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REVIEW ARTICLE

Macrolide resistance mechanisms in *Enterobacteriaceae*: Focus on azithromycin

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ABSTRACT

From its introduction in 1952 onwards, the clinical use of macrolides has been steadily increasing, both in human and veterinary medicine. Although initially designed to the treatment of Gram-positive microorganisms, this antimicrobial family has also been used to treat specific Gram-negative bacteria. Some of them, as azithromycin, are considered in the armamentarium against *Enterobacteriaceae* infections. However, the facility that this bacterial genus has to gain or develop mechanisms of antibiotic resistance may compromise the future usefulness of these antibiotics to fight against *Enterobacteriaceae* infections. The present review is focused on the mechanisms of macrolide resistance, currently described in *Enterobacteriaceae*.

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General introduction

History

In 1942, proactinomycin was isolated from *Streptomyces gardneri* (Gardner & Chain, 1942). Although this was the first member of the macrolide family to be isolated, the macrolide era was not considered to begin until the description of picromycin, a natural antibiotic produced by *Streptomyces felleus* (Brockmann & Henkel, 1950). Indeed, this era actually began with the introduction into clinical practice of erythromycin [isolated from a *Saccharopolyspora erythraea* culture, formerly known as *Streptomyces erythraeus* (Labeda, 1987)] in 1952 (McGuire et al., 1952).

During the 1950s, a long series of macrolides was isolated from natural sources, like carbomycin in 1952 (Tanner et al., 1952); griseomycin, leucomycin and methymycin in 1953 (Donin et al., 1953; Hata et al., 1953; Van Dijck et al., 1953); angolamycin, narbomycin, oleandomycin and spiramycin in 1955 (Corbaz et al., 1955a, b; Pinnert-Sindico et al., 1955; Sobin et al., 1955); miamycin in 1957 (Schmitz et al., 1957) and lankamycin in 1960 (Gäumann et al., 1960) among others. Subsequently, modifications in the macrolide basal structure led to the development of new subgroups of macrolides such as the azalides, with azithromycin

being the first and most representative member (Mutak, 2007), or ketolides such as telithromycin (Ackermann & Rodloff, 2003; Zuckerman, 2004) (Figure 1). New antibiotic macrolides with other number of carbon atoms (e.g. fidaxomicin a new 18C macrolide) have been recently introduced in clinical practice (Vaishnavi, 2015).

Structure and classification

The macrolide structure possesses a characteristic macrolactonic ring which is attached to a series of sugars, aminosugars and other side chains (Figure 2). In some cases, the macrolide is in fact a pool of closely related molecules as in the case of erythromycin, the major component of which is the so-called erythromycin A. However, traces of other variants presenting minimal differences in chemical composition are also present, such as erythromycin B, C and D which are, in fact, erythromycin synthesis intermediates (Reeves et al., 1999; Zhang et al., 2011).

Different classifications schemes have been elaborated. The most widely used are probably those related to the size of the macrolactonic ring or those based on structural modifications. Thus, in the first aforementioned classification, three main subgroups (14C, 15C and 16C) have been defined according to

Figure 1. Chronology of the macrolide history (1940-onwards). In the upper-side of the temporal line are indicated the years of discovering/synthesis of a series of representative macrolides. In all cases it is reported the data of the most ancient report found in the literature. In the lower-side of the temporal line are indicated a series of macrolide’s history milestones.

Figure 2. Chemical structure of different macrolides.

Table 1. General classification of the main macrolide antibiotics based on the number of carbons in the macrolactonic ring.

	Macrolide antibiotics				
	14C		15C	16C	
	Natural	Semisynthetic		Natural	Semisynthetic
Macrolides ^b	Erythromycin (SA) ^a	Clarithromycin (IA)	–	Spiramycin (SA) ^{a,c}	Miocamycin (SA)
	Oleandomycin (SA)	Roxithromycin (IA)	–	Josamycin (IA)	Rokitamycin (SA)
	Megalomicin	Flurithromycin (IA)	–	Rosaramicin (SA) ^d	Tilmicosin (LA) ^a
	–	Troleandomycin (SA)	–	Tylosin (SA) ^a	Tildipirosin (LA) ^a
	–	Dirithromycin (LA)	–	Mycinamicin	Tylvalosin (SA) ^a
	–	–	–	Carbomycin (SA) ^e	–
	–	–	–	Leucomycin (IA) ^f	–
	–	–	–	Midecamycin (SA)	–
Azalides	–	–	Azithromycin (LA)	–	–
	–	–	Gamithromycin (LA) ^a	–	–
	–	–	Tulathromycin (LA) ^a	–	–
Ketolides	Picromycin ^g	Telithromycin (IA)	–	–	–
	–	Cethromycin (IA)	–	–	–
	–	Modithromycin (LA)	–	–	–
	–	Solithromycin (IA)	–	–	–
Fluoroketolides	–	–	–	–	–

SA: short-acting (mean half life elimination <4 h); IA (mean half life elimination 4–24 h); LA (mean half life elimination >24 h).

^aUsed in veterinary practice in the European Union (erythromycin and spiramycin are also approved for human uses).

^bThose with a macrolactonic ring resembling erythromycin.

^cAlso named foromicidin.

^dAlso named rosamicin.

^eAlso named magnamycin.

^fAlso named kitasamycin.

^gPicromycin was reisolated by different authors several times in the 1950s being named as amaromycin, albomycetin and argomycin (Vanek & Majer, 1967).

the number of carbon atoms in the macrolactonic ring (Table 1 and Figure 2). Regarding structural modifications, four different classes have been described: those classically referred to as macrolides, such as erythromycin, which were the molecules first described; azalides, like azithromycin, that incorporate a nitrogen in the macrolactonic ring resulting in higher basicity and increasing both acid stability and bioavailability (Mutak, 2007); and ketolides, which possess a keto group at the C3 position of the lactone ring instead of the cladinose sugar found in erythromycin, enhancing their activity spectrum and improving their acid stability, and of which telithromycin is a representative class member (Ackermann & Rodloff, 2003; Schlünzen et al., 2003; Zhanel et al., 2002); finally, ketolides such as solithromycin, which incorporate a fluor atom in C2 (fluoroketolides), are currently under development (Llano-Sotelo et al., 2010; Putnam et al., 2010). Additionally, subclasses such as triamilides, which are represented by tulathromycin, a tribasic derivative azalide, have also been proposed (Letavic et al., 2002). Other classifications proposed are based on generations or mean plasma elimination half-life; thus, four generations are considered, with erythromycin representing the first, leucomycin the second, azithromycin the third and telithromycin the fourth (Morar et al., 2012). Regarding the mean plasma elimination half-time, three groups have been proposed: short- (<4 h), intermediate- (4–24 h) and long-acting (>24 h)

macrolides, with erythromycin, clarithromycin and azithromycin being representative members of each class, respectively (Adriaenssens et al., 2011). Macrolide resistance has also classically been classified following phenotypical criteria. Thus, phenotypes are usually classified as: MLS_B (resistant to macrolides, lincosamides and streptogramin B), either inducible (iMLS_B) or constitutive (cMLS_B); M (resistant to macrolides, but not to lincosamides or streptogramin B); ML (resistant to macrolides and lincosamides) and MS_B (resistant to macrolides and streptogramin B). Nonetheless, this classification is limited to Gram-positive microorganisms since some of these antimicrobial families have not been used to treat Gram-negative bacteria.

Moreover, natural or semisynthetic non-antibiotic macrolides with other numbers of carbon atoms with anti-inflammatory and immunomodulatory or immunosuppressor activities have been introduced into clinical practice. Some examples of these non-antibiotic macrolides are tacrolimus, a 23-membered macrolide lactone isolated from *Streptomyces tsukubensis* (Kino et al., 1987), used as an immunosuppressor in organ transplantations as well as in the treatment of diseases such as atopic dermatitis (Lubitz et al., 2006; Rodriguez-Cedeira et al., 2012); sirolimus (also named rapamycin), a 31-membered macrolide lactone indigenous of *Streptomyces hygroscopicus* (Vézina et al., 1975) with similar clinical uses (Maschan et al., 2014; Sutherland et al., 2014) and EM900, a erythromycin-derived

synthetic 12-membered macrolide lactone, which, among others, is currently under investigation (Otsu et al., 2011; Sugamata et al., 2014; Sugawara et al., 2011, 2012).

It should be noted that some natural macrolide-related molecules act as toxins, such as mycolactones, which are 12C-membered macrolide lactones. These molecules are produced by different members of the genus *Mycobacterium*, including *Mycobacterium marinum*, *Mycobacterium pseudoshottsii*, *Mycobacterium liflandii* (Hong et al., 2008; Kishi, 2011; Mve-Obiang et al., 2005; Ranger et al., 2006) or *Mycobacterium ulcerans*, the most relevant at human clinical level due to the production of a series of mycolactones considered to be responsible for the unique characteristics of Buruli ulcer (Adusumilli et al., 2005). At present, at least nine different *M. ulcerans* mycolactones have currently been described. They are produced in different proportions based on the geographical origin of the strain, and then, probably due to other more in depth differences, with mycolactone A/B being the most classically described and the predominant in isolates recovered from Africa (Hong et al., 2008).

Mechanism of action

Macrolides act at the 50S ribosomal subunit, binding in a reversible manner to the NPET of the ribosome, near the PTC. Interactions are established by hydrogen bonds with specific bases at domain V of 23S rRNA, including positions A2057, A2058, A2059, C2611 (*Escherichia coli* numbering of 23S rRNA is used throughout the manuscript) which are common in all macrolide-ribosome interactions (Kannan & Mankin, 2011). Other positions in 23S rRNA may play a role in macrolide-ribosome interactions based on the specific macrolide considered. Erythromycin, as well as macrolides possessing a cladinose sugar attached to C3, also interacts with C2610 and G2505. Other macrolides, such as josamycin or carbomycin, which possess a longer disaccharide C5 side chain, also interact with positions A2451 and C2452 where the PTC-A site is located (Kannan & Mankin, 2011).

Meanwhile, it has been reported that positions G748, A751 and A752 located within the loop of helix 35 of domain II are involved in tylosin-23S rRNA interactions in Gram-positive microorganisms (Hansen et al., 2002; Liu & Douthwaite, 2002a; Poehlsgaard & Douthwaite, 2005). Similarly, the role of position G745 in the tylosin-ribosome interactions in *E. coli* has been described (Liu & Douthwaite, 2002b), while positions G748 and A752 are reportedly involved in the interactions between ketolides and ribosomes

(Novotny et al., 2004). Additionally, both L4 and L22 proteins are implicated in this ribosome-macrolide interaction (Chittum & Champney, 1994; Gabashvili et al., 2001). Thus, according to the classical view of macrolide action, macrolides binding to the NPET clog up the ribosomal tunnel, thereby blocking the general protein synthesis in the early elongation steps (Figure 3) (Gamerding & Deuerling, 2012). However, it has been shown that the binding of macrolides like erythromycin to NPET leaves sufficient space to allow peptide elongation (Tu et al., 2005). In fact, it has recently been reported that peptide translation is not fully aborted in the presence of macrolides. Thus, in *E. coli*, residual translation in the presence of high concentrations of up to 100-fold the MIC of either erythromycin or telithromycin, is around 6% and 20–25%, respectively (Kannan et al., 2012). Additionally, the same authors have shown that peptide elongation arrest may take place at later stages of translation, resulting in the formation of large truncated proteins (Kannan et al., 2012). Telithromycin possesses higher bactericidal effects than erythromycin, which may be related to this higher residual translation and with the high number of nonfunctional proteins, which randomly interrupt biochemical pathways leading to the accumulation of potentially toxic metabolic intermediates or depletion of essential cofactors which may trigger a lethal cellular response (Kannan et al., 2012; Kohanski et al., 2007). Further studies have shown that the binding of macrolides and NPET allosterically affects the structure of PTC modifying the orientation of U2585 and A2602 (Sothiselvam et al., 2014). These data support the communication between NPET and PTC and that peptide arrest is related to the presence of specific three amino acids motifs, the most relevant being [K/R] X [K/R] or more generally [-+] X [+]. It is of note that the number of motifs resulting in protein stalling in the presence of erythromycin is higher than in the case of telithromycin (Kannan et al., 2014). Interestingly, some of these motifs may be found in the arrest zones of most of the leader peptides involved in macrolide-inducible resistance (see section *Methylases*) (Kannan et al., 2014; Ramu et al., 2009).

Thus, an alternative has been proposed to the classical view of the full transcription blocking the macrolide mode of action. In this model, macrolide binding to the NPET only results in partial lumen blocking, despite the pass constriction generated by the L4 and L22 proteins, not being sufficient to abolish peptide synthesis by itself. In this model, macrolide action is mediated by a selective inhibition of peptide bond formation

Figure 3. Interaction between macrolide and ribosome. (A) Transversal vision of a ribosome. The amino acids are ensambled in the PTC, being send to the cytoplasm through the tunnel. In the scheme are represented the position in which are located L4 and L22. (B) The interaction of the macrolide with the ribosome results in a blocking of the protein synthesis.

between specific combinations of donor and acceptor substrates (Kannan et al., 2014).

Additionally, it has been proposed that in concomitance with the aforementioned blocking of protein synthesis some macrolides, such as josamycin, may also indirectly inhibit peptide elongation through a peptidyl-tRNA drop-off leading to depletion of the tRNA isoacceptors (Lovmar et al., 2009b). Moreover, macrolides may also affect the normal assembly of the 50S subunit, resulting in incomplete, ribosomes that are degraded (Chittum & Champney, 1985).

Clinical use

These agents have classically been used in the treatment of infections related to Gram-positive microorganisms, such as respiratory tract infections associated with

Streptococcus pneumoniae, *Mycoplasma pneumoniae* or *Legionella pneumophila* among other microorganisms, soft tissue infections by *Staphylococcus aureus* or *Streptococcus pyogenes* or other infections such as those by *Helicobacter pylori* (Saraya et al., 2014; Uğurlu et al., 2014; Zuckerman, 2004). Furthermore, macrolides rank among the treatments considered for different infections due to Gram-negative microorganisms, such as *Bordetella pertussis*, *Bartonella* spp., *Chlamydia* spp., *Neisseria gonorrhoeae*, *Treponema* spp. or *Campylobacter* spp. (Allos, 2001; Anonymous, 2013; Cramer & Heininger, 2008; Jimenez et al., 2015; Mitjà et al., 2015; Rolain et al., 2004; Ruiz et al., 2007; Stoner, 2007; Zuckerman, 2004), and have shown activity against some protozoa such as *Plasmodium* spp. or *Toxoplasma gondii* (Lee et al., 2011; Pereira et al., 2011). Additionally, despite not showing good activity against non-fermenter Gram-negative microorganisms such as *Pseudomonas*, macrolides like azithromycin have demonstrated potential to disaggregate biofilms by this microorganism, and thus, their synergistic effect together with antipseudomonal antibiotics are currently under study to avoid and eradicate *Pseudomonas* biofilms (Saini et al., 2015). Moreover, the antiviral action of some macrolides, such as leucomycin, has recently been observed thereby opening the door to research on new macrolides with improved antiviral action (Sugamata et al., 2014).

Macrolides have been extensively used, that, in some countries as USA, macrolides rank among the most frequently prescribed antibacterial agents to outpatients, with azithromycin in the top position (Hicks et al., 2013).

Macrolides have not classically been considered for the treatment of *Enterobacteriaceae* infections due to the low levels of activity observed against these microorganisms (Stock & Wiedemann, 1999, 2000) related to their poor membrane permeability (Mao & Putterman, 1968). However, some macrolides have shown potential for use in the treatment of *Enterobacteriaceae* infections, with azithromycin, which possesses excellent clinical parameters against *Enterobacteriaceae* (Butler & Girard, 1993; Gordillo et al., 1993; Pons et al., 2013), being the most widely used. This fact has allowed its inclusion in the armamentarium to treat both diarrheogenic and systemic infections due to different *Enterobacteriaceae*, such as *Shigella* spp. or *Salmonella enterica* serovar Typhi (Erdman et al., 2008; Trivedi & Shah, 2012), and it is also used in the treatment of children and traveler's infections as an alternative to the usual antimicrobial agents (Basualdo, 2003; DuPont, 2009). Interestingly, unlike other macrolides, has shown no interactions with a series of drugs including antiretroviral agents such as

zidovudine (Chave et al., 1992; Nahata, 1996). This fact, together with its broad spectrum and safety, has led to azithromycin being proposed and considered as an alternative to cotrimoxazole in areas with endemic HIV1 in low and middle-income countries when antibiotic prophylaxis is needed in HIV patients (Van Oosterhout et al., 2005).

Recently the use of azithromycin in mass treatment to eradicate either trachoma or yaws has been proposed and evaluated (Jimenez et al., 2015; Mitjà et al., 2015). Interestingly, in a clinical trial on the eradication of trachoma developed in Ethiopia it was observed that the survival rate of children in the azithromycin arm was better than that of children receiving a placebo (Keenan et al., 2011). This finding might be related to the antibacterial activity, the long post-antibiotic effect plus the anti-inflammatory properties of azithromycin, which might break the vicious circle of diarrhea – gut inflammation – nutrient malabsorption – weakened immune status – diarrhea; directly diminishing the number of diarrhea cases due to the good activity of azithromycin against the most relevant diarrheogenic pathogens, but also reducing gut inflammation, thereby favoring nutrient absorption, and consequently, improving the immune status. In the dark size, this mass use of azithromycin will most likely result in the selection of macrolide-resistant microorganisms, as has been described in Tanzania (Seidman et al., 2014). Long-term follow up of these cohorts is needed in order to determine the long-term effect on the general levels of macrolide resistance.

Clarithromycin, a 14C semisynthetic erythromycin derivative, is not currently considered for the treatment of *Enterobacteriaceae* infections. However, animal models have demonstrated its usefulness in the treatment of experimental sepsis and acute pyelonephritis, either alone or in combination with amikacin (Giamarellos-Bourboulis et al., 2005a, b). This finding has been related to the potent antiinflammatory and immunomodulatory properties of clarithromycin but not to its antibacterial activity.

Regarding other macrolides, new fluoroketolides such as solithromycin have also shown good *in vitro* activity against clinically relevant *Enterobacteriaceae* (Putnam et al., 2010). However, their utility in the treatment of infections by these microorganisms needs to be clearly established with further studies.

On the other hand, the clinical use of an antibacterial agent is not only delimited by its specific spectrum of activity or the presence of resistant microorganisms but also by the frequency and relevance of adverse events. Regarding macrolides, mild gastrointestinal disorders (including diarrhea, vomiting, nausea and other symptoms) or cardiac events in specific patients at-risk have

been reported among other adverse events (Ayele et al., 2011; Georgopapadakou, 2014; Mortensen et al., 2014). Nevertheless, the most serious adverse event reported is irreversible hepatotoxicity associated with the use of telithromycin, which in some cases results in patient death. The possible development of such a severe adverse event led the FDA to restrict its use in 2007 and to make a black-box warning in 2010 (Brinker et al., 2009; Georgopapadakou, 2014).

Veterinary use

Macrolides as well as other antimicrobial agents have been largely used in veterinary practice for both the treatment of pets and, to a greater extent, livestock. In fact, a recent report of the CDC considered that approximately 80% of the antibacterial agents are addressed to veterinary use, with 90% of these being used as growth promoters (CDC, 2013).

Regarding livestock, since the initial introduction in some regions of spiramycin into veterinary practice in the 1960s, a series of macrolides have been used as growth promoters, prophylactic agents or as treatments when necessary (CMPVU, 2011; Sgoiffo Rossi et al., 2010; Torres et al., 2013). Some of these macrolides such as erythromycin have also been used in human medicine while others including tylvalosin, tildipirosin or gamithromycin have exclusively been used in veterinary applications.

In some countries, including those belonging to the EU, the use of antimicrobial agents as growth promoters has been forbidden since 1998, while other uses have been drastically reduced because of the general increased levels of antibiotic resistance registered during the last two decades (CMPVU, 2011). Similarly, the FDA has recently proposed a reduction of veterinary consumption of antibacterial agents (FDA, 2013). Along the same line, the WHO has included certain macrolides (erythromycin, azithromycin and clarithromycin), together with other antimicrobial agents, on the list of essential medicaments, in order to promote control actions to preserve their clinical usefulness so far as possible (WHO, 2013a, b). However, despite actions and regulations in different countries, the illegal use of antibiotics in veterinary medicine continues to be reported (Sheridan et al., 2014).

Mechanisms of resistance

Following the introduction of erythromycin into clinical practice the presence of macrolide resistance was reported as early as 1952 when the first *Staphylococcus*

Table 2. Mechanisms of macrolide resistance presents in *Enterobacteriaceae*.

Generic mechanism	Specific mechanism	No genes	Chrom	TMMR
Target mutations	23S rRNA mutations	1	1	–
	Ribosomal protein alterations	2	2	–
Methylation	Erm-like	6	–	6
	Cfr-like	1	–	1
	RlmA-like	1	1	–
Pseudouridylation ^a	RluC	1	1	–
Decreased uptake	Efflux pumps	≥18	≥14	4
	Outer membrane alterations	NA ^b	NA ^b	–
Macrolide modification	Esterases	2	–	2
	Phosphotransferases	4	–	4
Other	Short peptides	NA	NA	–
	Peptidyl tRNA hydrolase overexpression	1	1	–

Chrom: chromosomal encoding; TMMR: transferable mechanisms of macrolide resistance; NA: not applicable.

^aPossible effect on 16C macrolides.

^bResistance mediated by specific mutations or alterations at chromosomal genes expression.

exhibiting erythromycin resistance was isolated (Lepper et al., 1953; Nakajima, 1999).

As aforementioned, *Enterobacteriaceae* are intrinsically resistant to the vast majority of macrolides in relation to the natural low macrolide permeability through the outer membrane due to their hydrophobic nature (Vaara, 1993a). The enhanced activity of azithromycin against *Enterobacteriaceae* has been associated with better permeability related to its increased basic character, which favors higher intracellular uptake of this antibiotic (Farmer et al., 1992; Retsema et al., 1987).

A MIC of 16 mg/L has been proposed as the ecological resistance breakpoint for *Salmonella* spp. for azithromycin (Sjölund-Karlsson et al., 2011). Nonetheless, to date no clinical resistance breakpoint has been established for the members of the *Enterobacteriaceae* family by either CLSI or EUCAST (CLSI, 2013; EUCAST, 2015). However, the isolation of highly azithromycin-resistant *Enterobacteriaceae* is increasing worldwide and the emergence of azithromycin-resistance has been observed among different members of this family in different geographical areas (Baker et al., 2015; Boumghar-Bourtchai et al., 2008; Bowen et al., 2015; Le Hello et al., 2013; Ochoa et al., 2009; Sjölund Karlsson et al., 2013; Vlieghe et al., 2012).

Up to now, different mechanisms of macrolide-resistance have been identified in *Enterobacteriaceae*, some of which are encoded within the bacterial chromosome, like target alterations or chromosomal efflux pumps and others able to be transferred amongst microorganisms, encoded within transferable elements (Table 2). These latter mechanisms are of special concern at a clinical level, since their easy dissemination among microorganisms, resulting in a continuous interchange of genetic material. In this context, the acquisition and maintenance of a TMMR may be enhanced in an environment with a high presence of macrolides due to competitive advantages. Besides, indirect selection may also be made, because some TMMR are present in

genetic structures that also include other antibiotic resistance determinants such as *dfrA* genes, involved in resistance to trimethoprim, *aacA* or *aadA* genes, involved in aminoglycoside resistance or β -lactamase genes, involved in β -lactam resistance, among others (Dolejska et al., 2013; Du et al., 2012; Ho et al., 2011; Pons et al., 2015; Sáenz et al., 2010). Thus, the TMMR may be fixed in the bacterial genetic background by the use of unrelated antimicrobial agents, or by the presence of heavy metals or antibiotic traces in the environment as has been described in other cases (Stepanuskas et al., 2006; Vien et al., 2012).

It is interesting to note that in microorganisms in which only one TMMR has been identified and no other mechanism of macrolide-resistance has been detected, the levels of resistance vary greatly (Nguyen et al., 2009). This may be related to the presence of specific alleles which might have different inactivation levels, with the presence of unidentified macrolide-resistance mechanisms or with the gene expression levels. In this latter line, different TMMR or multiple copies of the same TMMR may be present in the same or in different genetic structures within a microorganism (Baker et al., 2015; Katayama et al., 1998; Poirel et al., 2011; Soge et al., 2006). Moreover the final copy numbers of a specific gene also depend on its precise location (e.g. long plasmids are present with a low copy number, while small plasmids are likely to have a high copy number).

Three different targets have currently been described at the ribosomal level; the 23S rRNA, which is by far the most extensively studied, and two ribosomal proteins: L4 (encoded at *rpID* gene) and L22 (encoded at *rpIV* gene) which are located near the tunnel entrance. However, not all alterations in either 23S rRNA or the L4 and L22 proteins result in cross-resistance to all members of the macrolide family, with some being affected by specific alterations (Ettayebi et al., 1985). Chromosomal efflux pumps have been reported to

have a relevant role in macrolides resistance (Gomes et al., 2013b; Wehmeier et al., 2009). In addition, a series of less studied chromosomal encoded mechanisms, such as the presence of short peptides encoded in “mini-genes” able to affect the susceptibility levels to macrolides have also been described (Tenson et al., 1996; Tripathi et al., 1998) despite not taking part in the development of macrolide resistance in clinical *Enterobacteriaceae*. The development of chromosomal mutations involved in macrolide-resistance may have implications in other biological processes such as the virulence power. Thus, has been described that the presence of the L22 Δ 82–84 results in impaired production of Ag43 (Yap & Bernstein, 2013), which is involved in autoaggregation and biofilm formation.

Regarding TMMR, mechanisms able to result in target modifications, antibiotic inactivation or antibiotic pump out have been described in *Enterobacteriaceae* (Roberts, 2001). In 1999, a proposal devoted to bringing order to the nomenclature of these genes was published (Roberts et al., 1999). This proposal established that new genes need to possess amino acid identity $\leq 79\%$ with previously described genes, resulting in the presence of numerous alleles for most of the genes currently described. However, it should be taken into account that the presence of exact alleles may be influenced by local factors. Geographical and epidemiological studies may be useful to determine the prevalence of specific alleles in different regions. Moreover, the presence of different alleles might result in different affinities and abilities to act against macrolide targets or macrolide antibiotics, and consequently, in different levels of macrolide resistance similar to what has been described in other antibiotic-resistant determinants (Salverda et al., 2010). In fact the introduction of single amino acid changes *in vitro* in MphB results in different enzymatic activity levels (Taniguchi et al., 1999).

Newly identified sequences need to be submitted to the webpage <http://faculty.washington.edu/marilynr/> (Roberts, 2001) in order to be validated and appropriately named prior to inclusion in any article or repository such as GenBank. Unfortunately, as also occurs with other sequences (Pons et al., 2013; Valkiūnas et al., 2008) often the sequences are directly submitted to GenBank resulting in records of incorrectly named, as well as non-communicated genes, which may lead to erroneous analysis and conclusions.

Regarding TMMR it is of note that commensal and nonpathogenic microorganisms may act as hidden TMMR reservoirs. In this respect, gut-commensal *Enterobacteriaceae* may play a role as a reservoir of resistance genes that may be transferred to pathogenic

microorganisms (Nguyen et al., 2009; Seidman et al., 2014). Some studies have shown a higher prevalence of TMMR among Gram-negative microorganisms compared to Gram-positive recovered from healthy children (Luna et al., 2002; Ojo et al., 2004), reinforcing the relevance of these antibiotic-resistance reservoirs.

Target alterations

23S rRNA base substitutions

Although substitutions in 23S rRNA genes (encoded at *rrn* operons) have been extensively sought in both Gram-negative and Gram-positive microorganisms, their clinical relevance is species-dependent due to the presence of a variable copy number of *rrn* locus in the bacterial chromosome. This mechanism of resistance is therefore of special relevance in microorganisms with only one or two *rrn* loci (Leclercq, 2002; Vester & Douthwaite, 2001) also being described in microorganisms with three or four *rrn* loci (Chisholm et al., 2010; Saito et al., 2012). *Enterobacteriaceae* such as *E. coli*, *Salmonella* spp. or *Shigella* spp. have up to seven *rrn* loci (Anderson & Roth, 1981; Condon et al., 1995; Ellwood & Nomura, 1980; Hinojosa-Ahumada et al., 1991) or even more in the case of *Klebsiella* spp., which seems to possess up to eight *rrn* loci (Wang et al., 2008). Thus, in *Enterobacteriaceae*, the relevance of these mechanisms as being responsible for macrolide resistance is low since the development of macrolide resistance due to mutations at 23S rRNA requires the alteration of several of the encoding *rrn* loci. In this line, studies on unrelated microorganisms like *N. gonorrhoeae* or *Moraxella catharralis*, both of which have four *rrn* loci, have shown that a single *rrn* loci mutation (25% of *rrn* loci) has no effect on the MIC of macrolides, while the involvement of two loci results in low levels of macrolide resistance. Thus, at least three *rrn* loci (75% of *rrn* loci) mutated at positions A2058 or A2059 are needed to achieve high levels of macrolide resistance, while the presence of three or four *rrn* loci with the mutation C2611U only results in low-moderate levels (Chisholm et al., 2010; Saito et al., 2012). This is why the vast majority of studies have been developed *in vitro*, and sometimes only incomplete information about the extension of the effects of specific macrolides of all 23S rRNA alterations on *Enterobacteriaceae* is available.

Although data regarding *Enterobacteriaceae* are scarce, probably due to their low clinical relevance, data obtained in other microorganisms possessing a single copy of the *rrn* operon have shown possible fitness loss as well as lesser virulence of azithromycin-resistant microorganisms due to specific 23S rRNA base changes

Table 3. 23S rRNA gene positions involved in alterations in the susceptibility to macrolides in *Enterobacteriaceae*.

	23S rRNA modifications affecting macrolide activity														
	Increased resistance											Increased susceptibility			
	Resistance			Variable effect								H.S.		N.I.	
	2058	2059	1219-1230	745	754	752	2057	2504	2609	2503	2611	2032	2062	2503	2610
wt	A	A	–	A	U	A	G	U	A	A	C	G	A	A	C
Base change	C,G,U	C,G	–	–	A ^a	C,G,U ^b	A ^c	A,G	C ^d	–	A,G U ^e	A,C,U ^f	C,G,U	G	U
Deletion	–	–	Δ ^g	–	–	Δ ^b	–	–	–	–	–	–	–	–	–
Insertion	–	–	–	–	–	A ^b	–	–	–	–	–	–	–	–	–
Methylation	Y ^h	–	–	Y ⁱ	–	–	–	–	–	Y ^j	–	–	–	–	–
Pseudouridylation	–	–	–	–	–	–	–	Y ^k	–	–	–	–	–	–	–

Highlighted in gray: alterations not described in *Enterobacteriaceae*, albeit with a potential effect on specific 16C macrolides.

H.S.: hypersusceptibility; N.I.: non-induction of *erm* genes; wt: wild type; Y: yes; Δ: deletion.

^aEffects on the MIC of specific ketolides. Its effect on erythromycin MIC is controversial.

^bLow level of telithromycin resistance; in combination with A2058G telithromycin susceptibility is restored, but resistance to tylosin is enhanced.

^cResistance to 14C macrolides; no effect on 16C macrolides; also implies resistance to chloramphenicol.

^dResistance to telithromycin and cethromycin, but increased susceptibility to erythromycin and azithromycin.

^eResistance to erythromycin; does not affect 16C macrolides.

^fHypersusceptibility to erythromycin; resistance to chloramphenicol and lincosamides.

^gRelease of the promoter of the “mini gene” present in the 23S rRNA gene.

^hMediated by the Erm family.

ⁱMediated by RlmA¹. In combination with a monomethylation at position 2058 results in resistance to tylosin.

^jMediated by Cfr. Predictable resistance to specific 16C macrolides.

^kMediated by RluC. Predictable resistance to specific 16C macrolides.

(Binet et al., 2010; Hao et al., 2009). In the case of *E. coli*, the A2062G change has shown to strongly affect normal bacterial growth (Cochella & Green, 2004) while other substitutions such as C2610G, or A2503C, A2503U result in non-viability which does not support cell growth in the absence of wild-type ribosomes in *E. coli* (Vázquez-Laslop et al., 2010, 2011).

The substitutions of 23S rRNA related to the development of macrolide resistance most extensively described in *Enterobacteriaceae* and in other Gram-positive or Gram-negative microorganisms are those affecting positions A2058 or A2059 (Chisholm et al., 2010; Marvig et al., 2012; Saito et al., 2012). Additionally, other alterations have been described in *Enterobacteriaceae*, such as those affecting positions A745, A752, U754, G2057, A2032, A2062, A2503, U2609, C2610 or C2611, which are able to affect the activity of macrolides either by increasing the levels of resistance, or resulting in enhanced activity. Moreover, an association has been described between a specific 23S rRNA deletion (Δ1219–1230) and the development of erythromycin resistance (Douthwaite et al., 1985; Krokidis et al., 2014; Novotny et al., 2004; Vázquez-Laslop et al., 2008, 2010, 2011; Vannuffel et al., 1992; Weisblum, 1995) (Table 3). Alterations present in domain II of the 23S rRNA affect specific macrolides like tylosin and ketolide antibiotics but not erythromycin or azithromycin. Thus, amino acid changes, deletions or insertions at position A752 result in low levels of telithromycin resistance (Novotny et al., 2004). Curiously, the concomitant presence of any A752 alteration in

combination with the presence of A2058G results in the loss of telithromycin resistance but with an increase in tylosin resistance (Novotny et al., 2004). Meanwhile, the U754A alteration has a slight effect on the MIC of some ketolides, such as telithromycin, but not on that of others as in the case of the new ketolide K1835 (Krokidis et al., 2014). Regarding this specific substitution, its effect on the MIC of erythromycin remains controversial, with some authors describing a slight effect on its MIC levels (Xiong et al., 1999), while others do not observe any effect (Krokidis et al., 2014).

Mutations at position A2062 have not been associated with diminished interactions between macrolides and ribosomes but rather with the inability to induce *erm*(C) expression (Vázquez-Laslop et al., 2008, 2010) which might probably be extended to the induction of other *erm* genes. In this respect, the effect of the C2610U mutation has been described, which, despite affecting a base involved in the interaction with some macrolides (see below), does not avoid binding, resulting in a loss of the ability of erythromycin and other cladinose-possessing macrolides to induce the expression of the *erm* genes (Vázquez-Laslop et al., 2011). No other substitution at this specific point results in macrolide resistance. Interestingly, this base substitution has been associated with very modest increases in macrolide resistance in *S. pneumoniae* (Canu et al., 2002). A similar loss of induction of some *erm* genes has been observed in *E. coli* in the presence of the A2503G mutation (Vázquez-Laslop et al., 2010). Despite its effect on the viability of *E. coli*, in unrelated microorganisms,

such as *Mycobacterium smegmatis* the presence of the A2503U substitution resulted in resistance to 16C macrolides analyzed (Li et al., 2011).

These 23S rRNA substitutions may produce different levels of macrolide resistance as shown in other microorganisms (Chisholm et al., 2010) leading to dichotomic effects in which a specific substitution results in increasing resistance to some macrolides, with higher susceptibility to others. Although alterations in A2058 or A2059 affect all macrolides, most likely because these positions are directly involved in the interactions between all macrolides and ribosomes (Kannan & Mankin, 2011), substitutions like G2057A do not result in resistance to 16C macrolides, but do affect 14C macrolides (Ettayebi et al., 1985) (Table 3). Similarly, it has been shown that the C2611U alteration results in erythromycin resistance but does not affect 16C macrolides such as spiramycin or tylosin (Vannuffel et al., 1992). In species such as *M. pneumoniae* or *S. pneumoniae* it has been found that C2611A and C2611G mutations result in a similar pattern of resistance (Pereyre et al., 2004; Tait-Kamradt et al., 2000), and may thus predict a similar scenario in *Enterobacteriaceae*. Meanwhile, the U2609C mutation has a differential effect, resulting in slightly higher erythromycin and azithromycin susceptibility levels but an increase in the telithromycin and cethromycin resistance (Garza-Ramos et al., 2001).

Additionally, 23S rRNA is also the target of phenicol agents. In this respect, the development of cross-resistance between 14-membered macrolides and chloramphenicol has also been described in the presence of a G2057A mutation in *E. coli* (Ettayebi et al., 1985). Interestingly, other 23S rRNA alterations, such as those affecting positions G2032, G2032A, G2032C and G2032U, have been associated with the development of resistance to chloramphenicol and lincosamides resulting in hypersusceptibility to some macrolides including erythromycin (no data regarding azithromycin has been found) (Douthwaite, 1992).

Lastly, it should be taken into account that data regarding interactions between 23S rRNA and macrolides cannot be directly extrapolated between microorganisms, and thus, the possible impact of these interactions should be confirmed in experimental studies (Mankin, 2008).

Ribosomal protein alterations

Similar to studies on 23S rRNA, those on the role of ribosomal protein alterations in the development of macrolide resistance in *Enterobacteriaceae* have mainly been

developed by mutagenesis or studying spontaneous macrolide-resistant mutants *in vitro*. In *E. coli*, the involvement of alterations in ribosomal proteins in the acquisition of macrolide resistance was first described in the late 1960s early 1970s (Apirion, 1967; Wittmann et al., 1973). Currently, the effect of alterations, including punctual mutations, insertions and deletions, in two ribosomal proteins (L4 encoded in the *rpID* gene and L22 encoded in the *rpIV* gene) on the MIC of macrolides has been described (Table 4).

Punctual amino acid substitutions reducing the binding capacity of macrolides reportedly mainly affect the L4 positions Q62, K63, G64 or G66, as well as L22 position K90 (Chittum & Champney, 1994; Diner & Hayes, 2009; Zaman et al., 2007). In addition, a long series of less frequent changes such as those affecting positions 65 of L4 or 88 of L22 were described *in vitro* by Diner & Hayes (2009) (Table 4). It has been shown that the L4 K63E alteration may prevent the binding of macrolides to 23S rRNA, resulting in a smaller cross-section of the tunnel entrance that does not allow the penetration and subsequent binding of macrolides (Gabashvili et al., 2001). No data how the remaining point mutations at L4 affect macrolide-ribosome interactions are available in the literature, but the aforementioned model might be extrapolated. Interestingly, all of these alterations reportedly result in reduced duplication time (Zaman et al., 2007) and a decrease has been demonstrated in the rates of peptidyltransferase activity in the specific case of the alteration of L4 K63E mutants (Wittmann et al., 1973). Additionally, a series of secondary alterations have been reported including the L4 amino acid substitution T65P. Although this substitution is unable to produce macrolide resistance by itself, if concomitant with some G64 amino acid substitutions (G64C/V/A/S/R/D) results in higher values of erythromycin resistance (Diner & Hayes, 2009). Regarding L22, the amino acid changes at position K90 result in differential effects against macrolides, increasing the levels of erythromycin resistance but not affecting tylosin (Diner & Hayes, 2009); the reason for this differential effect remains unknown.

Moreover, a series of alterations have been described in other points, as shown in *Shigella flexneri*, with a L4 amino acid change H165Q concomitant with the L22 L46Q alteration. However, no univocal relationship with the development of resistance has been determined (Gomes et al., 2013a).

Insertions and deletions have also been reported in both ribosomal proteins. In studies analyzing the L4 protein of *E. coli*, insertions affecting macrolide-resistance have been described, including, among others, an insertion of six amino acids (EVTGSG) after codon 56

Table 4. L4 amino acid codon alterations involved in macrolide-resistant *Escherichia coli*.

Codon	wt	Amino acid substitution	Deletion	Insertion	Reference
Alterations described in L4 in macrolide-resistant <i>E. coli</i> strains					
56–57	–	–	–	56:EVTGSG:57	(Zaman et al., 2007)
62	Q	D, E, F, I, K, L, M, R, V, W, Y	–	–	(Diner & Hayes, 2009; Zaman et al., 2007)
63	K	A, C, D, E, G, H, I, L, M, P, Q, R, S, V	–	–	(Chittum & Champney, 1994; Diner & Hayes, 2009; Zaman et al., 2007)
63–64	KG	–	Δ63–64	63:TWRQ:64	(Chittum & Champney, 1994; Diner & Hayes, 2009; Zaman et al., 2007)
64–65	GT	–	Δ64–65	–	(Diner & Hayes, 2009)
64	G	I, L, P	Δ64	–	(Diner & Hayes, 2009)
65	T	D	Δ65	–	(Diner & Hayes, 2009)
65–66	TG	–	Δ65–66	65:IEG:66	(Diner & Hayes, 2009)
65–67	TGR	–	Δ65–67	–	(Diner & Hayes, 2009)
66	G	C, D, S, R	–	–	(Diner & Hayes, 2009; Zaman et al., 2007)
66–67	GR	–	Δ66–67	–	(Diner & Hayes, 2009)
67–68	RA	–	Δ67–68	–	(Diner & Hayes, 2009)
72–73	–	–	–	72:RARSGS:73	(Zaman et al., 2007)
Alterations described in L22 in macrolide-resistant <i>E. coli</i> strains					
82–84	MKR	–	Δ82–84	–	(Chittum & Champney, 1994; Zaman et al., 2007)
84–85	RI	–	–	84:IMPRAS:85	(Gomes et al., 2013a)
85	I	L	–	–	(Diner & Hayes, 2009)
87	P	T	–	–	(Diner & Hayes, 2009)
88	R	P	–	–	(Diner & Hayes, 2009)
90	K	F, G, L, M, P, V, W, Y	–	–	(Diner & Hayes, 2009)
90–91	KG	–	Δ90–91	–	(Diner & Hayes, 2009)
91–92	GR	–	Δ91–92	91:NAD:92	(Diner & Hayes, 2009)
95–96	RI	–	Δ95–96	–	(Diner & Hayes, 2009)
96–98	ILK	–	Δ96–98	–	(Diner & Hayes, 2009)
99–100	–	–	–	99:IMPRASKGRADRILKR:100	(Zaman et al., 2007)
105–106	–	–	–	105:TV:106	(Zaman et al., 2007)

Only are reported those alterations described in the absence of other macrolide-mechanisms of resistance. Additionally a series of alterations have been detected together other mechanisms of macrolide resistance (Diner & Hayes, 2009).

(insertion L4 56/6), an insertion of four amino acids (TWRQ) after codon 66 (L4 63/4) and an insertion of six amino acids (RARSGS) after amino acid 72 (L4 72/6) (Zaman et al., 2007). Some of these insertions such as L4 56/6 or L4 72/6 are derived from internal partial duplications of the L4 encoding gene. Additionally, deletions of the amino acids K63, G64 (Δ63–64) or G64 T65 (Δ64–65), among others, have also been reported (Diner & Hayes, 2009; Lovmar et al., 2009a). Similarly, it has also been reported that L22 insertions and deletions are involved in the development of macrolide resistance. Some of these insertions, such as the insertion of six amino acids (IMPRAS), located after amino acid 84 (L22 84/6), that of 15 amino acids (IMPRASKGRADRILKR) located after amino acid 99 (L22 99/15) or that of two amino acids (TV) located after amino acid 105 (L22 105/2), are duplications of the amino acid sequence of L22. Additionally, other insertions such as that of three amino acids (NAD) upstream from amino acid 91 (L22 91/3) have also been described (Diner & Hayes, 2009; Gomes et al., 2013a; Zaman et al., 2007), while described deletions affect different positions such as amino acids 82–84 (MKR) or 96–98 (ILK) (Diner & Hayes,

2009; Zaman et al., 2007) (Table 4). In the absence of antibiotic pressure, insertions easily revert, probably due to their associated fitness cost (Gomes et al., 2013a; Zaman et al., 2007). Indeed, the growth of bacteria carrying some of these insertions is slower than that of parental macrolide-susceptible isolates (Zaman et al., 2007).

It has been proposed that these alterations may affect the general structure of the ribosome, complicating the access of macrolides to their interaction point (Lovmar et al., 2009a; Zaman et al., 2007). However, in the case of the L22 deletion Δ82–84, the ability of erythromycin to bind the ribosome and the mechanism that underlies the effect over macrolides susceptibility are controversial. Zaman et al. (2007) showed that this deletion (as also observed for insertion L22 105/2) does not affect the ability of erythromycin to bind the ribosome. Furthermore, Gabashvili et al. (2001) reported that this deletion results in a broadened peptide exit tunnel and thus, in the non-inhibition of ribosomal activity despite macrolide-binding. Tu et al. (2005) proposed that this deletion may result in higher flexibility of the peptide loop, which is located in the peptide exit

tunnel constriction. Accordingly, Moore & Sauer (2008) have also shown that despite increases in different macrolide MIC levels, erythromycin efficiently binds with ribosomes carrying the L22 $\Delta 82-84$. Interestingly, this binding results in both complete ribosome translation inhibition and lower intracellular macrolide uptake. In the experimental deletion of TolC, similar values of macrolide susceptibility were achieved for both wild-type *E. coli* and for *E. coli* strains carrying the L22 $\Delta 82-84$ and a relation between the presence of L22 $\Delta 82-84$ and overexpression of the AcrAB-TolC system, able to extrude macrolides from bacteria, was proposed (Moore & Sauer, 2008). Contrary to aforementioned data, Lovmar et al. (2009a) described a strong effect of $\Delta 82-84$ on erythromycin binding, proposing that the enhanced flexibility might alter the conformations, hindering erythromycin transport through the tunnel. New data regarding the effect of $\Delta 82-84$ on the binding of macrolides to ribosome are needed to elucidate the exact mechanism of action.

On the other hand, it has been described that alterations in other ribosomal proteins, like those affecting positions 42 and 87 of the S12 protein involved in the acquisition of streptomycin resistance, may lead to increased susceptibility to other ribosome-targeting antibiotics such as tetracycline or chloramphenicol (Pelchovich et al., 2013). Although these specific substitutions do not seem to affect the action of macrolides, this phenomenon should be considered as feasible.

Methylation

The methylation of 23S rRNA was the first mechanism of macrolide resistance described (Roberts et al., 1999). This mechanism is related to the posttranscriptional addition of one or two methyl groups to an adenine, mainly at position A2058, of 23S rRNA by adenine-N6 methyltransferases. This modification is mediated by methylases encoded in the so-called *erm* genes (Roberts et al., 1999), resulting in the inability of macrolides to interact with ribosome. Although double methylation reportedly results in general higher levels of macrolide resistance and single methylation results in low levels of macrolide resistance (Vester & Long, 2009), recent reports have shown that the most modern fluoroketolides, such as solithromycin, are able to develop a weak interaction with ribosomes dimethylated at position A2058 (Llano-Sotelo et al., 2010). Although these studies were developed with *S. aureus* ribosomes, they can probably be extrapolated to Gram-negative microorganisms such as *Enterobacteriaceae*

since solithromycin possesses a high affinity towards *E. coli* ribosomes (Llano-Sotelo et al., 2010).

Furthermore, methylases able to act on other 23S rRNA positions have also been described in both Gram-positive and Gram-negative microorganisms. In the case of tylosin, the methylation at position A2058 does not by itself result in the development of resistance, but rather the concomitant methylation of other 23S rRNA positions, such as G745 or G748 in Gram-negative and Gram-positive microorganisms, respectively is needed (Liu & Douthwaite, 2002a, b). Thus, among others, Erm(32) (formerly TlrB or RlmA^{II}) encoded in the chromosome of members of the *Streptomyces* genus has been described to be able to methylate position G748 (Liu & Douthwaite, 2002a). In *E. coli* the presence of a related methylase, called RlmA^I (formerly RmA) has been described which acts by methylating position G745 (Liu & Douthwaite, 2002b). Nonetheless, when Erm(32) or RlmA^I has been cloned in *E. coli* no effect was observed on the macrolide MIC, including tylosin (Liu & Douthwaite, 2002a). However, in both cases, the concomitant monomethylation of A2058, which by itself is unable to affect tylosin activity, leads to the development of tylosin resistance (Liu & Douthwaite, 2002a). This finding is related to the different interaction points involved in the interaction of tylosin with 23S rRNA, as mentioned previously.

Some groups have attempted to design methylase inhibitors able to restore the macrolide activity of bacterial isolates carrying these genes (Hajduk et al., 1999), but no molecule is currently under development for therapeutic use in humans.

At least 39 *erm* genes have been described to date, with multiple alleles (Roberts, 2001) and mainly in Gram-positive microorganisms, some of which are present in the bacterial chromosome of antibiotic-producing microorganisms, mostly indigenous of *Streptomyces* spp. (Roberts, 2004). Regarding *Enterobacteriaceae*, a series of chromosomal encoding genes, such as the aforementioned RlmA^I gene, plus at least six different transferable *erm* genes – *erm*(B), *erm*(C), *erm*(D), *erm*(E), *erm*(F) and *erm*(42) – have currently been described (Harmer et al., 2015; Roberts, 2001) (Table 5). Five of these six transferable determinants act by dimethylating A2058 (Kim et al., 1993; Vester & Long, 2009; Weisblum, 1995), while the remaining determinant, the *erm*(42) gene, encodes a monomethyltransferase (Desmolaize et al., 2011a). In *Enterobacteriaceae*, these genes have been described in mobile elements such as plasmids carrying more than one *erm* gene, as observed in the *E. coli* plasmid pTN48 that sequentially carries an *erm*(B) plus an *erm*(C) gene, as well as another macrolide resistance determinant such as the *mphA* (Billard-

Table 5. Methylases described in *Enterobacteriaceae*.

Type	Genes	Position	Methylation	Genera
Transferable ^a	<i>erm(B)</i>	A2058	Dimethylation	<i>Citrobacter</i> , <i>Enterobacter</i> , <i>Escherichia</i> , <i>Klebsiella</i> , <i>Pantoeae</i> , <i>Proteus</i> , <i>Serratia</i> , <i>Shigella</i> .
	<i>erm(C)</i>	A2058	Dimethylation	<i>Escherichia</i> ,
	<i>erm(D)</i>	A2058	Dimethylation	<i>Salmonella</i>
	<i>erm(E)</i>	A2058	Dimethylation	<i>Shigella</i>
	<i>erm(F)</i>	A2058	Dimethylation	<i>Shigella</i>
	<i>erm(42)</i>	A2058	Monomethylation	<i>Salmonella</i>
	<i>cfr</i>	A2503	Monomethylation	<i>Escherichia</i> , <i>Proteus</i>
Chromosome	<i>rmlA</i> ^b	A745	Monomethylation	<i>Escherichia</i> , <i>Citrobacter</i> , <i>Klebsiella</i> , <i>Shigella</i> , <i>Salmonella</i>

^aIn some cases have been described within bacterial chromosome due to integration phenomena.

^bIts ability to methylate position 745 has only been confirmed in *E. coli*. Following the criteria of Roberts (Roberts, 2001; Roberts et al., 1999) only are indicated the genera in which amino acid identity levels with *E. coli* RmlA^I ranks between 80 and 100%. Other similar genes have been detected in other *Enterobacteriaceae* (e.g. *Enterobacter*, with an amino acid identity of 78% compared to RmlA^I of *E. coli*).

Pomares et al., 2011). It is of note that some of these genes might not be satisfactorily expressed in *Enterobacteriaceae* (Rasmussen et al., 1986).

Two types of Erm methylases have been described; those constitutively expressed and those with inducible expression when macrolides are present. When inducible, prior to the methylase encoding gene, such as *erm(B)*, *erm(C)* or *erm(D)*, a short ORF is present, encoding a peptide leader or a ORF leader, which is named as their respective *erm* gene, but followed by "L"; [e.g. *erm(CL)*]. Moreover, although not yet described in *Enterobacteriaceae*, some inducible *erm* genes, such as *erm(A)*, possess two ORF leaders (Ramu et al., 2009).

However, not all macrolides are able to induce *erm* genes. While 14C and 15C macrolides may induce the expression of methylases, those with 16C are unable to do so (Leclercq, 2002). Additionally, the selection of constitutive variants of theoretically inducible genes is possible (Hue & Bechhofer, 1992). Thus, constitutive expression of *erm(D)* is produced when specific mutations between amino acid codon positions 4 and 8 of *erm(DL)* are present (Hue & Bechhofer, 1992).

As mentioned previously, studies on inducible phenotype have mainly been developed using Gram-positive microorganisms; however, the inducibility of these genes has also been reported in *E. coli*. Thus, using a lacZ fusion methodology, Bailey et al. (2008), showed that an inducible *erm(C)* from *S. aureus* was also induced when cloned in *E. coli*. In the same study, it was also found that the spectrum of inducing antibiotics differs based on the presence of specific mutations in the ORF leader.

The *erm(C)* gene is probably the most extensively studied inducible *erm* gene. Nonetheless, its mechanism of induction may generally be extended to other *erm* genes, as well as other inducible macrolide-resistant determinants such as those encoding the transferable efflux pumps MefA and MsrA or the esterase encoding

gene *ere(A)*, among others (Ramu et al., 2009). The induction of *erm(C)* is mediated by a mechanism of translation attenuation regulation of gene expression that is ribosome stalling-dependent. Thus, a double hairpin that is present in the mRNA and encodes the aforementioned ORF and the methylase encoding gene has been described. This short peptide, which varies in size depending on the exact inducible *erm* gene (e.g. the *erm(CL)* has 19 aa), is constitutively expressed, while the RBS of the methylase gene is "hidden" in the second stem-loop structure (Ramu et al., 2009). When an inducer (e.g. erythromycin) is present, an alteration in the hairpin pattern is produced, resulting in the rearrangement of the structure in a single hairpin, releasing the RBS of the methylase encoding gene (Figure 4) which is then expressed (Ramu et al., 2009). To release the *erm(C)* RBS, transcription of the *erm(CL)* gene must be blocked precisely when the second stem-loop structure is unwound. Regarding the *erm(CL)* gene, this release takes place when the transcription is blocked and the ribosome is placed between positions 9 and 17 of its mRNA, once the ribosome has unwound the mRNA eight nucleotides before the P position (Takyar et al., 2005). The presence of punctual sequence alterations in the ORF leaders, leading or not to the premature ending peptides may result in the constitutive expression of its corresponding *erm* gene, which becomes non-inducible irrespectively of the presence or absence of macrolides. Thus, the presence of non-sense mutations in the amino acid codon 10 of *erm(BL)* abolishes the inducibility of the *erm(B)* gene (Min et al., 2008). Interestingly, the amino acid motif at positions 8–10 of *erm(BL)* is XDK (Ramu et al., 2009), which ranks amongst the most relevant for resulting in amino acid stalling following the newly proposed model of macrolide action (Kannan et al., 2014). Similarly the ORF leader of most other inducible macrolide resistance genes possesses some of the stalling-motifs described as account with the so-called RLR or RP peptides (Ramu et al.,

Figure 4. Model of the induction of *erm(C)* gene. In the upper side it is represented the non-induced conformation. In this, two hairpins are present in the mRNA (hairpin 1–2 and hairpin 3–4) being *erm(CL)* constitutively translated, while *erm(C)* no, because its RBS (represented in the position 4) is blocked within the second mRNA hairpin. In the lower side it is represented the induced conformation. During *erm(CL)* translation the interaction macrolide–ribosome results in the stalling of the transcription of the *erm(CL)* and in mRNA conformational alteration in which a different hairpin structure is present (hairpin 2–3) resulting in the liberation of the *erm(C)* RBS allowing the gene expression.

2009), suggesting a positive selection of these stalling-motifs in the evolution of the inducible macrolide-resistance mechanisms.

These processes must be able to recognize the presence of a macrolide. Thus, it has been proposed that specific 23S rRNA positions which act as sensors, like A2062 and U1782, as well as C2610 are involved in the recognition of the stalling cues by monitoring the structure of the antibiotic molecule. This might explain that the C2610U mutation results in a non-induction of the *erm* genes (Vázquez-Laslop et al., 2011).

Additionally, the presence of the *cfr* gene has recently been described in different reports regarding *Proteus vulgaris* and *E. coli* (Wang et al., 2011, 2012; Zhang et al., 2014, 2015). In *P. vulgaris* the gene was functional and encoded within the bacterial chromosome, in a structure with a certain degree of homology with the plasmid pKKS825 from *S. aureus* inserted within the *fimD* gene and flanked by IS26 sequences, demonstrating that its integration was not entirely stable (Shen et al., 2013; Wang et al., 2011). In *E. coli* isolates, this gene has been found within chromosome (Zhang et al., 2015) and plasmids, despite most being unable to be transferred by conjugation (Deng et al., 2014; Wang et al., 2012), and, up to now, only one *cfr* gene has been detected in *E. coli*, being

encoded within a conjugative IncA/C plasmid (Zhang et al., 2014). Recently, in *E. coli* the presence of a *cfr* gene, flanked by two IS26 elements in the same orientation, has been observed within a conjugative plasmid resembling the pEA3, from *Erwinia amylovora*, highlighting the risk of dispersion of the *cfr* gene within different bacterial hosts and genetic environments (Zhang et al., 2015). Supporting this risk, in several studies the *cfr* gene was flanked either by IS26 or IS256 sequences in all *E. coli*, although different genetic environments have been described (Deng et al., 2014; Wang et al., 2012; Zhang et al., 2014). Interestingly, in all the cases the *cfr* genes were detected in samples from domestic animals (pigs), and from Chinese farms. The *cfr* gene has been described in the methylation of A2503 in Gram-positive microorganisms, resulting in the acquisition of resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A antibiotics as well as to some 16C macrolides, such as josamycin (Long & Vester, 2012; Smith & Mankin, 2008). No data has been found regarding its effect on these macrolides, either regarding *E. coli* or *P. vulgaris*, but by being functional a similar effect might be predicted. Currently, it seems that the presence of this gene in Gram-negative microorganisms is testimonial. Wang et al. (2012) reported its presence in one (0.08%) of the 1230 isolates analyzed. However, this should be considered with caution and continued surveillance of its dissemination amongst *Enterobacteriaceae* should be followed. In this sense the broad-host-range of the IncA/C plasmids should be taken into account (Zhang et al., 2014).

Interestingly, as with other microorganisms, *Enterobacteriaceae* possess housekeeping genes which have a close phylogenetic relationship with the *cfr* gene. These genes belong to the *rlmN* family and are also able to methylate position A2503. Only a few studies have been addressed to determine the role of RlmN in the development of antimicrobial resistance, showing a null (Atkinson et al., 2013) or very modest role, with a maximum two-fold decrease in MIC (Toh et al., 2008), in the basal levels of ribosome-targeting antimicrobial agents such as phenicols, pleuromutilins, lincosamides or others. This different result has been related to its final product, which is a 2-methyladenosine, while that produced by the action of Cfr is an 8-methyladenosine (Atkinson et al., 2013).

Pseudouridylation

Although by far the most relevant mechanism of 23S rRNA modifications related to macrolide resistance is

the presence of methylations, other types of modifications may also be related to macrolide activity. In addition to modifications related to transferable genes, at least 25 positions of the *E. coli* 23S rRNA undergo post-transcriptional modifications. Of these, 14 are located in domain V, mainly being methylations (seven positions) and pseudouridylation (five positions) while the remaining two are 1-dihydrouridine and one predicted 2-thiocytidine (Andersen et al., 2004; Toh & Mankin, 2008).

Two of these 14 modifications affect positions which have been described in the development of macrolide resistance; that is, the aforementioned methylation at position 2503 and pseudouridylation of position 2504 (Toh & Mankin, 2008).

Although classically not considered as a mechanism of antibiotic-resistance, pseudouridylation of position 2504 mediated by RluC has been described in the intrinsic levels of resistance to different PTC-targeting antimicrobial agents, including lincosamides, oxazolidinones and pleuromutilins (Toh & Mankin, 2008).

A search of the literature did not find any study addressed to determining the effect of the inactivation of RluC on basal macrolide resistance levels. However, alterations at this position have been involved in the development of resistance to 16C macrolides in non-*Enterobacteriaceae* such as *M. smegmatis* (Li et al., 2011). Moreover, 16C macrolides such as josamycin reach the active PTC site. Thus, some authors have suggested that the lack of RluC activity may result in increased susceptibility to these macrolides (Kannan & Mankin, 2011).

Decreased antibiotic uptake

The bacterial wall is the border between the external media and the bacterial cytoplasm. At this border two kinds of customs are active: those involved in product importation and those devoted to product extrusion. Commercial efforts in this area are intense and continuous, but the facility to import or export specific products may vary depending on the microorganism. As account with all antibacterial agents, with macrolides this imbalance between intrusion and extrusion has a direct effect on the basal levels of resistance exhibited by each microorganism. Alterations in the normal border structure of the species may result in phenomena of hyper-susceptibility or in increased levels of antibiotic resistance (Vaara, 1993b).

The hydrophobic nature of macrolides has classically been considered the main cause underlying the intrinsic resistance to most of these antimicrobial agents (Vaara, 1993a). Nevertheless, until recently the role of efflux has been underestimated and misinterpreted.

Chromosomal efflux pumps

The physiological role of chromosomal efflux pumps is to pump out environmental toxins such as heavy metals or molecules that the bacteria needs to export as may be the case of siderophores (Martinez et al., 2009). Due to structural similarities or because products are recognized as toxic, chromosomal efflux pumps may extrude antibacterial agents from the bacteria, resulting in increased levels of antibacterial resistance.

Efflux pumps are classified into several families according to phylogenetic relationships, structure and mode of action. The most relevant bacterial efflux system families involved in antibiotic resistance are: ABC, MFS, MATE, RND and SMR (Li et al., 2015). For more in depth information about efflux pumps and efflux pump classifications see Li et al. (2015) as well as the web-pages: <http://www.tcdb.org/>; <http://www.membrane-transport.org/>.

Regarding chromosomal efflux pumps able to extrude macrolides from bacterial cytoplasm, the high number of uncharacterized efflux systems should be taken into account (Nishino & Yamaguchi, 2001). The current information on these systems is limited to efflux pumps in which the effects on macrolides have been tested and efflux pumps do not necessarily extrude related antibacterial agents with the same efficacy (Chollet et al., 2004). Thus, the information available on efflux systems is incomplete, albeit growing rapidly.

Similarly to what has been described for other antimicrobial agents (Ruiz et al., 2005; Sáenz et al., 2004), the effect of chromosomal efflux pumps over macrolides usually remains hidden due to the combination of specific bacterial permeation, basal efflux pump activity as well as the natural affinity for ribosomes, resulting in intrinsic macrolide susceptibility levels. Thus, the real relevance of these efflux pumps is only visualized if they are inactivated or inhibited (Gomes et al., 2013c; Sáenz et al., 2004), while their role in antimicrobial resistance at a clinical level is observed when they are overexpressed (Li et al., 2015). Efflux pump overexpression has been related to the development of azithromycin resistance in 19 *E. coli* azithromycin-resistant mutants obtained *in vitro* in which the use of PA β N, an inhibitor of RND-type efflux pumps, resulted in a four- to 16-fold decrease in the MIC (Gomes et al., 2013b). In *E. coli* and *Shigella* spp. the use of PA β N has shown a lower selection of azithromycin-resistant mutants (Gomes et al., 2013a), thereby demonstrating the potential use of combinations of efflux pumps inhibitors plus antibacterial agents to prevent or limit the development of antibacterial-resistant microorganisms. Interestingly, in these aforementioned *E. coli*

Table 6. Main Efflux Pumps involved in macrolide resistance in *Enterobacteriaceae*.

Microorganism	Efflux system ^a	Family	Macrolide substrate ^b		Reference
			Yes	No	
Chromosomal					
<i>E. coli</i>	AcrAB-TolC	RND	Ery, Azm	Tel	(Chollet et al., 2004; Li et al., 2015)
<i>E. coli</i>	MdtEF-TolC ^c	RND	Ery, Azm, Clar, Tel	–	(Bohnert et al., 2007; Nishino & Yamaguchi, 2001)
<i>E. coli</i>	MacAB-TolC ^d	ABC	14C, 15C	16C	(Kobayashi et al., 2001)
<i>E. coli</i>	YebQ	MFS	Ery	–	(Li et al., 2015)
<i>E. aerogenes</i>	EefFABC	RND	Ery	–	(Masi et al., 2005)
<i>S. marcescens</i>	SdeXY-TolC?	RND	Ery	–	(Chen et al., 2003)
<i>K. pneumoniae</i>	KexD ^e	RND	Ery	–	(Ogawa et al., 2012)
<i>K. pneumoniae</i>	KpnEF	SMR	Ery	–	(Srinivasan & Rajamohan, 2013)
<i>E. coli</i>	MdfA ^f	MFS	Ery	–	(Edgar & Bibi, 1997)
<i>E. coli</i>	bcr	MFS	Azm	–	(Li et al., 2015)
<i>E. coli</i>	emrAB-TolC	MFS	Azm, Jos	–	(Elkin & Mullis, 2007) (Jos)
<i>E. coli</i>	emrE-OmpW?	SMR	Ery	–	(Yerushalmi et al., 1995)
<i>E. coli</i>	MdtJ ^g	SMR	Ery	–	(Li et al., 2015)
<i>E. coli</i>	AcrEF-TolC	RND	Azm, Ery	–	(Nishino & Yamaguchi, 2001; Horiyama et al., 2010; Jellen-Ritter & Kern, 2001)
Transferable					
<i>Enterobacter</i> spp.	Msr(A)	ABC	14C, 15C, Ket ^h	16C ^h	(Ojo et al., 2006; Reynolds & Cove, 2005)
<i>Enterobacteriaceae</i>	Msr(D)	ABC	14C, 15C, Ket ^h	16C ^h	(Ojo et al., 2004; Reynolds & Cove, 2005)
<i>Enterobacteriaceae</i>	Msr(E)	ABC	14C, 15C	16C	(Desmolaize et al., 2011b)
<i>Enterobacteriaceae</i>	Mef(A)	MFS	14C, 15C	16C	(Ojo et al., 2004; Poole, 2005)
<i>E. coli</i> ⁱ	Mef(B)	MFS	14C, 15C ⁱ	16C ⁱ	(Liu et al., 2009)

Ery: erythromycin; Azm: azithromycin; Tel: telithromycin; Jos: josamycin; Clar: clarithromycin; ?: proposed association.

^aHomologs of some of these efflux pumps are present in other *Enterobacteriaceae*, such as AcrAB-TolC which is present in almost all *Enterobacteriaceae*, or KpnGH or KdeA which are the EmrAB and MdtA homologs in *Klebsiella pneumoniae* (Srinivasan et al., 2014). In these cases only the microorganism type is indicated.

^bOnly macrolides in which this has been established.

^cIn some manuscripts and GenBank sequences MdtEF is reported as YhiUV.

^dIn some manuscripts it is reported as YbjYZ.

^eAlthough KocC may act as an outer membrane protein (Ogawa et al., 2012), no exact data about the other two components have been found. Neither is a periplasmic protein nor an outer membrane channel encoded in its flanking regions.

^fAlso named CmlA.

^gIt has been proposed that its role in erythromycin resistance is not by direct extrusion but rather is mediated by the presence of a Leu at position 3 and Trp at position 5, which allow the nascent peptide to act as an E-peptide.

^hInferred from Gram-positive microorganisms (Reynolds & Cove, 2005).

ⁱInferred from Mef(A) substrates.

^jAlthough truncated, this gene has also been detected in other *Enterobacteriaceae* like *Salmonella* spp. (de Toro et al., 2011).

azithromycin resistant mutants, the overexpression of OmpW, an outer membrane protein with a controversial role in the development of resistance to antibacterial agents, was observed (Gomes et al., 2013a). The down-expression of OmpW has been observed in *E. coli* strains exhibiting resistance to ceftriaxone (Hu et al., 2005), while, as in the case of azithromycin, its overexpression has been observed in *in vitro* obtained tetracycline resistant *E. coli* strains (Zhang et al., 2008). Additionally, the overexpression of OmpW was previously observed in another spontaneous *Shigella boydii* azithromycin-resistant mutant obtained in the absence of PAβN (Gomes et al., 2013b). These data suggest the role of OmpW as an extrusion way of azithromycin as has been proposed for other substances (Gil et al., 2007). The overexpression of OmpW might be a collateral reflection of alterations of series of metabolic cascades. Supporting the role of OmpW in the extrusion of toxic

substances, Beketskaia et al. (2014) recently showed that in combination with EmrE, a SMR efflux pump, OmpW is involved in the extrusion of quaternary cationic compounds such as tetraphenylphosphonium (Bay et al., 2008). Although studies by Nishino & Yamaguchi (2001) did not observe any effect on the MIC of erythromycin with the cloning of EmrE, the overexpression of this efflux pump has been related to the abolishment of growth inhibition related to erythromycin (Yerushalmi et al., 1995).

To date, chromosomal efflux pumps belonging to different families have been described as being able to pump out macrolides (Table 6). Among these, the AcrAB-TolC like RND-type efflux pumps present in different members of the genus are probably the best characterized efflux pumps of *Enterobacteriaceae*. The effect of AcrAB-TolC on the extrusion of different macrolides has been shown by developing defective

mutants for the expression of some, or all, genes encoding this efflux pump. In a study on *Salmonella enterica* serovar Typhimurium with an inactivated *acrB* gene, Nikaido et al. (1998) observed a 64-fold decrease in the MIC of erythromycin (from 512 mg/L to 8 mg/L). These observations are consistent with the aforementioned effect of the PA β N (Gomes et al., 2013b) and with the 128-fold decreases in the MIC levels to erythromycin, clarithromycin and azithromycin (from 512 mg/L to 4 mg/L for the first two and from 64 to 0.5 for azithromycin) described by Wehmeier et al. (2009) when disrupting the *acrB* gene. As with other antimicrobial families, this kind of generic efflux pumps are not able to extrude all macrolide members from the bacterial cytoplasm, thereby demonstrating the inability of AcrAB-TolC to pump out telithromycin (Chollet et al., 2004). In addition to the expression levels, the presence of specific amino acid changes in AcrAB-TolC may result in differences in macrolide-affinity, leading to differences in the final MIC levels (Wehmeier et al., 2009). Thus, the introduction of a glycine in the amino acid position 616 of AcrB produces a slight decrease in the level of resistance of different macrolides (four-fold decrease in the MICs of erythromycin and eight-fold decrease in the MICs of azithromycin and clarithromycin) (Wehmeier et al., 2009). A similar effect has also been observed for punctual mutations in other efflux pumps such as MdtEF-TolC which is another RND-type efflux pump or MacAB-TolC, and ABC transporter. Thus, the MdtF amino acid change V610F also results in slight decreases in the ability to extrude some macrolides (Bohnert et al., 2007). A similar phenomenon occurs when the G353C substitution is present in the MacA subunit (Modali & Zgurskaya, 2011).

Other efflux pumps may be involved in erythromycin resistance in non-classical antibiotic-extrusion. MdtJ is an SMR efflux pump involved in the pumping out of different substances, such as deoxycholate (Li et al., 2015). Interestingly, despite the absence of data regarding its ability to extrude macrolides, it is considered to be able to facilitate the growth of *E. coli* in the presence of erythromycin (Li et al., 2015). This finding may be related to the presence of a Leu at position 3 and Trp at position 5, which allow its encoding mRNA to act as a E-peptide (see section on *Resistance mediated by Short Peptides*) (Macvanin et al., 2007).

Transferable efflux pumps

The main difference with respect to chromosomal efflux pumps arises from the fact that those which are

transferable usually, but not always, have a more specific substrate profile, resulting in the development of high levels of antibiotic resistance above the basal levels.

To date a long series of transferable efflux pumps have been described, albeit mostly in Gram-positive microorganisms (Roberts, 2001). At present, five transferable efflux pumps involved in macrolide resistance have been established in Gram-negative microorganisms, all being present in *Enterobacteriaceae*. Three belong to the ABC family: *msr(A)*, *msr(D)* and *msr(E)* while the remaining two belong to the MF family: *mef(A)* and *mef(B)* (Roberts, 2001). Other efflux pumps, such as an AcrR-MexAB CusC homologous RND efflux pump detected in a IncH1 plasmid from *Citrobacter freundii* (Dolejska et al., 2013; Li et al., 2015) might also be involved in macrolide extrusion, but further studies are needed to establish this. Additionally, other transferable efflux pumps, such as *vga(A)v*, which are involved in resistance to other ribosome-targeted antimicrobial agent compounds, have also been described in *Enterobacteriaceae* (Cassone et al., 2006).

Regarding the MF family efflux pumps, the *mef(A)* gene was first described in a clinical isolate of *S. pyogenes* (Clancy et al., 1996). However, at present it has been described in a variety of microorganisms, including *E. coli*, *Citrobacter* spp., *Klebsiella* spp., *Proteus* spp. and others (Ojo et al., 2004; Roberts, 2001). The *mef(A)* gene is frequently reported as *mef(E)* or *mef(A/E)* which is a specific allele of this gene possessing more than 90% of identity compared to the standard *mef(A)* sequence (Roberts, 2001).

Currently, the *mef(A)* gene ranks among the most frequently detected TMMR in both *Enterobacteriaceae* (Ojo et al., 2004) and Gram-positive microorganisms (Bley et al., 2011). MefA is able to extrude different macrolides of 14 and 15C but has no effect on those with 16C (Poole, 2005). MefA activity may be blocked by different efflux pumps inhibitors such as arsenate, CCCP or DDP (Clancy et al., 1996, 1997).

The *mef(B)* gene, with 38% of identity with *mef(A)*, has, to our knowledge, only been described in porcine related *E. coli* isolates. Liu et al. (2009) reported the presence of functional non-conjugative *mef(B)* genes, but located in mobilizable plasmids in two different genetic structures in a total of three *E. coli* isolates. Additionally, another two different genetic structures have also been described in two other isolates carrying a 254 bp fragment of the *mef(B)* gene truncated by the presence of a IS26. In all cases, the *mef(B)* gene was downstream of the atypical class 1 integron. Interestingly, 256 bp of a putative *mef(B)* gene truncated by IS26 has been described just after an atypical

class 1 integron in a *S. Typhimurium* (de Toro et al., 2011). No data have been found regarding the effect of inhibitory agents on MefB activity, but MefB most likely loses or diminish its macrolide efflux capacity in the presence of the same type of inhibitors as MefA.

Despite having been described in several *Enterobacteriaceae* the role of the three ABC transporters in the development of macrolide-resistance remains unsatisfactorily defined among *Enterobacteriaceae*, with scarce data being available.

The *msr(D)* gene has even been described in association with *mef(A)* as part of a common operon. Thus, the need to confirm the presence of this gene in all *mef(A)* positive isolates has been suggested (Roberts, 2008). It has been proposed that both MsrD and MefA (variant encoded in the *mef(E)* allele) interact *in vivo*, with the MefA macrolide pump out activity being enhanced. On the other hand, it has been suggested that MsrD by itself does not pump out macrolides (Nunez-Samudio & Chesneau, 2013).

Although previously sequenced (Gołebiewski et al., 2007; González-Zorn et al., 2005), the identification of the *msr(E)* gene as encoding an efflux pump involved in the development of macrolide resistance was achieved in a study on sewage treatment plant (Schlüter et al., 2007). Similarly to what has been described for the *msr(D)* gene, the *msr(E)* gene has been concomitantly reported with the *mph(E)* gene to be part of a common operon in both Gram-positive (Desmolaize et al., 2011b) and Gram-negative microorganisms, including *E. coli*, *Salmonella enterica* and *C. freundii* (Dolejska et al., 2013; Du et al., 2012; Gołebiewski et al., 2007; González-Zorn et al., 2005; Ho et al., 2011) of different origins, including livestock. It is of note that the *msr(E)* gene is frequently referred to as the *mel* gene, while the *mph(E)* is indicated as the *mph2* gene in most of the studies performed in Gram-negative microorganisms. As in the case of *mef(A)* and *msr(D)*, it seems that the presence of the *mph(E)* gene, has a modest role in the development of macrolide-resistance, while strongly enhancing the pump out of some macrolides via the MsrE efflux pump (Desmolaize et al., 2011b). These two genes have frequently been described within plasmids surrounded by IS26 sequences as part of the Tn1548, which also carry other antimicrobial resistance determinants, such as *ArmA*, facilitating its dissemination (Dolejska et al., 2013; Du et al., 2012; Ho et al., 2011). The presence of truncated *msr(E)* genes has also been observed with the IS26 inserted within it (Granier et al., 2011).

Regarding *Enterobacteriaceae*, the *msr(A)* gene has only been reported in an isolate of *Enterobacter* spp., in which no other of the macrolide-resistance mechanisms

sought was found (Ojo et al., 2006). This isolate presented high levels of erythromycin resistance, despite some reports indicating a loss of MsrA activity when cloned in *E. coli* (Matsuoka et al., 1999). Although no data have been found regarding this *Enterobacter* isolate, in other microorganisms, the *msr(A)* gene, as has been mentioned in the case of the *msr(D)* and *msr(E)* genes, is frequently present together with the *mph(C)* gene, another macrolide resistance gene belonging to the *mph* family (Matsuoka et al., 2003).

Permeability alterations

As mentioned previously, antibiotic bacterial intake is directly related to the basal level of antibiotic resistance. Thus, a barrier permeable to a specific antimicrobial agent favors cytoplasmic uptake, and the intrinsic level of bacterial resistance to this antimicrobial agent is lower.

In general, the membranes of Gram-negative bacteria are poorly crossed by macrolides, with this impermeability being classically considered as the most relevant cause of intrinsic macrolide-resistance of Gram-negative microorganisms (Vaara, 1993a). As mentioned previously, although this impermeability is a real factor, it is not strictly true, since the concomitant role of efflux pumps has been largely underestimated. Some macrolides such as azithromycin have better membrane penetration in relation to their higher basic character. It has been proposed that these macrolides are able to cross borders through bacterial membranes by a self-promoted uptake thanks to a direct interaction with divalent cation binding sites on LPS (Farmer et al., 1992). In support of this hypothesis, it has been shown that alterations in the expression of the *E. coli* outer membrane proteins *OmpC* and *OmpF* have no effect on the levels of azithromycin resistance (Farmer et al., 1992). Moreover, in studies analyzing the effect of different mutations affecting the structure of outer membrane of *E. coli* and *S. enterica*, lipopolysaccharide defective mutants showed increased susceptibility to macrolides. Thus, in *E. coli*, the presence of mutations resulting in lower LPS production, such as those in the *lpxA* gene, which lead to the production of approximately 30% less lipid A than control strains when grown at 30 °C, results in increases in the susceptibility to different macrolides, including erythromycin, azithromycin, clarithromycin and roxithromycin, which in the case of roxithromycin may be of up to 1024-fold with respect to the wild type isolates (Vaara, 1993b). The presence of mutations in the *lpxD* (formerly *firA*), *envA* genes or in the *rfa* operon, which are also involved in LPS biosynthesis results in similar effects on the MICs of macrolides (Vaara, 1993b).

As occurs in efflux pumps, the inhibition of which has been proposed as an alternative method to sensitize bacteria to antimicrobial agents (Bhardwaj & Mohanty, 2012), several reports have proposed the use of “permeabilizers” which favor the entrance of macrolides such as erythromycin within the *Enterobacteriaceae* cytoplasm. Along this line, Saha et al. (2008) analyzed the effect of the addition of 0.5 mg/L of ceragenin CSA-13 on the MICs of erythromycin of four *Enterobacteriaceae* and observed decreases greater than 32-fold in all the enterobacteria tested except *P. vulgaris*.

Antimicrobial agent modification

Bacterial macrolide modifications usually results in the loss of direct antibacterial activity. However, it should be considered that macrolides also possess a series of immunomodulation and anti-inflammatory properties which may also be affected. Moreover, as mentioned beforehand, a series of macrolide molecules without antibacterial activity have been designed for these purposes. However, no data on the effect of modification mechanisms against these non-antibiotic macrolides have been found in the literature.

Esterases

This mechanism of resistance was first described in 1984. It was detected in an *E. coli* strain (Barthélémy et al., 1984) which borne a 61-Kb plasmid, named pIP1100, belonging to the incompatibility group X (Andremont et al., 1986), carrying a gene encoding a 406 amino acid protein of 44.8 kDa (Ounissi & Courvalin, 1985) with macrolide-inactivating activity. The gene, named *ere(A)* because of its esterase activity, was rapidly detected in a variety of other *Enterobacteriaceae* such as *Klebsiella*, *Citrobacter* or *Enterobacter* (Arthur et al., 1986a, b, 1987), and has currently been described in at least eight different *Enterobacteriaceae* genera (Roberts, 2001). At present, two esterase encoding genes have been reported, the aforementioned *ere(A)* gene as well as the *ere(B)* gene (Arthur et al., 1986a), which is also widely disseminated among *Enterobacteriaceae* family members (Roberts, 2001). Both enzymes act by hydrolyzing the macrolactonic ring at the ester union present in their structure (Morar et al., 2012). However, the substrate profile of both esterases differs. While EreB confers resistance to various 14C and 15C macrolides but not to telithromycin, EreA has a more restrict substrate profile and does not recognize azithromycin or telithromycin (Morar et al., 2012).

Despite to be first described among *Enterobacteriaceae*, the DNA sequence of the *ere(B)* gene possesses a G + C content of 36%, thereby showing a specific codon usage different from that of *E. coli* and suggesting that the *ere(B)* gene has an exogenous *E. coli* origin, being proposed as proceeding from a Gram-positive cocci (Arthur et al., 1986b). On the other hand, the 50.5% G + C content of the *ere(A)* gene, together with its codon usage, suggests that this gene is indigenous of *E. coli* (Ounissi & Courvalin, 1985). Despite these differences, both enzymes act similarly, hydrolyzing the macrolactonic ring and inactivating macrolides.

It seems that punctual mutations in these esterase-encoding genes may affect their ability to inactivate macrolides. Thus, on cloning the *ere(A)* allele encoded within the plasmid pLQ1723, Biskri & Mazel (2003) isolated a mutant presenting the amino acid codon substitution L101S, showing a decrease in the MICs of erythromycin and clarithromycin from 25 and 20 mg/L to 1 and 4 mg/L, respectively. However, to our knowledge, no further studies on the effect of alteration at this amino acid codon have been performed. Similarly it has been shown that specific substitutions (H46A, E74A or H288A) in EreB result in a marked decrease in EreB ability to hydrolyze erythromycin (Morar et al., 2012). Indeed, the authors hypothesized that H46, which forms a hydrogen binding pair with E74, is directly involved in the activation of a water molecule, which acts as a nucleophile in the ester hydrolysis reaction (Morar et al., 2012). Although found at different positions (40 and 68), both amino acids are conserved in EreA.

An interesting difference between these two enzymes is the metal requirements of EreA, which was manifested by its inhibition in the presence of chelant agents (Morar et al., 2012).

Both *ere(A)* and *ere(B)* genes may be encoded within a type 1 and 2 integron environment, together with other antimicrobial gene resistance (Biskri & Mazel, 2003; Krauland et al., 2010; Takaya et al., 2006). In some cases the presence of insertion sequences which may facilitate the dissemination of these genes has been described (Biskri & Mazel, 2003).

Phosphotransferases

Macrolide resistance mediated by phosphotransferases was first described in *Streptomyces coelicolor* (Wiley et al., 1987), being detected in an *Enterobacteriaceae* in 1988 (O'Hara et al., 1988). To date, six different macrolide resistance phosphotransferase encoding genes have been described, being four of them, *mph(A)*,

Figure 5. Bottle brush model. The macrolide–ribosome interactions results in the blocking of amino acid translation. However if R-peptide is translated (A), results in the removing of the macrolide from ribosome (B), which recover its functionality (C). If the new translated peptide arrives to a minimum size (4–6 amino acids) does not allow the macrolide interaction and the protein is translated.

mph(B), *mph(D)* and *mph(E)* genes, described in *Enterobacteriaceae* (Roberts, 2001).

The phosphotransferases act by modifying the macrolide structure. Thus, it has been described that MphA and MphB act by phosphorylating the 2'-OH of the desosamine moiety (O'Hara et al., 1988, 1989). The inhibitory effect of different substances such as EDTA, divalent cations or iodine on the action of some phosphotransferases, such as MphB, suggests that the presence of Mg^{2+} is crucial for its activity (O'Hara et al., 1989).

On cloning three of the aforementioned genes [*mph(A)*, *mph(B)*, *mph(E)*] and the *mph(C)* gene in the *E. coli* AG100, Chesneau et al. (2007) showed that these phosphotransferases presented a different substrate profile. Thus, MphA was able to inactivate 14C, 15C and 16C macrolides, while MphB, MphC and MphE were unable to inactivate macrolides possessing 15C, such as azithromycin. However, other authors have indicated that MphA preferentially inactivates the 14C macrolides than those possessing 16C (O'Hara et al., 1989), while azithromycin affinity of MphB is reportedly higher than that of spiramycin (Taniguchi et al., 1999). This may be explained by the presence of different alleles or the effect of punctual mutations on phosphotransferase activity (Taniguchi et al., 1999).

As mentioned previously, the *mph(E)* gene has frequently been detected together with the *msr(E)* gene (see above). Regarding the remaining phosphotransferase-encoding genes, *mph(A)* is probably the most relevant, and has ranked among the most frequently detected TMMR in *Enterobacteriaceae*. Together with its ability to inactivate azithromycin this finding may have a direct impact on the usefulness of azithromycin against pathogenic *Enterobacteriaceae* and has been increasingly described in *Shigella* spp. including some recent azithromycin-resistant *Shigella sonnei* outbreaks (Boumghar-Bourtchai et al., 2008; Gaudreau et al., 2014; Heiman et al., 2014; Howie et al., 2010; Sjölund Karlsson

et al., 2013) and intercontinental spreaded *Shigella flexneri* 3a clone (Baker et al., 2015). The *mph(A)* gene has also been detected in a few isolates of the highly antibiotic-resistant *Salmonella enterica* serovar Kentucky, which is currently spreading from North Africa to different European countries (Le Hello et al., 2013). Additionally, the *mph(A)* gene has also been described in other clinically relevant *Enterobacteriaceae* such as *Klebsiella pneumoniae* (Sandegren et al., 2012). This gene has also frequently been detected in both hospital recovered and commensal *E. coli* of different geographical origins (Nguyen et al., 2009). It is interesting to note that in microorganisms in which only the *mph(A)* gene has been identified and no other mechanism of macrolide-resistance has been detected, the levels of resistance varies greatly (Boumghar-Bourtchai et al., 2008; Gaudreau et al., 2014; Lluque et al., 2015; Sjölund Karlsson et al., 2013). This may be related to both the presence of specific alleles which might have different inactivation levels, by the concomitant presence of unidentified macrolide-resistance mechanisms or by *mph(A)* gene expression levels. In the latter respect, the presence of a series of *mph(A)* gene copies has been described in the same genetic structure (Poirel et al., 2011).

Resistance mediated by short peptides

The presence of peptides able to confer erythromycin resistance was first reported in 1996 (Tenson et al., 1996). In this study a series of 23S rRNA fragments were randomly cloned and their effect on the erythromycin resistance was observed. The results showed that the overexpression of 23S rRNA fragments located between positions 1233–1348 of 23S rRNA was sufficient to select erythromycin-resistant strains. A more in depth analysis of the region showed which erythromycin resistance was linked to a region placed between positions 1235–1276. In this region five amino acid codons,

possessing their own Shine-Dalgarno sequence (positions 1235–1240), containing ORF (positions 1248–1265) encoding the peptide MRMLT were identified. Additionally, this peptide seemed to act in *cis* and then developed its protective action in the ribosome in which was transcribed (Tenson et al., 1996).

Further studies using random peptide libraries have shown a variety of synthetic short peptides of between three and six amino acids able to produce macrolide resistance as well as the inability of these peptides to affect the MIC of erythromycin when included in longer amino acid sequences (Tenson et al., 1997). However, the Shine-Dalgarno region is located in a hairpin at position 1198–1247, being difficult to access by RNA-polymerases (Dam et al., 1996) and probably being unexpressed in normal conditions.

Interestingly, it has been shown that peptides able to increase the levels of resistance to one macrolide, may have little or null effect against other macrolides (Tenson & Mankin, 2001; Tripathi et al., 1998; Vimberg et al., 2004), suggesting a direct interaction between these peptides and macrolides. Thus, some authors refer to these peptides based on the macrolide or type of macrolide to which resistance is conferred, such as for example the E-peptide if conferring resistance to erythromycin, or K-peptides if conferring resistance to ketolides, while other authors use the more generic term R-peptide (resistance peptide).

Based on the aforementioned data a model of action of these peptides (the bottle brush model) has been proposed (Figure 5). In this model when the R-peptide is translated in a ribosome with a macrolide in the macrolide binding pocket, on release from the peptidyl-tRNA or during the last translocation event it is able to interact with the macrolide, removing it from the ribosome. On removal of the macrolide, the ribosome can initiate a new protein translation. A translation of 4–6 amino acids can avoid the possibility of macrolides interacting with the ribosome, and the protein can successfully be translated (Tenson & Mankin, 2001; Tripathi et al., 1998). Lovmar et al. (2006) reinforced this model by analyzing the erythromycin dissociation rates with the use of the E-peptide MRLFV. Moreover, the same study showed that alterations in the size of the peptide possess a strong effect on the resistance levels conferred, suggesting that the Leu residue plays a key role in the dissociation of erythromycin. This finding may be in accordance with the high consensus of previously studied E-peptides selected using erythromycin, which usually presents at least one Leu at position 3 (Tenson et al., 1997). However, this E-peptide model of action cannot be extended to all macrolides. Thus, on studying the interactions between peptides and josamycin

(Lovmar et al., 2009b), peptide expression was found to not eject josamycin from the ribosome. Subsequently, the presence of another peptide mechanism of resistance was sought, and it was proposed that the interactions between peptides and josamycin results in the “sequestration” of josamycin in the ribosomes and in the elimination of the josamycin depletion of tRNA isoacceptors (Lovmar et al., 2009b).

This macrolide resistance mechanism has not been described at a clinical level. However, it has been proposed that $\Delta 1219$ –1230 in 23S rRNA may result in increasing levels of erythromycin resistance due to a destabilization of the hairpin at position 1198–1247, providing accessibility to the Shine Dalgarno sequence preceding the mini-gene and allowing its expression (Tenson et al., 1996).

In close relationship with this mechanism it has been proposed that the transcription of mRNAs possessing a Leu or Ile encoding codon at position 3 and a GGG (Gly) or UGG (Trp) codons at position 4 or 5 might act in a similar manner, ejecting macrolides from PTC and restoring ribosome functionality (Macvanin et al., 2007).

Although a series of studies have been performed to characterize this mechanism of macrolide resistance and data regarding the effect on macrolide dissociation are available, no data have been found in the literature on the effect on MIC levels.

Overexpression of peptidyl t-RNA hydrolase

The involvement of peptidyl tRNA hydrolase in the development of macrolide resistance has been described in the specific case of josamycin (Lovmar et al., 2009b). Thus, josamycin action results in the depletion of tRNA isoacceptors by a peptidyl tRNA drop off. Lovmar et al. (2009b) proposed that the peptidyl tRNA hydrolase alters peptidyl-tRNAs (which are inactive in protein synthesis) to become deacylated tRNAs, able to carry amino acids, which may revert the josamycin-associated peptidyl tRNA drop off. On cloning the peptidyl tRNA hydrolase in an expression vector, the development of a low level of resistance to josamycin was confirmed and it was also shown how this method of action of resistance-peptides does not affect the action of erythromycin.

Conclusions

Although until recently macrolides were not considered for the first line treatment for *Enterobacteriaceae* infections, a few macrolides such as azithromycin are currently used in the standard treatment of infections due to *Shigella* spp. or *Salmonella* spp., among other

Enterobacteriaceae (Basualdo, 2003; DuPont, 2009; Erdman et al., 2008; Trivedi & Shah, 2012). Moreover, several clinical trials have established a relationship between mass treatments with azithromycin and enhanced child survival.

Unfortunately, a great variety of macrolide resistance mechanisms have been described in the *Enterobacteriaceae* family. Furthermore, studies addressed to determining if the presence of specific modifications in macrolide resistance mechanisms also affects macrolides designed as immunomodulators or anti-inflammatories are needed. The presence of these macrolide resistance mechanisms demonstrates both the potential of punctual chromosomal mutations or specific alterations in protein expression in the development of macrolide resistance phenotypes, and the high level of promiscuity of the bacterial world, allowing the transfer of genetic material among distantly phylogenetically related microorganisms. In addition, and more importantly, the relevant role of nonpathogenic microorganisms from both human and environmental microbiota as an antibiotic resistance storehouse must be addressed since these microorganisms may gain, maintain and deliver genes to transient microorganisms. Moreover, there is an urgent need to develop and implement effective antibiotic surveillance worldwide to thereby allow continued use of macrolides and other antimicrobial agents before their utility is definitively lost.

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