) *mexCD-oprJ* was described in 1996 as the efflux pump whose overexpression was responsible for the *nfxB*-type MAR phenotype (Poole *et al*., 1996a), (iii) in 1997, *mexEF-oprN* was shown to confer antibiotic resistance in *nfxC*-type mutants (Kohler *et al*., 1997; 1999), (iv) *mexXY* was studied in *E. coli* in 1999 for its ability to cause antibiotic resistance in conjunction with TolC or OprM (Mine *et al*., 1999), (v) *mexJK* was characterized in 2002 (Chuanchuen *et al*., 2002), (vi) *mexHI-opmD* was reported for its MAR-associated phenotype in 2003 (Sekiya *et al*., 2003), (vii) *mexVW* was found in 2003 and could be selected for its overexpression in MAR mutants (Li *et al*., 2003), (viii) *mexPQ-opmE*, and (ix) *mexMN* in 2005 revealed the two latest RND efflux pumps characterized to date (Mima *et al*., 2005). A great number of these efflux pumps are composed by a three-gene operon (*mexAB-oprM*, *mexCD-oprJ*, *mexEF-oprN*, *mexHI-opmD* and *mexPQ-opmE*), being the first gene the MFP, the second one the efflux protein, and the third gene the outer membrane protein. The other four pumps are just a common two-gene operon (*mexXY*, *mexJK*, *mexVW* and *mexMN*). Despite the lack of a final gene encoding for a porin, it does not mean that it is not needed. In fact, all these four pumps can work together with OprM (Mine *et al*., 1999; Chuanchuen *et al*., 2002; Li *et al*., 2003; Mima *et al*., 2005) or even with a hitherto uncharacterized porin (Chuanchuen *et al*., 2002; Li *et al*., 2003). This may be why a promoter-like sequence has been found upstream from the *oprM* gene permitting weak expression to ensure the functioning of the other two-gene efflux pumps in the case that MexAB is not functional (Zhao *et al*., 1998; Masuda *et al*., 2000). However, not all these pumps are able to extrude quinolones; there are two exceptions: MexJK-OprM (Chuanchuen *et al*., 2002) and MexMN-OprM (Mima *et al*., 2005), although in latter case is doubtful due to a low level of expression of the pump.

All these operons have their own promoter in the upstream region, and in some cases the presence of a regulatory gene upstream from the promoter has been

demonstrated. *MexR* is transcribed divergently from *mexAB* and acts as a local repressor for this operon by binding to the promoter (Poole *et al*., 1996b). Two new ORFs have recently been characterized as additional repressors of the pump. The *nalC* gene is a member of the TetR/AcrR family located upstream from a two-gene operon. It has been suggested that the resulting proteins of this operon may modulate MexR levels and cause an indirect negative effect over the *mexAB-oprM* genes (Cao *et al*., 2004). NalD is another member of the TetR/AcrR family, which binds to a second promoter of the *mexAB-oprM* operon, causing a repressor effect by a direct mechanism, such as MexR (Sobel *et al*., 2005a; Morita *et al*., 2006). Overexpression of this pump has been implicated in the acquisition of quinolone resistance among clinical isolates, and several studies have reported a link between the presence of mutations in any of the regulatory loci and an increase in MexAB-OprM (Oh *et al*., 2003; Llanes *et al*., 2004; Henrichfreise *et al*., 2007).

MexCD-OprJ, the second efflux pump characterized, does not seem to be expressed during growth under laboratory conditions, thus no role in intrinsic resistance has been related to it (Poole *et al*., 1996a; Li *et al*., 2000). Upstream from this operon, an ORF, *nfxB*, transcribed divergently has been reported to encode a repressor protein of the pump (Poole *et al*., 1996a). The overexpression of the *mexCD-oprJ* operon confers extrusion ability regarding quinolones, macrolides, lincomycin, novobiocin, penicillins (except carbenicillin and sulbenicillin), cephems (except ceftazidime), flomoxef and meropenem, and with lower specificity tetracyclines and chloramphenicol (Vila and Martínez, 2008). This phenotype appears in clinical strains as a consequence of mutations within *nfxB* gene, with two amino acid changes: Asp-56–Gly and Ala-124–Glu, being the most frequently found (Higgins *et al*., 2003; Henrichfreise *et al*., 2007). However, some cases have reported high levels of MexCD-OprJ not linked with a mutation in *nfxB* (Oh *et al*., 2003). Interestingly, *P. aeruginosa* clinical strains isolated from cystic fibrosis patients, which have evolved to a quinolone-resistant phenotype, show MexCD-OprJ overexpression as a predominant mechanism (Jalal *et al*., 2000). Alternatively, increased levels of this pump have also been shown to appear owing to inactivation of the *mexAB-oprM* genes, in an attempt to compensate for the loss of the main efflux pump activity. However, in this situation, the levels produced are not as high as those reached by a mutation within *nfxB*; thus the final phenotype shows higher susceptibility levels to all antibiotics in comparison with strains that have a functional MexAB-OprM (Li *et al*., 2000). Following this inverse correlation, the MexAB-OprM levels decrease when MexCD-OprJ is overexpressed (Li *et al*., 2000).

MexEF-OprN is the third most relevant efflux pump described to date with regard to quinolone resistance. This efflux pump, which can extrude fluoroquinolones, chloramphenicol, trimethoprim and tetracycline (Vila and Martínez, 2008), has a particular genetic structure: upstream from the *mexE* there is no repressor gene transcribed divergently, but there is an ORF, called *mexT*, encoding a protein, which belongs to the LysR family of transcriptional activators, and is transcribed in the same direction as the other three genes. MexT is essential for the *mexEF-oprN* activation (Kohler *et al*., 1999). In addition, adjacent to and activated by *mexT* is a further gene, transcribed divergently, termed *mexS* [previously *qrh* (Kohler *et al*., 1999)], which does encode a regulatory protein, as it is, in fact, an oxidoreductase, but can confer the MAR phenotype by enhancement of MexEF-OprN overexpression (Sobel *et al*., 2005b). The *mexEF-oprN* is not expressed under standard growth in laboratory conditions. Moreover, its expression is not activated in the presence of the antibiotics susceptible to be pumped out (Kohler *et al*., 1999). However, wild-type strains can show a diverse degree of expression in basal conditions (Li *et al*., 2000). The operon can be activated by either a mutation in *mexT* (Maseda *et al*., 2000) or a mutation in *mexS* (Sobel *et al*., 2005b; Henrichfreise *et al*., 2007). Similar to what occurs with MexCD-OprJ, MexEF-OprN overexpression is also a predominant mechanism in *P. aeruginosa* clinical strains that have been isolated from cystic fibrosis patients with a quinolone-resistance phenotype. In addition, it is possible to find both pumps overexpressed at the same time (Jalal *et al*., 2000).

The first two-gene operon characterized was *mexXY*. The substrate specificity of this pump relies on exporting quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, aminoglycosides, penicillins (except carbenicillin and sulbenicillin), cephems (except cefsulodin and ceftazidime) and meropenem (Vila and Martínez, 2008). Upstream from *mexX*, an adjacent gene, *mexZ*, has been characterized, which is transcribed in the opposite direction and encodes a member of the TetR/AcrR family (Ramos *et al*., 2005). MexZ is a repressor protein that binds to the pump promoter and inhibits MexXY expression. In contrast with the other members of this family, MexZ does not interact with the respective multidrug transporter substrate to turn on the corresponding operon, although it is known that drugs activate the efflux pump (Matsuo *et al*., 2004). An implication in contributing to intrinsic resistance has been reported, although no significant role in extruding quinolones has been shown in wild-type strains (Masuda *et al*., 2000). The prevalence of overexpression in MexXY can be important as it is found as an efflux pump mechanism implicated in conferring the quinolone-resistance phenotype among clinical *P. aeruginosa* isolates (Oh *et al*., 2003). Some studies have

revealed that this pump can be activated in a great percentage of the clinical isolates studied (Llanes *et al*., 2004; Henrichfreise *et al*., 2007), and characterization of the *mexZ* gene mutations appears to be the genetic variation responsible for the overexpression. In addition, it has been suggested that an interplay between the different efflux pumps may take place (Li *et al*., 2000; Sobel *et al*., 2005b).

The *mexHI-opmD* and *mexPQ-opmE* are the two other three-gene operons currently described in *P. aeruginosa*. When overexpressed '*in vitro*', MexHI-OpmD can extrude norfloxacin, nalidixic acid, kanamycin, spectinomycin, carbenicillin, tetracycline, chloramphenicol and rifampicin (Sekiya *et al*., 2003), whereas MexPQ-OpmE confers resistance to quinolones, tetracycline, erythromycin, kitasamycin, rokitamycin and chloramphenicol (Vila and Martínez, 2008). The three other remaining two-gene operons, *mexJK*, *mexVW* and *mexMN*, work in conjunction with OprM, but only MexVW has been reported to confer quinolone resistance in '*in vitro*' assays, as well as tetracycline, chloramphenicol and erythromycin (Vila and Martínez, 2008); whereas MexJK only extrudes erythromycin and tetracycline, and MexMN chloramphenicol and thiamphenicol (Vila and Martínez, 2008). To date, none of these five pumps has a known ORF encoding a regulatory protein of the operon, and no constitutive expression in basal growth or implication in conferring quinolone-resistance phenotype by means of its overexpression has been detected.

• *Acinetobacter baumannii.* The intrinsic resistance in *A. baumannii* that let it become the paradigm of multiresistant bacteria has been attributed to low number and size of the porins simultaneously with low-level constitutive expression of efflux pump(s). The main porin contributing to intrinsic resistance characterized so far has been HMP-AB, a homologue of OmpA from *Enterobacteriaceae* and OprF from *P. aeruginosa* (Vila *et al*., 2007). The first efflux pump characterized was found to be encoded in the *adeABC* operon, which encodes three consecutive proteins (AdeA, AdeB and AdeC) forming an RND efflux pump (Table 5), showing homology with the three-gene operons described in *P. aeruginosa*. The substrates susceptible to extrusion are: aminoglycosides, tetracyclines, fluoroquinolones, erythromycin, chloramphenicol, trimetoprim and cefotaxime (Magnet *et al*., 2001). The essential role for efflux functioning does not seem to be equally played by every member of the pump, as inactivation of *adeB* leads to a decreased MIC to all these antimicrobial agents, whereas the inactivation of *adeC* does not seem to have any consequence in the MAR phenotype, meaning that AdeC can be replaced by another porin (Magnet *et al*., 2001; Marchand *et al*., 2004). Expression of the *adeABC* operon is regulated by the *adeRS*, a two-component regulatory system which is co-transcribed in the opposite direction (Koretke *et al*., 2000). Gentamicin-resistant strains acquired '*in vitro*' showing a MAR phenotype were studied and two different kinds of mutations appeared: in the amino acid codon a Thr-1533–Met in AdeS and in the amino acid codon Pro-1163–Leu in AdeR. These results have suggested that MAR phenotype appears as a consequence of *adeABC* overexpression, which in turn may be a response when mutations in the regulatory loci are present (Marchand *et al*., 2004). The clinical implication of the overexpression of this pump was shown as strains recovered from two different outbreaks. In one group, MAR mutants showed a high level of expression of *adeABC* genes; while in the second group, the MAR phenotype was not linked with this efflux pump overexpression (Higgins *et al*., 2004).

adeIJK is the second RND efflux pump characterized in *A. baumannii* (Table 5). It extrudes β-lactams, chloramphenicol, tetracyclines, erythromycin, fluoroquinolones, trimetoprim, fusidic acid, novobiocin, lincosamides and rifampin. It has not been possible to clone these genes in plasmids probably because expression is toxic. This pump is found in all laboratory and clinical isolates, as well as the corresponding homologues in the other *Acinetobacter* species, implying a potential role in intrinsic resistance (Damier-Piolle *et al*., 2008). AbeM is another efflux pump characterized in *A. baumannii* that belongs to the MATE superfamily, which shows high homology with YdhE of *E. coli*. The substrates susceptible to be pumped out with this efflux pump are fluoroquinolones and gentamicin, whereas those with lower affinity include kanamycin, erythromycin, chloramphenicol and trimetoprim. No clinical data are available at present (Su *et al*., 2005).

• *Stenotrophomonas maltophilia.* In this group of microorganisms *Stenotrophomonas maltophilia* need special attention because quinolone resistance acquisition is not related to mutations in the *gyrA* and/or *parC* genes (Ribera *et al*., 2002a; Valdezate *et al*., 2002). Quinolone resistance is acquired by high efficiency of efflux pumps, which reduces intracellular quinolone concentrations to a level at which the quinolone targets are not under challenge (Ribera *et al*., 2002b; Valdezate *et al*., 2005) (Table 2). Up today, two efflux systems have been identified in this pathogen (Table 5): SmeDEF (Alonso and Martínez, 2000) and SmeABC (Li *et al*., 2002).

Studies of the prevalence of *smeDEF* and *smeABC* overexpression have been carried out. It has been shown that *smeDEF* overexpression may occur in approximately 32% of the clinical isolates tested (Alonso and Martínez, 2001; Chang *et al*., 2004) whereas *smeABC* overexpression can be found in 59% (Chang *et al*., 2004). Both overexpressions are related to quinolone-resistant strains.

Table 2. The most frequent amino acid substitutions found in GyrA and ParC of non-fermentative Gram-negative bacilli.

Microorganism	Amino acid change				MIC (mg l^{-1}) CIP
	GyrA		ParC		
Pseudomonas aeruginosa wt	Thr-83	Asp-87	Ser-80	Glu-84	
	Ile				2
	Ile		Leu		8
	Ile			Lys	32
	Ile	Gly/Asn	Leu		128
Acinetobacter baumannii wt	Ser-83	Asp-87	Ser-80	Glu-84	
	Leu				4–64
	Leu		Leu		32–128

MIC, minimum inhibitory concentration; CIP, ciprofloxacin.

Microorganisms lacking Topo IV. Campylobacter jejuni, *Corynebacterium* spp., *Helicobacter pylori* and other microorganisms lack Topo IV. In these microorganisms a single mutation in the *gyrA* gene produces a high level of resistance to ciprofloxacin whereas a double mutation in the *gyrA* gene is necessary to produce a high level of resistance to moxifloxacin (Ruiz *et al*., 2005; Sierra *et al*., 2005a) (Table 3).

Although this species can become ciprofloxacin-resistant only with the acquisition of one point mutation in *gyrA*, efflux mechanisms have also been described. The main efflux pump found in *C. jejuni* was dubbed CmeABC. This system can export fluoroquinolones, erythromycin, rifampin, tetracycline, chloramphenicol, β-lactams and ethidium bromide (Lin *et al*., 2002). Its levels of expression have not only been detected in wild-type strains but also in both kinds of MAR mutants, those selected '*in vitro*' by exposure to the antibiotic and clinical isolates. These results suggest that this efflux pump plays a role in intrinsic resistance to the antibiotics that can be transported due to its constitutive expression, as well as in conferring high levels of fluoroquinolone resistance (Luo *et al*., 2003; Lin *et al*., 2005). In addition, the inactivation of this pump leads to an increase in the susceptibility to fluoroquinolones in wild-type strains (Lin *et al*., 2002) and decreases the resistance phenotype below the clinical break points in resistant strains (Luo *et al*., 2003). Among clinical isolates it is possible to find several situations: (i) strains that show the maximum levels of ciprofloxacin resistance as a consequence of *cmeB* overexpression in addition to a *gyrA* mutation (64–128 mg l^{-1}), (ii) only a *gyrA* mutation (8–64 mg l^{-1}), (iii) only *cmeB* overexpression (2–32 mg l^{-1}), and (iv) MAR phenotype not affecting ciprofloxacin, so that there is no *gyrA* mutation and no *cmeB* overexpression (0.25–0.5 mg l^{-1}) (Pumbwe *et al*., 2004). The CmeR regulates the *cmeABC* expression. This repressor belongs to the TetR/AcrR family of regulators (Ramos *et al*., 2005). Mutations affecting the normal function of this local repressor have also been shown in the substrate binding region (Pumbwe *et al*., 2004) and in the *cmeABC* promoter (Lin *et al*., 2005).

A second RND efflux system encoded by *cmeDEF* contributes to the intrinsic resistance in *C. jejuni* (Akiba *et al*., 2006). However, its overexpression in *C. jejuni* clinical isolates does not contribute to the ciprofloxacin efflux (Pumbwe *et al*., 2004; Ge *et al*., 2005).

Gram-positive bacteria. The first mutation associated with fluoroquinolone resistance in *S. aureus* is usually found in the *parC* gene (*grlA* gene in this microorganims) (Table 4), hence Topo IV is considered the primary target for fluoroquinolones. A mutation at the amino acid codon Ser-80 (changing to Phe) of the *grlA* gene produces a MIC of norfloxacin of 4 mg l^{-1}, whereas a double mutation at the amino acid codon Ser-80 of the *grlA* gene plus a mutation at the amino acid codon Ser-84 (changing to Leu) increases the MIC of norfloxacin to 16 mg l^{-1} and three mutations, two in the *grlA* gene and a third in the *gyrA* gene, generate a MIC of norfloxacin of 128 mg l^{-1} (Table 4) (Sierra *et al*., 2002). However, in *Streptococcus pneumoniae* the primary target can be both DNA gyrase and Topo IV, depending on the fluoroquinolone (Pan and

Table 3. The most amino acid substitutions found in GyrA and ParC of different microorganisms lacking topoisomerase IV.

Microorganism	Amino acid change		MIC (mg l^{-1})	
	GyrA		CIP	MOX
Campylobacter spp. wt	Thr-86	Asp-90		
	Ile		> 32	0.38–2
	Ile	Asn	> 32	> 32
Corynebacterium spp.	Ser-87	Asp-91		
	Phe		1	0.19
		Tyr	3	0.5
	Phe	Ala	> 32	6

CIP, ciprofloxacin; MIC, minimum inhibitory concentration; MOX, moxifloxacin.

Table 4. Amino acid substitutions found in GyrA and ParC of different Gram-positive cocci.

Microorganism	Amino acid change				MIC (mg l^{-1}) NOR
	GyrA		Parc		
Staphylococcus aureus wt	Ser-84	Asp-88	Ser-80	Glu-84 (GrlA)	
			Phe		4
	Leu		Phe		16
	Leu		Phe	Lys	128
Streptococcus pneumoniae wt	Ser-83	Asp-87	Ser-79	App-83	
			Tyr/Phe		8
	Tyr		Tyr		64

MIC, minimum inhibitory concentration; NOR, norfloxacin.

Fisher, 1999; Sierra *et al*., 2005b). In addition to point mutations in the genes encoding the protein targets, acquisition resistance by intra- or interspecific recombination has been shown (Balsalobre *et al*., 2003; de la Campa *et al*., 2004; Stanhope *et al*., 2005). However, in clinical isolates point mutations are more frequent than recombinant ones (Ferrándiz *et al*., 2000).

Drug efflux transporters of Gram-positive bacteria mainly belong to MFS superfamily, although members of SMR and ABC families have also been characterized. Furthermore, members of MATE family have also been observed although they were believed to exist only in Gram-negative bacteria (Poole, 2000b).

• *Staphylococcus aureus.* The first efflux pump characterized from a norfloxacin-resistant clinical strain was NorA (Table 5), a member of the MFS superfamily that when overexpressed from a plasmid yielded a higher MIC for norfloxacin and ciprofloxacin. The role of NorA in quinolone-susceptible strains became clear after its inactivation, as the resulting strain showed an eightfold decrease in the MIC of norfloxacin in addition to a threefold increase of the same antibiotic internal accumulation (Yamada *et al*., 1997). The mechanisms by which *norA* is overexpressed in clinical isolates have been reported. The first mutation identified linked to this phenotype was a mutation in the *norA* promoter most likely resulting in prevention of repressor binding (Ng *et al*., 1994). In other cases high levels of NorA resulted from increased stability of its mRNA (Fournier *et al*., 2001; Kaatz *et al*., 2005b). Increased levels of NorA were also linked to the two-component regulatory system ArlSR (Koretke *et al*., 2000). Inactivation of ArlS resulted in an increase in NorA production (Fournier *et al*., 2000). The third option is the presence of a transcriptional regulator, MgrA (formerly NorR), which has been shown to bind specifically to the *norA* promoter. It was previously assumed to play a positive role in *norA* expression (Truong-Bolduc *et al*., 2003); however, a new study has shown that in fact this regulator causes a negative effect on NorA levels, suggesting that the first role as an activator could be due to high levels of the MgrA protein (Kaatz *et al*., 2005b). Recently, another regulator, dubbed NorG, has been proposed. This protein is able to bind to its own promoter, meaning that it can be autoregulated, and to *norA* promoter although it has not resulted in any increase in *norA* transcripts. Intriguingly it cannot bind to *mgrA* promoter, whereas MgrA can bind to *norG* promoter, indicating that MgrA may play a more global effect than NorG does (Truong-Bolduc and Hooper, 2007).

Table 5. Fluoroquinolone efflux transporters characterized to date and their clinical implication.

Microorganism	Efflux pumps that can extrude fluoroquinolones in a reproducible manner[a]				
	RND	MFS	MATE	ABC	SMR
Escherichia coli	**AcrAB**, AcrEF	MdfA	YdhE		
Salmonella enterica	**AcrAB**, AcrEF				
Klebsiella pneumoniae	**AcrAB**	KmrA, KdeA			
Pseudomonas aeruginosa	**MexAB-OprM**, **MexCD-OprJ**, **MexEF-OprN**, **MexXY**, MexVW, MexHI-OpmD, MexPQ-OpmD		PmpM		
Stenotrophomonas maltophilia	**SmeDEF**, **SmeABC**				
Acinetobacter baumannii	**AdeABC**, AdeIJK		AbeM		
Campylobacter jejuni	**CmeABC, CmeDEF**				
Staphylococcus aureus		**NorA**, **NorB**, **NorC**, **SdrM**	MepA		
Streptococcus pneumoniae		**PmrA**		**PatA, PatB**	

a. Boldface type indicates those efflux pumps found overexpressed in fluoroquinolone-resistant clinical isolates.

Alternatively, other pumps have been described with ability of extruding quinolones. This can be the case of NorB, NorC, MepR and SdrM (Table 5). NorB is a member of the MFS superfamily and pumps out norfloxacin and ciprofloxacin as NorA does, but in addition it can recognize moxifloxacin and sparfloxacin as substrates, causing, when overexpressed, an eightfold increase in the MICs of the first two antibiotics and a fourfold increase in the others. It has been reported a low-level expression in wild-type strains suggesting that it may play a role in determining susceptibilities in quinole-susceptible strains as NorA does (Truong-Bolduc *et al*., 2005). This pump is negatively regulated by MgrA, which binds directly to the *norB* promoter which contains less binding motifs than those found in *norA* promoter resulting in a weaker interaction (Truong-Bolduc *et al*., 2005). In addition, NorG has been shown to bind directly to *norB* promoter and cause a positive effect by increasing *norB* transcripts. However, inactivation of *norG* in a wild-type strain does not lead to any change in the MIC of fluoroquinolones (Truong-Bolduc, and Hooper, 2007).

NorC is another MFS member and shows substantial homology with NorB protein. Its overexpression causes a fourfold increase in the MIC of norfloxacin and moxifloxacin and reproducible twofold increase in the MIC of ciprofloxacin and sparfloxacin, resulting in the same substrate profile as NorB. Its regulation is based on the negative effect that MgrA causes over its transcription (Truong-Bolduc *et al*., 2006).

The overproduction of the MFS SdrM leads to a twofold increase in the MIC of norfloxacin. No basal expression has been detected in standard laboratory conditions suggesting that activation must occur in order to detect it. No clear role of MgrA on SdrM expression has been reported (Yamada *et al*., 2006).

The last efflux pump is a MATE superfamily member called MepA (Table 5). This protein is encoded within a three-gene operon *mepRAB*. The first gene encodes for a transcriptional regulator with strong homology with MarR, which acts as a repressor of its own transcription and MepA expression. The second gene encodes the efflux pump, while the third gene codifies for a protein with no homology to any protein with known function. Two '*in vitro*' selected fluoroquinolone-resistant mutants overexpressed this pump as a consequence of mutations within the *mepR*-encoding region in one case, leading to a truncated protein and a consequent lack of repressor activity; however, no detectable nucleotide mutation was found in the operon of the second strain, suggesting that another regulatory loci must be implicated in conferring increased levels of this pump. In both mutant strains *mepR* and *mepRAB* transcripts can be detected (Kaatz *et al*., 2005a).

There are few reports about the prevalence of the overexpression of these efflux pumps in clinical isolates. A recent report has analysed the prevalence of all known efflux pumps (with exception of SdrM) and suggests that around 50% of the clinical strains evaluated had at least one efflux system implicated in quinolone resistance. Increased expression of NorA and NorB were the most predominant mechanisms, although overexpression of NorC and MepA were also observed. The concomitant overproduction of several pumps (20% of the effluxing strains) was also described, being the increased levels of NorB and NorC what predominated. Mutations such as point mutations, insertions or deletions, in *mepR*, *norA*, *norB* and *norC* promoters, were described in overexpressing strains respectively. In addition, mutations in structural genes, such as those mutations leading to a MepR-truncated protein, were also found. However no mutation affecting MgrA protein levels was seen (DeMarco *et al*., 2007).

• *Streptococcus pneumoniae.* Efflux pump as a mechanism of fluoroquinolone resistance in *S. pneumoniae* is not well elucidated. To date, only two efflux pumps have been characterized (Table 5). The first one is PmrA, a protein belonging to the MFS superfamily showing substantial homology with NorA, and whose overexpression leads to an increase in the MIC of norfloxacin and ciprofloxacin which can decrease up to fourfold upon reserpine addition. Furthermore, ethidium bromide accumulation is decreased in the strain that overexpresses this efflux system but it totally reverts to wild-type levels when *pmrA* has been inactivated, suggesting its role as an efflux system (Gill *et al*., 1999). Its prevalence among clinical isolates has been reported; however, a clear role has not been elucidated as it shows variable levels of expression among norfloxacin-resistant and -susceptible strains, as well as resistant strains do not always show a phenotype affected by reserpine. In general, it appears in addition to QRDR mutations and it is not well associated with strains showing a high level of norfloxacin resistance. Furthermore, susceptible strains showing increased levels of PmrA without an effect of reserpine have been found (Piddock *et al*., 2002).

Its basal expression in laboratory strains and fluoroquinolone-susceptible clinical isolates let think about a possible role in determining the intrinsic susceptibilities of these agents in standard conditions (without drug exposure). However, its role in acquiring fluoroquinolone resistance can be limited (Piddock *et al*., 2002; Avrain *et al*., 2007).

The second efflux system characterized is that formed by two ABC transporters, PatA and PatB. Yet, it is not clear if they could interact so as to constitute a heterodimeric efflux system as it happens in other Gram-positive bacteria, or maybe they are two independent systems that both contribute to the final multidrug-resistance phenotype. Its

overexpression has only been found in resistant mutants, where its inactivation has led to a loss of multidrug-resistance phenotype. However, reserpine only seems to inhibit PatA contribution to resistance, whereas PatB would not be affected. Intriguingly, when *patA* has been inactivated, an increase in *patB* expression occurs, but not vice versa (Marrer *et al*., 2006; Avrain *et al*., 2007; Garvey and Piddock, 2008). Among clinical isolates high levels of both PatA and PatB have been found as a mechanism of resistance, instead of PmrA overexpression, around 25% of the isolates evaluated (Garvey and Piddock, 2008). Currently, it is thought that maybe PmrA is not the main efflux system characterized, as it seems that overexpression of PatA/B is more prevalent among clinical isolates. However other systems could be implicated and not necessarily reserpine-susceptible.

Plasmid-mediated quinolone resistance

In 1998, the first plasmid-mediated mechanism of resistance to quinolones was described in *K. pneumoniae* (Martínez-Martínez *et al*., 1998). This was due to the *qnrA* gene, which encodes for a pentapeptide repeat protein (Tran and Jacoby, 2002; Tran *et al*., 2005a,b). As expected from its structure, Qnr determinants did not seem to produce a change in intracellular quinolone accumulation nor did it cause drug inactivation. The direct effects of the Qnr have been studied using DNA-supercoling assays. At least when performed '*in vitro*', Qnr protects the DNA gyrase from the inhibition of ciprofloxacin. This protection is dependent on Qnr concentration and is inversely proportional to ciprofloxacin concentrations (Tran *et al*., 2005a). Moreover, Topo IV, the secondary target of quinolones in *Enterobacteriaceae*, also seems to be protected from quinolones by Qnr.

However, the expression of the Qnr peptide results in low-level quinolone resistance. Since the first report of this mechanism of resistance to quinolones, a large number of studies addressed to find this gene in different collections of clinical isolates have been reported. Up to the present, three *qnr* genes have been identified: the *qnrA* gene found in *K. pneumoniae*, and later found in other *Enterobacteriaceae*; *qnrS*, first described in *Shigella flexneri* (Hata *et al*., 2005) and the *qnrB* gene located on plasmids found in *K. pneumoniae*, *Citrobacter koseri*, *E. cloacae* and *E. coli* (Jacoby *et al*., 2003).

After the first prevalence survey of the *qnrA* gene in 350 Gram-negative isolates in which this gene was not found (Jacoby *et al*., 2003), several reports suggested that this plasmid was widely distributed and was present in all clinically relevant *Enterobacteriaceae* (Wang *et al*., 2003; 2004; Cheung *et al*., 2005; Jeong *et al*., 2005; Jonas *et al*., 2005; Mammeri *et al*., 2005; Nazik *et al*., 2005; Robicsek *et al*., 2005). Although these genes have not been found in non-fermenting Gram-negative bacilli such as *P. aeruginosa* and *A. baumannii*, it is important to point out that the *qnrS* gene has been found in *Aeromonas* spp. isolated from both environment and clinical samples (Cattoir *et al*., 2008; Sánchez-Céspedes *et al*., 2008).

The *qnrA* gene has recently been identified in the chromosome of the water-borne species *Shewanella algae* (Poirel *et al*., 2005a). The G+C content of the *qnrA*-like gene of *S. algae* matches that of the genome exactly, suggesting that this microorganism may be the origin of the *qnrA* gene. Moreover, *Vibrionaceae* may also constitute a reservoir for Qnr-like quinolone-resistance determinants (Poirel *et al*., 2005b). However, a *qnr*-like gene has recently been found in *Enterococcus faecalis*, suggesting that the expression of this gene may explain the intrinsic resistance of *E. faecalis* to fluoroquinolones (Arsène *et al*., 2007).

If Qnr is the only mechanism of resistance to quinolones present, the MIC of ciprofloxacin will increase only to 0.25 mg l^{-1}, hence being considered susceptible. Although the action of Qnr results in low-level quinolone resistance, this reduced susceptibility facilitates the selection of mutants with higher-level resistance (Martínez-Martínez *et al*., 1998). It is thought that this low level of resistance to the antibacterial agent makes it possible for bacteria populations to raise concentrations that facilitate the occurrence of secondary mutations and thus the high level of resistance.

Recently, a new mechanism of transferable quinolone resistance has been reported: enzymatic inactivation of certain quinolones. The *cr* variant of *aac(6′)-Ib* encodes an aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin by N-acetylation of its piperazinyl amine (Robicsek *et al*., 2006). Aac(6′)-Ib-cr has two amino acid changes, Trp-102–Arg and Asp-179–Tyr, which together are necessary and sufficient for the ability of the enzyme to acetylate ciprofloxacin. The *aac(6′)-Ib* gene encodes a common aminoglycoside acetyltransferase responsible for resistance to the aminoglycosides such as kanamycin, amikacin and tobramycin. A plasmid containing this new variant of *aac(6′)-Ib* was cloned resulting in MICs to kanamycin of 64 μg/ml, as expected, and a three- to fourfold increase in the MIC of ciprofloxacin in *E. coli* DH10B. This new variant was then called *aac(6′)-Ib-cr* for ciprofloxacin resistance (Robicsek *et al*., 2006). Not only Aac(6′)-Ib-cr has been described to cause low-level ciprofloxacin resistance, but it also acts additively together with Qnr to generate ciprofloxacin resistance. In fact, when both *qnrA* and *aac(6′)-Ib-cr* are present in the same bacteria, the level of resistance to ciprofloxacin is increased fourfold more than that conferred by *qnrA* alone, with a MIC of ciprofloxacin of 1.0 μg ml^{-1}, a value near the clinical break point for susceptibility. In addition, the presence of *aac(6′)-Ib-cr* alone substantially increased the frequency of

selection of chromosomal mutants upon exposure to ciprofloxacin. Moreover, the *aac(6′)-Ib-cr* gene has also been frequently located in the same genetic element as the bla_{CTX-M} gene (Pitout *et al.*, 2008). Park and colleagues (2006), on analysing 313 *Enterobacteriaceae* with a MIC of ciprofloxacin ≥ 0.25 mg l^{-1}, found that 14% carried the *aac(6′)-Ib-cr* gene.

A new plasmid-mediated quinolone-resistance mechanism has recently been described (Yamane *et al.*, 2007). This new mechanism consists of a gene named *qepA* that encodes for an efflux pump. QepA showed high similarity with members of the Major Facilitator Superfamily responsible for resistance to hydrophilic quinolones such as norfloxacin and ciprofloxacin. This gene is located in a 10 kb region flanked by two copies of IS*26*. Recently, after analysis of the prevalence of the *qepA* and *qnr* genes in a collection of 751 *E. coli* clinical isolates, Yamane and colleagues (2008) found only two isolates (0.3%) that carried this gene and they did not find any isolate carrying the *qnr* genes. Following the recent discovery of resistance by target protection and enzyme inactivation, efflux represents a third new plasmid-mediated mechanism of resistance to fluoroquinolones. Neither of these latter mechanisms affects the action of nalidixic acid.

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