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REVIEW

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Mechanisms of bacterial resistance to macrolide antibiotics

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Abstract Macrolides have been used in the treatment of infectious diseases since the late 1950s. Since that time, a finding of antagonistic action between erythromycin and spiramycin in clinical isolates[1] led to evidence of the biochemical mechanism and to the current understanding of inducible or constitutive resistance to macrolides mediated by *erm* genes containing, respectively, the functional regulation mechanism or constitutively mutated regulatory region. These resistant mechanisms to macrolides are recognized in clinically isolated bacteria. (1) A methylase encoded by the *erm* gene can transform an adenine residue at 2058 (*Escherichia coli* equivalent) position of 23S rRNA into an ^{6}N, ^{6}N-dimethyladenine. Position 2058 is known to reside either in peptidyltransferase or in the vicinity of the enzyme region of domain V. Dimethylation renders the ribosome resistant to macrolides (MLS). Moreover, another finding adduced as evidence is that a mutation in the domain plays an important role in MLS resistance: one of several mutations (transition and transversion) such as A2058G, A2058C or U, and A2059G, is usually associated with MLS resistance in a few genera of bacteria. (2) M (macrolide antibiotics)- and MS (macrolide and streptogramin type B antibiotics)- or PMS (partial macrolide and streptogramin type B antibiotics)-phenotype resistant bacteria cause decreased accumulation of macrolides, occasionally including streptogramin type B antibiotics. The decreased accumulation, probably via enhanced efflux, is usually inferred from two findings: (i) the extent of the accumulated drug in a resistant cell increases as much as that in a susceptible cell in the presence of an uncoupling agent such as carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol (DNP), and arsenate; (ii) transporter proteins, in M-type resistants, have mutual similarity to the 12-transmembrane domain present in efflux protein driven by proton-motive force, and in MS- or PMS-type resistants, transporter proteins have mutual homology to one or two ATP-binding segments in efflux protein driven by ATP. (3) Two major macrolide mechanisms based on antibiotic inactivation are dealt with here: degradation due to hydrolysis of the macrolide lactone ring by an esterase encoded by the *ere* gene; and modification due to macrolide phosphorylation and lincosamide nucleotidylation mediated by the *mph* and *lin* genes, respectively. But enzymatic mechanisms that hydrolyze or modify macrolide and lincosamide antibiotics appear to be relatively rare in clinically isolated bacteria at present. (4) Important developments in macrolide antibiotics are briefly featured. On the basis of information obtained from extensive references and studies of resistance mechanisms to macrolide antibiotics, the mode of action of the drugs, as effectors, and a hypothetical explanation of the regulation of the mechanism with regard to induction of macrolide resistance are discussed.

Key words Macrolide · Antibiotic · Resistance · Bacteria · Decreased accumulation ·*erm* · Efflux · Inactivation

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Introduction

Macrolide antibiotics[2] consist of a large lactone ring (aglicone of 12–16 carbon atoms) to which one or more sugars (which can be amino sugars, non-nitrogenous sugars, or both) are linked. The antibiotics, inhibit protein synthesis by acting on the 50S subunit of the 70S ribosome.[3] New insights into the structure-activity relationship of macrolides antibiotics, including semisynthetic macrolides such as azalides, have been given by Bryskier et al.[4]

In a broad sense of the word, the term "macrolides", in relation to the resistance mechanism to the drugs has been considered to include all lincosamide and streptogramin type B antibiotics, because they have a similar mode of action, despite being chemically distinguishable from each other (Fig. 1). In order to distinguish, the narrow meaning of the term "macrolide antibiotics" from the wider mean-

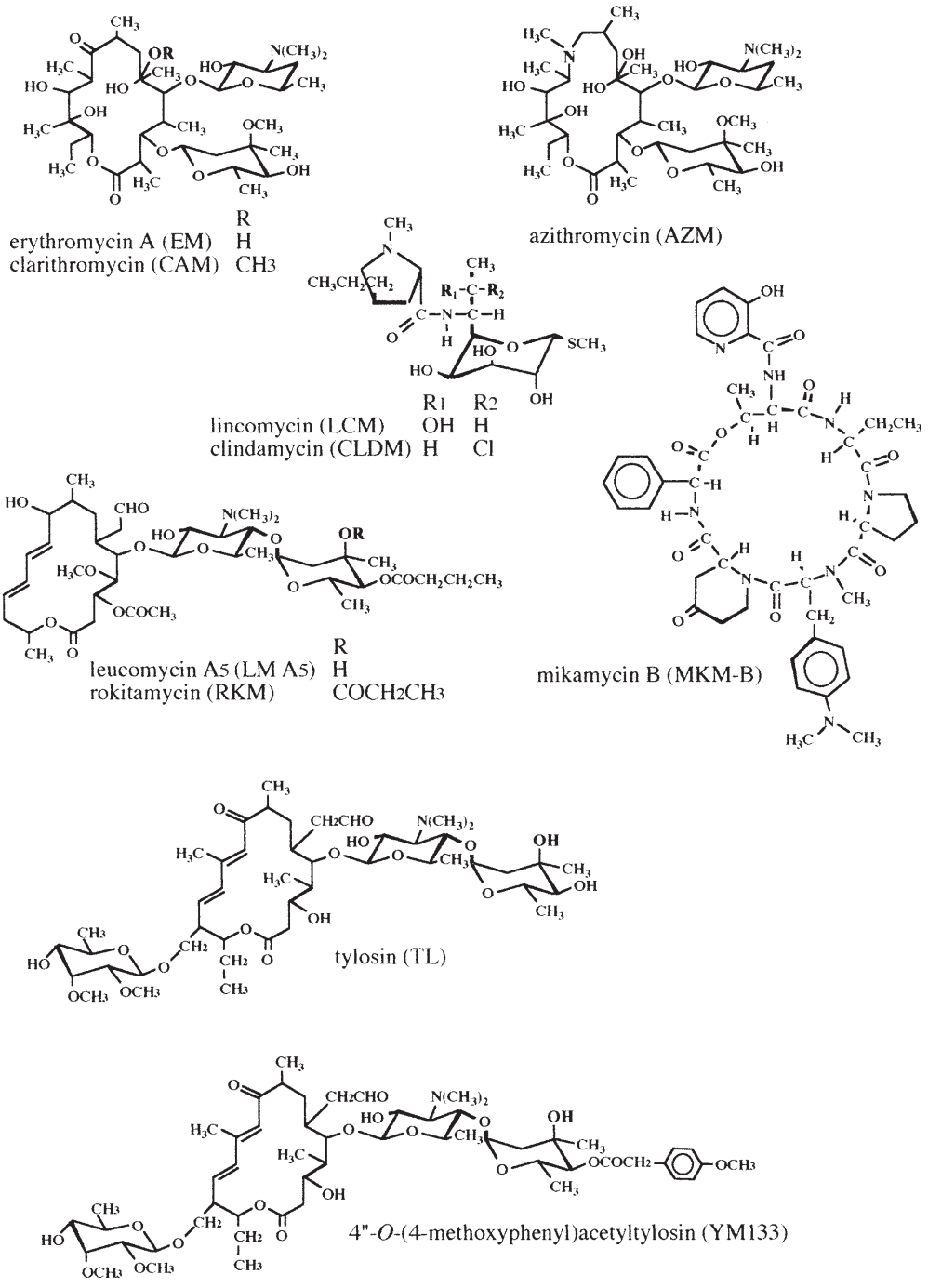

Fig. 1. Chemical structures of macrolide, lincosamide, and streptogramin type B antibiotics. Macrolide antibiotics (M), EM, CAM, AZM, LM A_5, RKM, TL, YM133; Lincosamide antibiotics (L), LCM, CLDM; Streptogramin type B antibiotic (S), MKM-B

ing, the former narrow group is hereafter referred to as macrolide antibiotics, while the latter are referred to as macrolides or MLS (macrolide, lincosamide, and streptogramin type B) antibiotics (Figs. 1 and 2).

The MLS antibiotics have a narrow spectrum of activity that includes gram-positive cocci (e.g., staphylococci, streptococci, and enterococci) and bacilli, and gram-negative cocci. After the β-lactam antibiotics, macrolide antibiotics are often used as a safe remedy against infection by one of these bacteria, because they fail to give rise to severe adverse effects. Gram-negative bacilli are usually intrinsically resistant to MLS antibiotics, but certain gram-negative bacteria, including *Haemophilus*, *Bordettella*, *Legionella*, *Campylobacter*, *Chlamydia*, and *Treponema* spp. are susceptible to them. The β-lactam antibiotics, and quinolone antimicrobial agents, are not active against *Mycoplasma* spp., whereas a macrolide antibiotic, such as erythromycin, is effective against the bacteria.

In the year 1952, erythromycin was first obtained from *Saccharopolyspora erythreus* (formerly *Streptomyces erythreus*).[5] Shortly after unsuccessful erythromycin treatment (for only 7–10 days) in two patients with acute

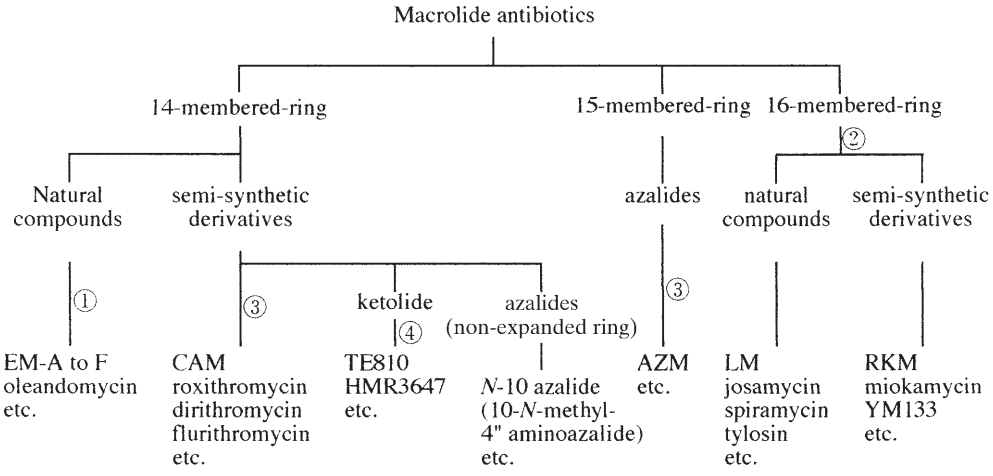

Fig. 2. Some macrolide antibiotics.[4] Symbols ①, ②, ③, and ④ indicate first, second, third, and fourth generation macrolide antibiotics, respectively[76]

bacterial endocarditis,[6] resistance to erythromycin emerged in two strains of bacteria, in particular in *Staphylococcus aureus*.

In the two bacteria, with acquired erythromycin-resistance, obtained in clinical isolates in 1952 in the United States, it is too late to determine retrospectively whether the kind of resistant genotype (*erm* or *msr*) in the two *S. aureus* strains could be specified. The *erm* gene encodes a methylase that catalyses dimethylation of a specific adenine residue in 23S rRNA and the *msr* gene encodes for a cell-membrane protein which acts as an active erythromycin-efflux pump.

Despite being unable to determine the specific genotype in erythromycin-resistant *S. aureus*, Westh et al.[7] have recently reported that the *erm* gene was present in 98% of erythromycin-resistant strains isolated from blood between 1959 and 1988 in Denmark. Accordingly, the resistant strains from the blood of the two endocardial patients must have borne the *erm* gene.

MLS resistance due to modification of the drug target, a specific adenine residue of 23S rRNA, is wide spread and has been found in *Staphylococcus* spp.,[1,8–11] *Streptococcus* spp.,[12,13] *Corynebacterium diphtheriae*,[14] *Clostridium* spp.,[15–17] *Bacillus* spp.,[18–22] *Lactobacillus* spp.,[23] *Propionibacterium* spp.,[24] *Bacteroides fragilis*,[25–27] *Escherichia coli*,[28] and *Klebsiella* spp.[29] In other antibiotic-producing bacteria, such as *Streptomyces* spp.,[30–37] *Micromonospora* sp.[38] *Saccharopolyspora* sp.,[39] and *Arthrobacter* sp.[40] macrolide resistance mediated by *erm* genes, has been described in detail, together with cooperative determinants coding for a transport ATPase.[41]

This article reviews the biochemical mechanism and the genetic basis of resistance to MLS antibiotics by target modification, the decreased macrolide accumulation usually related to enhanced efflux, and inactivation of the antibiotics. Careful attention is focused on clinically isolated bacteria, especially *S. aureus*. In addition, important developments in macrolide antibiotics will be briefly featured, and on the basis of their resistance mechanism to macrolides, the mode of their action will be discussed as well.

Resistance in clinical isolates

Three mechanisms of resistance to macrolides in bacteria are known: (1) modification of the antibiotic target, mediated by the so-called *erm* gene (Table 1);[41] (2) enhanced efflux mediated by the *msr*, *erp*, *mef*, and *mre* genes (Table 2);[42,43] (3) inactivation of macrolide antibiotics by erythromycin esterase encoded by the *ere* or the *ere*-like gene,[44] by streptogramin B hydrolase encoded by the *vgb* gene, by macrolide phosphotransferase encoded by the *mph* gene, and by lincosamide nucleotidyltransferase encoded by the *lin* gene (Table 3).

Alteration of the MLS target site

Erythromycin is an inhibitor of bacterial protein synthesis; however, the transfer of *N*-acylamino residues is usually stimulated by erythromycin under certain conditions.[45–48]

The stimulating effect of erythromycin on peptidyltransferase is thought to depend on several structural factors, such as the number of amino acid residues on the donor tRNA, the hydrophobicity of the aminoacyl portion of the donor tRNA, and the size of the amino acid side chain.[48,49] In addition to such factors, this stimulatory effect of erythromycin seems to be related to another factor, the amount of the macrolide antibiotic present.

In fact, in poly (A)-dependent polylysine synthesis by cell-free extracts containing S100 (105000*g* supernatant) from *E. coli* Q13 and ribosomes from *S. aureus*,[50] stimulation of 15% to 50% has occurred in the presence of small amounts of macrolides (for example, 0.4μg or less of the drug/ml, corresponding to about 0.5μM or less), compared with synthesis in the absence of erythromycin or spiramycin. The extent of the stimulation by erythromycin was greater than that by spiramycin at the same low concentration of the drugs. On sodium dodecylsulfate-polyacrylamide gel electrophoresis, the molecular size of

Table 1. MLS resistance due to target modification, mediated by *erm* genes, and due to mutation at several sites in the peptidyl transferase circle of 23S rRNA domainV from clinical isolates

Resistance mechanism (resistant phenotype): Host	Gene	References
Target modification (MLS)		
Staphylococcus aureus	*ermA*	110
	ermB	13
	ermC	64,95
	ermGM	111
Streptococcus epidermidis	*ermM*	112
Streptococcus sanguis	*ermAM*	12
Enterococcus fecaris	*ermB*-like	13
	ermAMR	113
Lactobacillus reuteri	*ermGT*	23
Corynebacterium diphtheriae	*ermCD*	114,115
Clostridium perfringens	*ermP*	15
	ermQ	16
Clostridium difficile	*ermZ*	17
Propionibacterium spp.	ND	24
Escherichia coli	*ermBC*	28
Klebsiella spp.	*ermAM*-like	29
Bacteroides fragilis	*ermF*	25
	ermFS	26
	ermFU	27
Target mutation (MLS or ML)		
Helicobacter pylori	A2058G[a]	60,116
	A2059G	
Propionibacteria spp.	G2057A[a]	78
	A2058G	
	A2059G	
Mycobacterium intracellulare	A2058G[a]	66
	A2058C	
	A2058U	

MLS, Macrolide, lincosamide, and streptogramin type B antibiotics; ND, not determined; ML, macrolide and lincosamide antibiotics

[a] For example, G2057A, A2058G, and A2059G correspond to G → A and A → G transitional mutations at positions related with *E. coli* in 23S rRNA positions 2057, 2058 and 2059. In this connection these positions correspond to *H. pylori* positions 2141, 2142 and 2143, respectively

the product stimulated in the presence of erythromycin gave the same polylysine as that in the absence of the drug (unpublished data). This finding may be consistent with a concept that the antibiotics, as low-molecular-weight secondary metabolites, have played unique and important biochemical roles in the evolution of living forms on earth:[51] macrolides, low-molecular-weight effectors, may have stimulated peptide-bond formation on ribozyme-like protoribosome, which was made of RNA alone, as tentatively suggested by Crick.[52]

Ribosomal RNA, a ribozyme that is able to act as a transpeptidase, appears to be one of the most important components of living cells. Most mutational changes of the conserved base sequence in 23S rRNA would become unfavorable for ribosomal function, and in the course of evolutionary events, would lead to cessation of cell growth and, probably, to cell death before long. Therefore, the reason that target site modification such as dimethylation of a specific adenine residue in 23S rRNA emerges in isolates clinically resistant to macrolides rather than either target site mutation such as deletion or inversion or mutation affecting ribosomal protein L4 or L12, which also gives

Table 2. Macrolide resistance due to decreased accumulation (enhanced efflux) in staphylococci and streptococci

Resistant phenotype	Host	Genotype	Reference
MS	*Staphylococcus epidermidis*	*msrA*	83,85
PMS	*Staphylococcus aureus*	*msrSA*	91
PMS	*Staphylococcus aureus*	*msrSA′*	96,97
M	*Staphylococcus epidermidis*	*erpA*	85,112
M	*Streptococcus pyogenes*	*mefA*	88,117
M	*Streptococcus pneumoniae*	*mefE*	89
M	*Streptococcus agalactiae*	*mreA*	90

PMS, Partial macrolide and streptogramin type B antibiotics; MS, macrolide and streptogramin type B antibiotics; M, macrolide antibiotics

Table 3. Macrolide, lincosamide, and type B streptogramin resistance in clinically isolated bacteria due to inactivation by hydrolytic degradation and modification of the drugs by certain transferases

Resistant phenotype[a]	Enzyme	Gene	Host	Refenence
Degradation				
M_d[a]	Erythromycin esterase type I	*ereA*	*E. coli*	118
M_d	Erythromycin esterase type II	*ereB*	*E. coli*	119
M_d	14- and 16-Membered macrolide esterase	*ere*-like	*S. aureus*	44
S_d	Streptogramin B hydrolase	*vgb*	*S. aureus*	120
Modification				
M_m[a]	Macrolide 2′-phosphotransferase (14-membered ring macrolides only)	*mphA*	*E. coli*	121
M_m	Macrolide 2′-phosphotransferase (14- and 16-membered ring macrolides)	*mphB*	*E. coli*	122
M_m	Phosphotransferase?[b]	*mphBM*	*S. aureus*	97
M_m	Macrolide 2′-phophotransferase (14-membered, rather than 16-membered ring macrolides)	*mphK*[c]	*E. coli*	123
L_m	3-Lincomycin, 4-clindamycin-*O*-nucleotidyltransferase	*linA*	*S. haemolyticus*	124
		linA′	*S. aureus*	124

[a] Subscript letters "d and m" represent degradation and modification, respectively

[b] ?, Putative enzyme

[c] The *mphK* gene differs from the *mphA* gene at only five amino acid positions

rise to macrolide-resistance,[53] appears to be related to an evolutionary concept from the prebiotic RNA world.[51]

The resistance mechanism consisting of modification of the antibiotics target mediated by the *erm* gene, is especially prevalent in clinical gram-positive isolates in comparison with gram-negative ones. In *S. aureus*, macrolide-resistant strains of the bacteria are clinically isolated at a frequency of about 50%. In Hokkaido, the northern part of Japan, most resistant isolates (more than 90%) had MLS-resistant phenotypes (unpublished data).

Methylation of domain V

In terms of the basic principle of the secondary structure, *E. coli* 23S rRNA has six domains.[54] Macrolide antibiotics interact with two regions (in domains II and V) of 23S rRNA,[54–56] and the domains are thought to play an important role in RNA in terms of translation, especially peptidyltransferase activity.[57–60]

In clinically isolated strains of *S. aureus* and some other bacteria, modification involving methylation of A2058, which corresponds to an adenine residue at position 2058 that is based on the *E. coli* numbering system,[61,62] has been accomplished in domain V by adenine-N^6-methyltransferase specified by an *erm* gene.

The adenine residue at position 2058 underwent dimethylation by methyltransferase which was produced transcriptionally (in the case of *ermK*),[22] or posttranscriptionally (in the case of *ermC*)[63,64] or both,[65] or mutationally.[20,22,60,66]

Whether the regulation of *erm* gene expression, in terms of MLS resistance, is sustained inducibly or constitutively, the gene codes for an enzyme, Erm (erythromycin resistance methylase). Methylation prevents MLS antibiotics from binding to the internal loop in domain V of the 23S rRNA, probably as a consequence of a conformational change in the RNA, leading to high resistance to MLS antibiotics, since the binding sites of these drugs overlap.[41,67–69] This kind of resistance mechanism also appears to create a phenotypically decreased accumulation of macrolide antibiotics in resistance cells.[70,71]

Apart from clinical isolates, *erm* genes were isolated from soil bacteria, such as *Bacillus licheniformis* (*ermD* and *K*), *Bacillus sphaericus* (*ermG*), *Bacillus subtilis* (*ermIM*), and *Bacillus anthracis* (*ermJ*), as well as *Saccharopolyspora erythreus* (*ermE*, erythromycin producer), *Arthrobacter luteus* (*ermR* or *ermA'*, *AR*, erythromycin producer), and *Streptomyces fradiae* (*ermSF*). Extensive studies of *erm* alleles and their regulation of macrolide resistance have been reviewed.[41,72–76]

In addition to the base methylation, point mutations (A2142G, A2143G) within the peptidyltransferase region in domain V of RNA from clarithromycin-resistant *Helicobacter pylori* have been found to give rise to MLS resistance clinically. The residues at these positions 2142 and 2143 correspond to adenine residues at positions 2058 and 2059 of *E. coli* 23S rRNA, respectively.[60,77] This class of resistance has been reported in 23S rRNAs obtained from clinical isolates of *Mycobacterium intracellulare* and *Propionibacterium* spp.[66,78]

Mutation of domain II

The contribution of domain II to erythromycin resistance (dependent on the amount of E-peptide encoded by position 1198 to 1247 nucleotides in 23S rRNA), in terms of mutation, has been confirmed in the domain of *E. coli* 23S rRNA.[79]

23S rRNA domain II deletions were responsible for erythromycin resistance in *E. coli*: a clone that mediates erythromycin resistance has been obtained from a certain plasmid containing the *rrnB* operon of the bacteria, the plasmid that was exposed to a hydroxylamine mutagen.[55,79,80]

Deletion of 12 nucleotides (positions 1219–1230), observed within a conserved rRNA hairpin structure between nucleotides 1198 and 1247 in domain II of the *E. coli* 23S rRNA gene, conferred erythromycin resistance. This 12 nucleotide sequence is located upstream of an open reading frame which encodes the peptide MRMLT, 'E-peptide'. The expression of the pentapeptide in vivo renders *E. coli* cells resistant to erythromycin. Curiously, such a deletion and other engineered deletions did not affect the binding of erythromycin to the mutant ribosomes, as assayed by footprinting in vivo. In contrast with this, point mutations at the central loop in domain V gave rise to a marked decrease in the ribosome-drug interaction.[68,81]

These findings have been interpreted to indicate that erythromycin resistance mutation in domain II – affecting the stability of a secondary rRNA structure, the hairpin, in which the Shine-Dalgarno sequence of the rRNA-encoded E-peptide ORF is sequestered – caused an increase in the peptide, disrupted a functional interaction between domains II and V, and thereby suppressed the action of macrolides, including erythromycin, oleandomycin, and spiramycin, but not clindamycin and chloramphenicol, without preventing their binding.[79,82]

Accordingly, the effect of this type of mutation in domain II appears to mediate activation of E-peptide expression. However, at present, no clinical bacterial isolate, with such a resistance mechanism to macrolides is known.

Decreased macrolide accumulation

In recent years, new resistance phenotypes (MS [macrolide and streptogramin type B antibiotics] or partial macrolide and streptogramin type B antibiotics [PMS], and M [macrolide artibiotics]) were observed in clinical isolates of staphylococci and streptococci. Ross et al.,[42,83,84] and Goldman and Capobianco[85] have reported that MS-resistant strains of *Staphylococcus epidermidis* were resistant to 14-membered ring macrolides and streptogramin type B, but sensitive to 16-membered ring macrolide and lincosamide antibiotics.

Two resistant phenotypes (the M-type and the MS- or PMS-type) due to decreased macrolide accumulation have been identified in clinical isolates of staphylococci and streptococci (Table 2).

It is generally considered that transporters which mediate multidrug efflux can be characterized according: (1) whether they are conducted by proton motive force (PMF) or by ATP and (2) whether they consist of a single protein that has any one of 4-, 12-, or 14-transmembrane-spanning domains,[86,87] or whether they constitute a more complex multicomponent transporter.[76] The more complex transporters, in addition to a multidrug efflux protein (MexB, for example) contain a membrane fusion protein such as MexA and an outer membrane protein such as OprM.[86]

The four classes of transporters (PMF-dependent single or complex, and ATP-dependent single or complex transporters) do not correspond with the two examples of macrolide efflux transporters observed in clinical isolates, i.e., (i) M-type and (ii) MS- or PMS-type. These groupings are based on the variety of antibiotics: first, the M phenotype is characterized as usually resistant to 14- and 15-membered macrolides (respectively, erythromycin and azithromycin), and in addition to these, occasionally being resistant to 16-membered macrolides (spiramycin and tylosin), but susceptible to clindamycin and streptogramin type B antibiotics. The respective genes, *mefA* from *Streptococcus pyogenes*[88] and *mefE* from *S. pneumoniae*,[89] and *mreA* from *S. agalactiae*[90] were cloned, functionally expressed, and sequenced. A comparison of the deduced amino acid sequences between the *mefA* and *mefE* genes revealed that the two genes were 90% identical. Further analysis of their amino acid sequences disclosed the presence of 12-transmembrane domains. However, the amino acid sequence deduced from the *mreA* base sequence was significantly different from both of them. Although MreA has short recurrent hydrophobic regions of about ten amino acids, it may associate transiently with the cell membrane, or perhaps with specific membrane proteins.[90]

MefA, MefE, and MreA are thought to be driven by proton motive force, since the decreased macrolide accumulation via their mediation was increased to the same accumulation level as that in corresponding susceptible streptococci in the presence of some uncouplers, such as carbonylcyanide-*m*-chlorophenylhydrazone (CCCP); 2,4-dinitrophenol (DNP); and arsenate.

Another resistance group, MS-phenotype *Staphylococcus epidermidis*, has been studied by Ross et al.[83] The MS resistance group showed inducible resistance to 14-membered ring macrolides and to streptogramin type B antibiotics, but susceptibility to 16-membered ring macrolides and lincosamides. A subcloned 1.9-kb DNA sequence from one strain of *S. epidermidis*, which was located on the 31.5-kb plasmid, contained the gene *msrA*, which conferred MS resistance. The sequence revealed an open reading frame which encoded a 488-amino acid protein (MsrA) whose regulation is mediated by translational attenuation,[42] a mechanism of regulation similar to that which regulates *ermC* (Fig. 3).

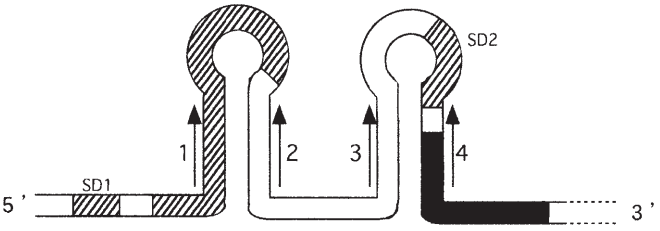

Fig. 3. Schematic representation of alternative conformations of the mRNA from the inducible *ermC* gene from pE194. Adjacent to the *ermC* structural gene for methylase is an open reading frame encoding a 19-amino-acid leader peptide (*diagonally shaded areas*). In this conformation, the 5′ end of the corresponding mRNA presents a set of four inverted repeats (*arrows*), the four repeats being paired as 1:2 and 3:4; the set 3:4 sequesters SD2 and the initiation codon for the methylase, by base pairing in the absence of erythromycin. Thereby SD2 and the codon are not accessible to the ribosomes, and only the sequence corresponding to the leader peptide is translated through SD1, which is not impeded (translational attenuation). When present, erythromycin binds to ribosomes, including those involved in the synthesis of the leader peptide, and causes them to stall. Ribosome stalling probably gives rise to conformational rearrangements in the mRNA and displacement of the stem-loop structure. Then SD2, being free, can be recognized by ribosomes for the initiation of translation of the methylase (*black area*). The methylase is synthesized either by ribosomes that are not complexed to erythromycin, or by those that are methylated by enzyme that may stimulatively be produced, in the presence of a low concentration of erythromycin, because of the spontaneous and transitory mRNA rearrangements.[125] *SD*, Shine-Dalgarno sequence, a sequence for the formation of the correct preinitiation complex between a 30S ribosomal subunit and an mRNA

As shown in Fig. 3, it is thought that a similar regulation mechanism in the *msrA* gene to the mode of inducible ErmC production is governed by a leader sequence of about 300 nt, which encodes, in sequence, an upstream ribosome binding sequence (RBS or SD1), GGAGG, a putative eight-amino acids leader peptide, MTASMRLK, and a noncoding region that contains four inverted complementary repeat sequences. Finally, the MsrA open reading frame (ORF) is preceded by its own RBS, AGGAG, which could be sequestered by a secondary structure of the leader region. If the upstream leader peptide sequence is occupied by a stalled erythromycin-ribosome complex, the ORF is presumed to become available.

Based both on the similarity between the amino acid sequence of the 488-amino-acid MS transporter, MsrA, and the sequences of ATP-binding casette (ABC) transporters, and on the result showing reduced erythromycin accumulation in the presence of an uncoupler (arsenate or dinitrophenol), Ross et al.[42] inferred that the MS transporter mediated the efflux of erythromycin and streptogramin type B antibiotics by consuming energy from ATP.

Phenotypic resistance to 16-membered ring macrolides or clindamycin does not appear to be seen in MS strains. However, the PMS strain of *S. aureus* reported by Jánosi et al.[91] was described as an inducible co-resistant to erythromycin and type B streptogramin but susceptible to lincosamide antibiotics. Later, it was found that the strain conferred resistance to mycinamicin, a 16-membered ring

macrolide, in addition to 14-membered ring macrolides such as erythromycin and oleandomycin, and streptogramin type B antibiotics. Matsuoka et al.[92] showed that the N-terminal PMS-resistance sequence (MsrSA) of the PMS-resistant *S. aureus* strain was identical to that of MsrA from *S. epidermidis* to the extent of 31 amino acids, that a cloned 5.04-kb DNA sequence contained the *msrSA* gene which conferred PMS resistance, and that its sequence revealed the same ORF which encodes a 488-amino acid protein whose regulation is mediated by translational attenuation, as is the ORF in the *msrA* sequence, except for four nucleotides (unpublished data). Consequently, it is reasonable that the MS phenotype *S. epidermidis* strain would also show resistance to mycinamicin, when MS resistance is induced by a suitable concentration of erythromycin (in the case of *S. aureus*, 1.35 μg/ml was required, for example).[92]

The PMS-resistant specificity of staphylococci whose PMS resistance manifested itself is usually restricted to 14-membered macrolides and streptogramin type B antibiotics, and occasionally to 16-membered macrolides, such as mycinamicin I and II. It is worth noting that these drugs all have common a physicochemical feature, i.e., a high pKa value, of 8.5 to 9.0, higher than the pH value of culture media (7.5), since a protonated macrolide is less permeable than a non-protonated macrolide,[92,93] or since a protonated macrolide is known to be harder to bind to ribosome.[94]

As described above, the genetic mechanisms which regulate MLS resistance usually occur in terms of a translational attenuator (*ermC*),[64,95] and, occasionally, in terms of transcriptional attenuation in resistance which is mediated by the *ermK* gene. With regard to translational attenuation, the induction of MLS and MS or PMS resistance by the presence of erythromycin is thought to result from the stalling of an erythromycin-ribosome complex on the DNA sequence of the leader (control) peptide. However, there may also be another possible explanation, that erythromycin acts as an accelerating effector that interacts with a peptidyltransferase, but not as an inhibitor which renders ribosomes stalling on the enzyme in the presence of the drug at a certain dose (about 7×10^{-8}M, corresponding to 0.05 μg/ml) insufficient to inhibit protein synthesis even in susceptible bacterial cells. The more vigorously the translation of leader peptide by erythromycin-ribosome complex is performed, the more SD2 and first two codons of ErmC methylase must come to increase the unsequestered chance, the more frequently must they be recognized by other erythromycin-ribosome complexes or ribosomes shielded from erythromycin attack, because of the reduced amount of the drug present in cytoplasm, and the more must initiation of methylase translation be promoted by the complex or by drug-free ribosomes. An altered ribosome composed of a dimethylated adenine residue in 23S rRNA then gives rise to resistance to MLS antibiotics. As the amount of such ribosomes is increased, the ribosomes will more frequently bind to the SD1 region and again normally undergo translational attenuation, because they no longer bind to MLS antibiotics. This alternative hypothesis also accounts for the fact that resistant and sensitive ribosomes can coexist even in cells whose resistance was sufficiently induced by erythromycin as an inducer. In fact, in terms of quantitative analysis of dimethyladenine present in 23S rRNA, about half of the ribosomes present in sufficiently erythromycin-induced *S. aureus* cells were sensitive (i.e., unaltered using). The induction of MLS resistance mediated by the *ermA* gene was performed on cells 0.05 μg erythromycin/ml for more than 20 h at 37°C (unpublished data).

Recently the plasmid, pMS97, residing in a strain of *S. aureus* clinically isolated in 1971 in Japan, studied by Matsuoka et al.[96,97] was found to carry not only PMS- and MLS-resistance determinants, but also the *mphBM* gene, probably encoding macrolide phosphotransferase. In clinical facilities, it may be difficult to isolate *S. aureus*, strains that are able to inactivate a macrolide antibiotic by this enzyme, because they produce phosphatase that may render the drug active (unpublished data). Thereby, the detection of a genotype such as *mphBM* may be required in clinically isolated *S. aureus*.

The emergence of such a multi-resistant isolate may be a result of exposure to selective pressure for the resistant *S. aureus* strain to macrolide antibiotics, since, in Japan, several varieties of macrolide antibiotics had been used from the late 1960s to 1970s.

The plasmid pMS97 has attracted our interest from the viewpoint of individual origin and assemblage of these three resistant genes and the regulation mechanism of macrolide resistance: where has each of them come from? Have they ever constituted a cluster themselves? How do they regulate the expression of drug resistance, independently or cooperatively?

Enzymatic inactivation of macrolide antibiotics

Enzymatic mechanisms that inactivate macrolides appear to be fairly rare in clinical isolates, compared with the mechanism of phenotypically decreased macrolide accumulation due to either the target site (i.e., ribosome modification) or enhanced macrolide efflux.

As shown in Table 3, any one of the phenotypic inactivations of MLS can be specifically distinguished by the inactivation enzymes as a corresponding substrate.

Important developments in macrolide antibiotics

Based on improvements, such as increased acid-stability, improved pharmacokinetics, a broader spectrum of action, and increased effectiveness against erythromycin-resistant strains, four generations of macrolides are distinguishable,[76] and these are summarized in Table 4. The four generations are also indicated in Fig. 2.

First generation macrolide antibiotics contain 14-membered ring macrolides, such as erythromycin, oleandomycin, and megalomicin (Table 4). They differ in

Table 4. Four generations of macrolide antibiotics[76]

Generation	Representative macrolide	Remarks
First	Erythromycin Oleandomycin Megalomicin	14-Membered ring macrolides. Emergence of inducible MLS resistance
Second	Carbomycin Leucomycin Spiramycin Rokitamycin	Semi-synthetic and 16-membered ring macrolides. Emergence of constitutive MLS resistance and efflux type resistance
Third	Clarithromycin Roxithromycin Dirithromycin Azithromycin	Semi-synthetic, acid-stable, broader-spectrum 14-membered ring macrolides. Chemical stability (improved pharmacokinetics) and broader spectrum of action. Emergence of mutation-resistant *Mycobacterium* spp. and *Helicobacter pylori*
Fourth	Ketolides HMR3647 TE810	Acid-stable 14-membered ring macrolides which do not induce MLS resistance

the extent of inducer ability, but they all are able to act as inducers of MLS resistance in most inducible-macrolide-resistant isolates of *S. aureus*: quantitative values for induction of resistance (100% for erythromycin, 28% for oleandomycin, and 77% for megalomicin, for example) refer to the capability of the resistant cells to grow in the presence of a high concentration of 16-membered ring macrolide (rokitamycin), as relative inducibility (unpublished data). These macrolides can also act as inducers toward efflux-based resistant strains bearing *msr* genes.

Drugs belonging to second-generation macrolides (Table 4) remain potent against *S. aureus* that show inducible resistance to MLS antibiotics, unless the bacteria were exposed to any one of the 14-membered ring macrolides, such as erythromycin, oleandomycin, and megalomicin. Second generation drugs, however, give rise to a mutation into constitutive MLS or PMS resistance in *S. aureus* strains that show inducible resistance to MLS or PMS (for example, mycinamicin, a 16-membered ring macrolide, for the latter phenotypic resistance) antibiotics.

Rokitamycin, a semi-synthetic derivative of leucomycinA_5 (Fig. 1), has a unique property that enables the drug to bind irreversibly to ribosome, bringing about a bactericidal effect on susceptible *S. aureus* strains, despite the lower affinity of the drug to ribosome than that of erythromycin.[98]

Third generation macrolides (Table 4) increase the acid stability of the 14-membered ring macrolides, and include a 15-membered ring macrolide, azithromycin. Thereby their pharmacokinetics are improved. The antibacterial spectrum of these macrolides is broadened. Thus, a recent development in macrolides has proceeded with a modification to erythromycin A.

For example, a clinical modification at C6, e.g., *O*-methylation (clarithromycin) or at C9, e.g., some 9-ether oxime derivatives (roxithromycin and dirithromycin) afford stabilization of the 14-membered ring macrolide in acidic media, even when the modified drugs are orally administered. Drugs with an expanded erythromycin A-lactone ring (e.g., azithromycin) are also more stable in acidic media and display better anti-gram-negative activity than erythromycin A.

The substitution of L-cladinose at C3 in erythromycin A with a keto group produces a ketolide, such as HMR3647 (formerly RU-66647), which is associated with an increase in acid stability and a new characteristic different from that of erythromycin: the semi-synthetic drug does not induce MLS resistance, unlike the 14-membered ring macrolides produced naturally by antibiotic producers.[99]

Despite having the same spectrum of action as macrolides, HMR3647 has better in vitro activity against gram-positive microorganisms, including oxacillin-resistant *Staphylococcus* spp. and vancomycin-resistant enterococci.[99,100]

From these studies on modifications to erythromycin A, a great deal of important and interesting information about undertaking pharmacokinetic improvements, broadening the antibacterial spectrum, and developing potency can be drawn. For details, see Weisblum[76] and Bryskier et al.[4]

Macrolides may mimic an aminoacyl-tRNA

According to Mao and Putterman's[101] basic study of the intermolecular complex of erythromycin and ribosome, including their chemically modified derivatives, it has been proposed that seven hydrogen bonds (2′-hydroxyl, 3′-dimethylamino, 11-, 12-hydroxyl, 9-carbonyl, 3″-methoxy, and 6-hydroxyl groups) with six adjacent nitrogenous bases (because of the inclusion of two pairs of hydrogen bonds with one of the bases) of the nucleotides (probably in 23S rRNA) an required to form an erythromycin-ribosome complex.

In addition to these seven hydrogen bonds concerned with interaction between erythromycin and ribosome, a

ketone residue (-O-CO-) present in the lactone of the antibiotic may be required to bind to ribosome, since dimethylation or a mutation (Table 1) at A2058 in 23S rRNA prevents ribosomes from binding to the drug, rendering ribosomes resistant not only to macrolides, but also to lincosamides and type B streptogramins.

This suggests that there must be a ketone residue common to all MLS antibiotic structures, without exception. The residue may be available for forming a complex between any one of the antibiotic residues and the nitrogenous base of ribosomal RNA: that is, residues, -CO-O- for macrolides (Fig. 4B, BB, and C); -CO-NH- for lincosamides; -CO-O- or an unspecified ketone residue, for which -CO-NH- or -CO-NR- is required, for type B streptogramins.

Extensive recent studies in MLS-resistance, which is mediated by an *erm* gene, in clinical isolates, have disclosed that a macrolide antibiotic binds to somewhere around peptidyltransferase in the ribosome. Noller and coworkers[57] have shown that protein-depleted 23S rRNA had peptidyltransferase activity, but it was difficult to eliminate the last traces of protein without losing the transferase activity. Recently, in terms of omission and addition tests using six domains of 23S rRNA synthesized individually by T7 RNA polymerase, Nitta et al.[58] demonstrated conclusively that the six domains were capable of stimulating peptide bond formation.

In addition, on the basis of studies of macrolide-resistant mechanisms in clinical isolates, it seems that the functional characteristic of macrolide antibiotics is that they act as an effector in the presence of a small amount of the drug. Wilhelm et al.[102] have presumed that macrolide antibiotics and lincomycin may mimic peptidyl-tRNA by binding to some ribosomal region, through not only the sugar moieties but also through the ester region of the lactone ring. In lincomycin, this binding may be via the peptidyl linkage, because the linkage is similar to the juncture between tRNA chains and polypeptides, rather than the ester region present in the macrolides. The model proposed by Wilhelm et al. in that the macrolides were considered as analogues of acyl-tRNA, agreeds with our interpretation of the drug function.

As shown in Fig. 4, however, our model concerning erythromycin and leucomycinA_5 is different from that of Wilhelm et al.[102] our speculation that aminoacylated ribose at the 3′ end of tRNA may in some way resemble a 3′-dimethyl amino-glycoside of macrolides (or, probably, 3′-methoxy-glycoside of lankamycin) disagrees with Wilhelm and co-workers' concept that a substituted N^9-ribosyl glycoside (R_2=N-sugar), at position N9 of the purine ring present in the 3′ end of the ribonucleotide, may mimic an amino sugar [$(CH_3)_2$=N-sugar] in the usual macrolides.[75] Based on our hypothesis, bis-glycosides such as erythromycin (Fig. 4B) may be able to stimulate peptide bond formation at a low drug concentration, i.e., probably a ratio of erythromycin to ribosome of one or less than one to one (unpublished data), since, for example, it is possible that an α-amino residue of Lys-$tRNA_{Lys}$ at A site may be moved proximately toward the carbonyl residue of fMet-$tRNAf_{Met}$ at P site, through negative-charge repulsion by the dimethylamino residue of erythromycin. Consequently, this could facilitate the transfer of nonbonding electrons on the α-amino residue to the carbonyl carbon atom (Fig. 4B). Another sugar (cladinose) present in the lactone ring of erythromycin may resemble formylmethionyl-free ribose, the aminoacyl residue of which was removed from fMet-$tRNA_{fMet}$ (Fig. 4B). The cladinose residue present in an erythromycin molecule may compete with the amino-acid free ribose at the 3′ end of $tRNA_{fMet}$. Thereby, the sugar in the drug bound to ribosome may bring about a conformational change of E site on the ribosome, facilitating the removal of the aminoacyl-free $tRNA_{fMet}$ from the site (Fig. 4BB). In the presence of large amounts of erythromycin, the drug binds to the transpeptidase region or to the vicinity of the same region in the ribosome in a steady state, inhibiting translocation[103,104] during the elongation step of protein synthesis (Fig. 4BB). Similarly, 16-membered ring macrolides, including leucomycin A_5 are disaccharide-monoglycosides (Fig. 4C), unlike bis-glycosides such as erythromycin. One of them may occupy a putative region ranging from an amino sugar residue area between P and A sites, to a neutral sugar residue area between P and E sites. The presence of neutral sugar accompanied by dimethyl amino sugar, as shown in Fig. 4C, may greatly interfere, as a translation inhibitor, with the transpeptidase reaction, because of the disaccharide's bulk.[45,47,105] On the other hand, the reaction is required to proceed for the proximate approach of an NH_2 residue of lysine present in Lys-$tRNA_{Lys}$ to a carbonyl residue of formylmethionine-linked to the 3′ end of $tRNA_{fMet}$.

Consequently, the 14-membered ring macrolides, erythromycin and oleandomycin, which normally inhibit translocation, may preserve polyribosomes,[106,107] but the 16-membered ring macrolides, especially those containing at least one disaccharide-monoglycoside in their structures, such as leucomycin, spiramycin, carbomycin, and tylosin, may cause polyribosome degradation.[106,108]

Menninger and Otto[109] have advanced a hypothesis that macrolides stimulate the dissociation of peptidyl-tRNA from ribosomes during translocation from the A site to the P site. According to this supposition, the 14-membered macrolides could account for the stabilization of polyribosomes by the drugs, since they are inhibitors of translocation at their inhibitory concentrations. As described above, in fact, a low concentration of erythromycin may stimulate peptidyltransferase activity, with the drug acting as if it is a cofactor for the transferase (Fig. 4B), this would result in the promotion of poly (A)-directed polylysine synthesis (unpublished data). In contrast, 16-membered ring macrolides, including leucomycin, spiramycin, tylosin, and carbomycin, are known to preferentially inhibit ribosome peptidyltransferase activity, i.e., the puromycin reaction (puromycin can enter the A site on the ribosome, causing premature release of the puromycinyl peptide from the ribosome). The 16-membered macrolides would give rise to degradation of polyribosomes, since their binding to ribosome probably dissociates peptidyl-tRNA from ribosomes stimulated during translocation. However, clearer direct evidence is still required.

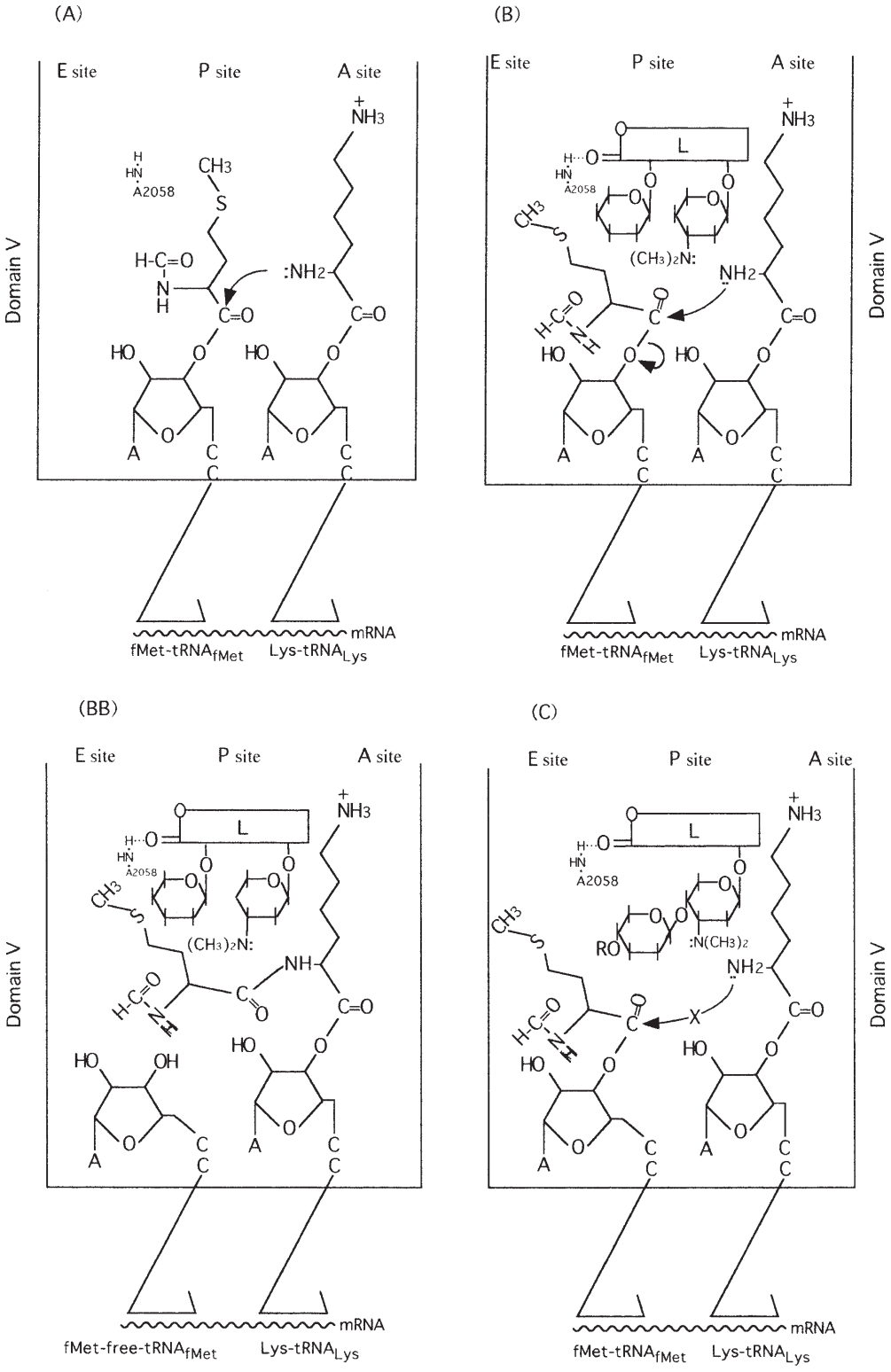

Fig. 4A–C. Peptide bond formation on peptidyltransferase center in domain V in the absence (**A**) and the presence of macrolide antibiotics, erythromycin (**B** and **BB**) and leucomycin A_5 (**C**). *L*, Lactone moiety; *R*, butylyl residue; *H_2NA2058*, 6-aminopurine residue (adenine) at position 2058 in 23S rRNA, since the methylation of residue A2058 in the RNA prevents ribosomes from binding to the drugs

Conclusion

Genes for resistance are not new creations. In terms of genetic studies on the origin of resistant genes to various antibiotics, there are also no exceptions to the genes responsible for resistance to macrolide antibiotics. This idea is supported by the findings that many kinds of clinical isolates that carry resistance determinant(s) to macrolide antibiotics rarely develop the same mechanism as drug-resistant mu-

tants which arise in vitro from treatment with a mutagen. In inducible MLS-resistant bacteria, their exposure to uninducible macrolides, such as, generally, 16-membered-ring macrolides, and, occasionally, certain 14-membered-ring macrolides (oleandomycin for a strain bearing the *ermA* gene, for example) gives rise to constitutive MLS-resistant mutants. Thereby imprudent overusage of antibiotics, because of antibiotic selective pressure, contributes to an increase in the numbers of resistance genes and of resistant bacteria, creating a paradoxical situation in that *Homo sapiens* is being challenged by drug-selected resistant bacteria to an everlasting struggle.

There are two major strategies that can be employed to prevent the emergence of macrolide-resistant bacteria: first, we should attempt to preserve the effectiveness of those antibiotics that are available, by determining in terms of rapid accurate diagnosis, which diseases they can still clear completely and which bacteria are still susceptible. Second, education must remove incorrect impressions and attitudes about antibiotics (as if they have almighty potency at any time, for example) in the minds of both consumers and prescribers.

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