

MINIREVIEW

Macrolide Resistance Conferred by Base Substitutions in 23S rRNA

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Resistance to all major groups of antibiotics has arisen hand in hand with their extensive use in medicine and animal husbandry, and macrolide antibiotics are no exception. The therapeutic utility of macrolides has been severely compromised by the emergence of drug resistance in many pathogenic bacteria. The molecular mechanisms by which bacteria become resistant are manifold, but in general these can be collectively characterized as involving either drug efflux, drug inactivation, or alterations in the drug target site. The target site for macrolides is the large (50S) subunit of the bacterial ribosome. Many cases of macrolide resistance in clinical strains can be linked to alteration of specific nucleotides in 23S rRNA within the large ribosomal subunit.

Macrolides are natural polyketide products of secondary metabolism in many actinomycete species (51, 140). Clinically useful macrolides consist of a 14-, 15-, or 16-member lactone ring (Table 1) that is generally substituted with two or more neutral and/or amino sugars (16). The structures of the 14- and 16-member-ring macrolides erythromycin and tylosin and of some semisynthetic erythromycin derivatives are shown in Fig. 1. The inhibitory action of erythromycin, and probably that of the other 14-member-ring macrolides, is effected at the early stages of protein synthesis when the drug blocks the growth of the nascent peptide chain (7, 140), presumably causing premature dissociation of the peptidyl-tRNA from the ribosome (85). The antimicrobial action of these drugs is compounded by their inhibition of the assembly of new large ribosomal subunits, which leads to gradual depletion of functional ribosomes in the cell (23). The mode of action of the 16-member-ring macrolides is less well characterized, although it is clear that they bind to the same region of the large subunit as the 14-member-ring macrolides and inhibit peptide bond formation in a more direct manner (reviewed in reference 140).

Shortly after the introduction of erythromycin in therapy in the 1950s, resistance to the drug was observed in bacterial pathogens (reviewed in reference 76). More disquieting was the observation that erythromycin-resistant strains were cross-resistant not only to all other macrolides but also to the chemically unrelated lincosamide and streptogramin B drugs. This phenomenon was first observed in *Staphylococcus aureus* and

came to be termed the macrolide-lincosamide-streptogramin B (MLS_B) antibiotic resistance phenotype. In these *S. aureus* strains, MLS_B resistance can be induced by exposure to low concentrations of erythromycin (151), which leads to expression of a methyltransferase enzyme (ErmC). ErmC specifically methylates 23S rRNA (74) at the N-6 position of adenosine 2058 (A2058) (*Escherichia coli* numbering) (121), which is a pivotal nucleotide for the binding of MLS_B antibiotics (see below). Subsequently, several dozen *erm* methyltransferase genes have been identified. Many of these are constitutively expressed, and their products all presumably methylate A2058. A new nomenclature system has recently been proposed for the different *erm* genes, which clarifies their phylogenetic relatedness (105). For a comprehensive account of the action of Erm methyltransferases, see the review by Weisblum (149).

Since the discovery of *erm* genes, another means of resistance involving alteration of rRNA structure has been identified. Under laboratory conditions, single base substitutions introduced into rRNA were shown to confer macrolide resistance. This form of resistance was first observed in the single rRNA (*rm*) operon of yeast mitochondria, which was mutated at position A2058 in the large-subunit rRNA (123). Shortly afterwards, similar phenotypes were obtained in *E. coli* by expression of mutant *rm* alleles from multiple-copy plasmids (see, e.g., references 120 and 143). About 6 years ago, reports of rRNA mutations conferring macrolide resistance in clinical pathogens began to appear in the literature. While it is conceptually gratifying to establish that the mutations appearing in pathogens are identical to those previously isolated in laboratory strains, the clinical implications of this are quite disturbing. The 23S rRNA mutations reported so far to cause macrolide resistance are shown in Table 2. Generally, pathogenic species that develop macrolide resistance through mutations at A2058 (or neighboring nucleotides) possess only one or two *rm* operons, such as in the case of *Helicobacter pylori* and *Mycobacterium* species. Resistance in bacteria with multiple *rm* operons, such as *Enterococcus*, *Streptococcus*, and *Staphylococcus* species, is generally conferred by Erm methylation of A2058 (Table 3) or by efflux (see e.g., references 70 and 110). However, there are cases of macrolide resistance by drug inactivation (reviewed in reference 150), and there are recent reports of macrolide resistance in *Streptococcus pneumoniae* strains conferred by mutations in ribosomal proteins L4 and L22 and in rRNA (129; P. Appelbaum, personal communication). Macrolide and ketolide resistance is additionally conferred in *E.*

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TABLE 1. Macrolide antibiotics and their derivatives discussed in this review

Antibiotic(s)	Phenotype designation	Mol wt	Lactone ring size	Description
ABT-773		765	14	Ketolide
Azithromycin	Azm	749	15	Azalide
Carbomycin	Cbm	842	16	Macrolide
Clarithromycin	Clr	748	14	Macrolide
Erythromycin A	Ery	734	14	Macrolide
Josamycin		828	16	Macrolide
Spiramycin I, II, III	Spi	843, 885, 899	16	Macrolide
Telithromycin	Tel	812	14	Ketolide
Tylosin	Tyl	916	16	Macrolide
Macrolides	M14		14	14-member ring only
	M16		16	16-member ring only
	Mac			All macrolides

coli by the expression of small, specific peptides (134), although the level of resistance is probably too low to be a problem in the treatment of clinical strains.

In the following sections of this review, we first look at the current state of knowledge of the bacterial ribosome target site for macrolide antibiotics. A detailed model of a drug target site

is a prerequisite for understanding the molecular mechanisms of drug binding and drug resistance and for rational design of new drugs. Our present state of knowledge, although far from being complete, supports the view that the macrolide target site is highly conserved within the ribosomes of all bacteria. We then direct attention to the pathogens, and in particular to *H. pylori*, that have been shown to attain resistance by rRNA mutation, and we consider the possibility of this form of resistance emerging in other pathogens. Finally, some suggestions are made regarding how future macrolide derivatives might be best equipped to combat bacteria with resistant rRNAs.

THE RIBOSOME TARGET FOR MACROLIDES

The drug binding site. Our knowledge of the tertiary structure of the ribosome has increased enormously within the last year. Models at resolutions approaching 5 Å have been obtained by X-ray crystallographic analysis of the small (30S) (29) and large (50S) subunits (11), as well as of the functional 70S ribosome complex of these two subunits (21). In addition to this, the structure of the ribosome at specific steps of protein synthesis has been deduced by cryoelectron microscopy (see, e.g., references 4 and 124), albeit at lower resolution. The

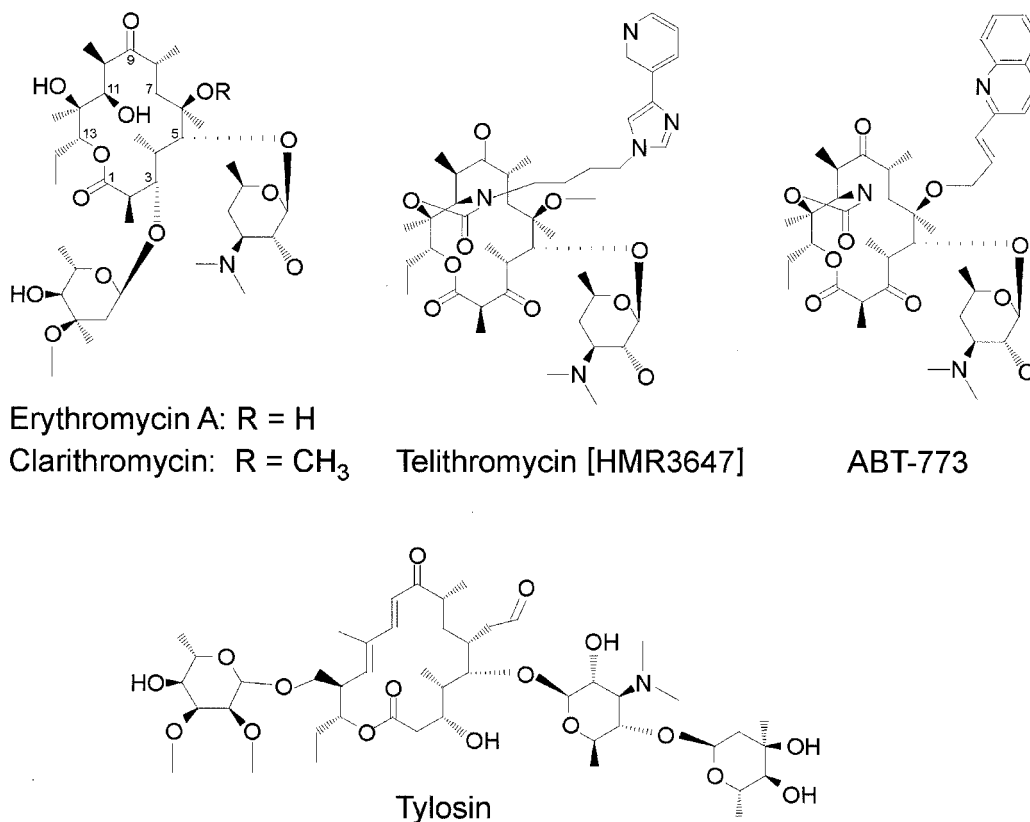


FIG. 1. Selected clinically important macrolide antibiotics and their derivatives. Two naturally occurring macrolides are shown: erythromycin A, which was the first therapeutic macrolide and possesses a 14-member ring, and tylosin, a 16-member-ring macrolide which has been used extensively in the farming industry both therapeutically and as a growth promoter. Clarithromycin is the 6-methoxy derivative of erythromycin and is presently the drug of choice in *H. pylori* eradication. The ketolides telithromycin and ABT-773 represent the most recent generation of drugs and are characterized by the 3-ketone group that substitutes the 3-cladinose sugar residue in erythromycin and clarithromycin. Both ketolides have a C-11-C-12 carbamate, which is extended by an alkyl-aryl group in the case of telithromycin. This extension enables telithromycin to make an alternative interaction with domain II of 23S rRNA (see text). Both ketolides are presently undergoing clinical trials, with ABT-773 in the early stage and telithromycin in the final stage of the process.

TABLE 2. 23S rRNA mutations reported to confer macrolide resistance

<i>E. coli</i> 23S rRNA position ^a	Organism ^b	Nucleotide(s)		Phenotype ^c	Reference(s)
		Wild type	Mutant		
754	<i>Escherichia coli</i>	U	A	Ery ^{lr} Tel ^{lr}	156
2057	<i>Chlamydomonas reinhardtii</i> chloroplast	G	A	Ery ^r Lin ^s	63
	<i>Escherichia coli</i>	G	A	Ery ^r M16 ^s Lin ^s S _B ^s	47
	Propionibacteria	G	A	Ery ^{lr} M16 ^s	111
2057+	<i>Escherichia coli</i>	G+G	A+A	Ery ^r Lin ^r	39
2032	<i>Helicobacter pylori</i>	A+G	G+A	Clr ^r Azm ^r Ery ^r	64
2058	<i>Brachyspira hyodysenteriae</i>	A	G, U	Ery ^r Tyl ^r Lin ^r	69
	<i>Chlamydomonas reinhardtii</i> chloroplast	A	G	Ery ^r Lin ^r	63
	<i>Escherichia coli</i>	A	G	Ery ^r Lin ^r	39, 143
		A	U	MLS _B ^r	120
	<i>Helicobacter pylori</i>	A	C	Clr ^r	125
				Mac ^r Lin ^r	94
				MLS _B ^r	148
				Cl ^a ^r	34
		A	G	Cl ^a ^r	142
				Mac ^r Lin ^p	94
				MLS _B ^p	148
				Cl ^a ^r	34
		A	U	MLS _B ^r	148
				Cl ^a ^r	34
	<i>Mycobacterium abscessus</i>	A	G	Clr ^r	146
	<i>Mycobacterium avium</i>	A	C, G, U	Clr ^r	90
	<i>Mycobacterium chelonae</i>	A	C, G	Clr ^r	146
	<i>Mycobacterium intracellulare</i>	A	C, G, U	Clr ^r	84
	<i>Mycobacterium kansasii</i>	A	U	Clr ^r	18
	<i>Mycobacterium smegmatis</i>	A	G	Clr ^r	113
	<i>Mycoplasma pneumoniae</i>	A	G	Ery ^{hr} Spi ^{mr} Tyl ^s Lin ^{hr}	79
	Propionibacteria	A	G	MLS _B ^r	111
	<i>Streptococcus pneumoniae</i>	A	G	MLS _B ^r	129
	<i>Sireptomycetes ambofaciens</i>	A	G	MLS _B ^r	98
	<i>Saccharomyces cerevisiae</i> mitochondrion	A	G	Ery ^r	123
	<i>Treponema pallidum</i>	A	G	Ery ^r	L. V. Stamm and H. L. Bergen, Letter, Antimicrob. Agents Chemother. 44:806-807, 2000
2059	<i>Helicobacter pylori</i>	A	C	Mac ^r Lin ^r S _B ^s	148
				Clr ^r	34
		A	G	Clr ^r	142
				Mac ^r Lin ^r	94
				Mac ^r Lin ^r S _B ^s	148
				Cl ^a ^r	34
	<i>Mycobacterium abscessus</i>	A	C,G	Clr ^r	146
	<i>Mycobacterium chelonae</i>	A	G	Clr ^r	146
	<i>Mycobacterium intracellulare</i>	A	C	Clr ^r Azm ^r	84
	<i>Mycobacterium avium</i>	A	C	Clr ^r Azm ^r	84
	<i>Mycobacterium smegmatis</i>	A	G	Clr ^r	113
	<i>Mycoplasma pneumoniae</i>	A	G	Ery ^{mr} Spi ^{hr} Tyl ^{lr} Lin ^{mr}	79
	<i>Streptococcus pneumoniae</i>	A	G	Mac ^r	129
	Propionibacteria	A	G	Mac ^{hr} Lin ^{lr}	111
2452	<i>Sulfolobus acidocaldarius</i>	C	U	Cbm ^r Lin ^r	1
2611	<i>Chlamydomonas moewusii</i> chloroplast	C	G	Ery ^r Spi ^{lr}	54
	<i>Chlamydomonas reinhardtii</i> chloroplast	C	G, U	Ery ^r Lin ^{mr}	63
	<i>Escherichia coli</i>	C	U	Ery ^r Spi ^s Tyl ^s Lin ^s	139
	<i>Streptococcus pneumoniae</i>	C	A, G	Mac ^r S _B ^s	129
	<i>Saccharomyces cerevisiae</i> mitochondrion	C	G	Ery ^r Spi ^r	122
	<i>Saccharomyces cerevisiae</i> mitochondrion	C	U	Ery ^s Spi ^r	122

^a Nucleotide positions are numbered according to the corresponding positions in *E. coli* 23S rRNA. Consistent use of the *E. coli* system facilitates comparison between the different organisms and avoids discrepancies in some of the other notation systems, such as that for *H. pylori* (132).

^b Pathogenic organisms are in boldface (the *E. coli* strains are nonvirulent laboratory strains).

^c The phenotypes conferred to the different types of macrolide antibiotics are given when these were specified in the original articles (the lack of a notation does not imply sensitivity but merely indicates that no specific phenotype was reported). Similarly, in some reports the levels of resistance are arbitrarily categorized, and when this is the case these are recounted here (r, resistant; s, sensitive; h, high; m, medium; l, low). Phenotype designations: Lin, lincosamides; S_B, streptogramin B group; MLS_B, macrolides, lincosamides, and streptogramin B. Other designations are given in Table 1.

TABLE 3. Macrolide resistance mechanisms found in some pathogens and their numbers of rRNA operons

Organism	Mechanism (reference[s] ^a)	No. of rRNA operons	Reference(s) for rRNA operons
<i>Brachyspira hyodysenteriae</i>	23S RNA mutation	1	157
<i>Mycoplasma pneumoniae</i>	23S RNA mutation	1	55
<i>Mycobacterium chelonae</i>	23S RNA mutation	1	146
<i>Mycobacterium abscessus</i>	23S RNA mutation	1	146
<i>Mycobacterium avium</i>	23S RNA mutation	1	84, 90
<i>Mycobacterium intracellulare</i>	23S RNA mutation	1	13, 84, 90
<i>Propionibacterium avidum</i>	23S RNA mutation	1	111
<i>Helicobacter pylori</i>	23S RNA mutation	2	17, 68, 133
<i>Propionibacterium granulosum</i>	23S RNA mutation	2	111
<i>Treponema pallidum</i>	23S RNA mutation	2	22
<i>Propionibacterium acnes</i>	23S RNA mutation	3	111
<i>Streptococcus pneumoniae</i>	23S RNA mutation	4	129; Tait-Kamradt et al., Abstr. ICMASKO V Meet.
<i>Corynebacterium diphtheriae</i>	<i>erm</i> (149)	NA ^b	
<i>Neisseria gonorrhoeae</i>	<i>erm</i> and efflux (61, 104)	4	14
<i>Enterococcus</i>	<i>erm</i> (67)	4–6	116, 117
<i>Lactobacillus reuteri</i>	<i>erm</i> (149)	NA	
<i>Bacillus anthracis</i>	<i>erm</i> (149)	NA	
<i>Bacteroides fragilis</i>	<i>erm</i> (149)	NA	
<i>Staphylococcus</i>	<i>erm</i> and efflux (43)	NA	
<i>Staphylococcus aureus</i>	<i>erm</i> (67)	6	145
<i>Streptococcus pneumoniae</i>	Ribosomal protein L4 (129; Tait-Kamradt et al., Abstr. ICMASKO V Meet.)	4	129; Tait-Kamradt et al., Abstr. ICMASKO V Meet.
<i>Streptococcus pneumoniae</i>	<i>erm</i> and efflux (73, 128)	4 or 6	10, 53, 129; Tait-Kamradt et al., Abstr. ICMASKO V Meet.
<i>Streptococcus agalactiae</i>	<i>erm</i> and efflux (28, 149)	6	37
<i>Streptococcus pyogenes</i>	<i>erm</i> and efflux (70, 128)	6	128
<i>Clostridium perfringens</i>	<i>erm</i> (149)	9	19

^a The references for the rRNA mutations are given in Table 2.

^b NA, not available (but data from another species of the same genus are given here or in Table 4).

macrolide binding site is presumably situated at the base of the deep cleft that provides access to the peptide exit channel of the large subunit (11, 21). This is at, or very close to, the location where the aminoacyl and peptidyl ends of tRNAs become aligned within the large subunit to catalyze the formation of peptide bonds. The X-ray crystallographers promise data at even better resolution in the near future, which will eventually reveal the molecular details of the antibiotic binding sites (see Addendum in Proof). For the moment, however, we must rely heavily on biochemical and molecular genetic data for our understanding of macrolide binding.

The site of peptide bond formation on the large ribosomal subunit (the peptidyl transferase center) is associated with the central loop in domain V of 23S rRNA (Fig. 2) (32, 93). The interactions of macrolides, and other MLS_B drugs, have been mapped here by chemical footprinting (39, 40, 62, 87, 99, 107, 138, 156). The 16-member-ring macrolides seem to make more extensive interactions in this rRNA region than the 14-member-ring macrolides (Fig. 2), which is undoubtedly related to the respective manner in which the drugs interfere with protein synthesis.

The interaction sites of erythromycin and ketolide derivatives have additionally been mapped to hairpin 35 in domain II of the rRNA (Fig. 2) (62, 156). A single molecule of erythromycin binds per large ribosomal subunit (reviewed in reference 140), and this holds true for the ketolide derivatives (62), indicating that the same drug molecule simultaneously contacts domains II and V of 23S rRNA. As these drugs are small relative to the ribosome, such interactions would be possible only

if the rRNA is folded so that hairpin 35 and the peptidyl transferase loop are adjacent. Evidence from other approaches, including phylogenetic comparisons of rRNA sequences (60) and RNA cross-linking (88), strongly supports the idea of contact between these two rRNA regions. In addition, mutations in ribosomal proteins L4 and L22 that confer erythromycin resistance in *E. coli* laboratory strains (27, 96, 152) presumably do so by perturbing the 23S rRNA structure. In the resistant L22 mutant, the configuration of the hairpin 35 loop is clearly affected (59). Most recent evidence indicates that the 16-member-ring macrolide tylosin also interacts with the peptidyl transferase and hairpin 35 loops. Two resistance determinants, *tlrA* and *tlrD* in the tylosin-producing actinomycete *Streptomyces fradiae*, encode Erm homologs that methylate A2058 (149), whereas a third resistance determinant, *tlrB*, encodes another type of methyltransferase that methylates G748 in the hairpin 35 loop (reference 80 and see Addendum in Proof).

The structure of the MLS_B drug binding pocket within the large ribosomal subunit is defined by the tertiary configuration of 23S rRNA. Hairpin 35 and the peptidyl transferase loop seem to be the main, although not the sole, components of this binding pocket. Nucleotide 2032 within the loop of 23S rRNA hairpin 72 is also implicated. Mutations at this nucleotide confer resistance to lincosamides (31, 39) but increase sensitivity to erythromycin (39) and perturb the peptidyl transferase loop structure (41). Also, several nucleotides within helices radiating from the peptidyl transferase loop interact with the aminoacyl end of tRNA (92), which places these regions near the

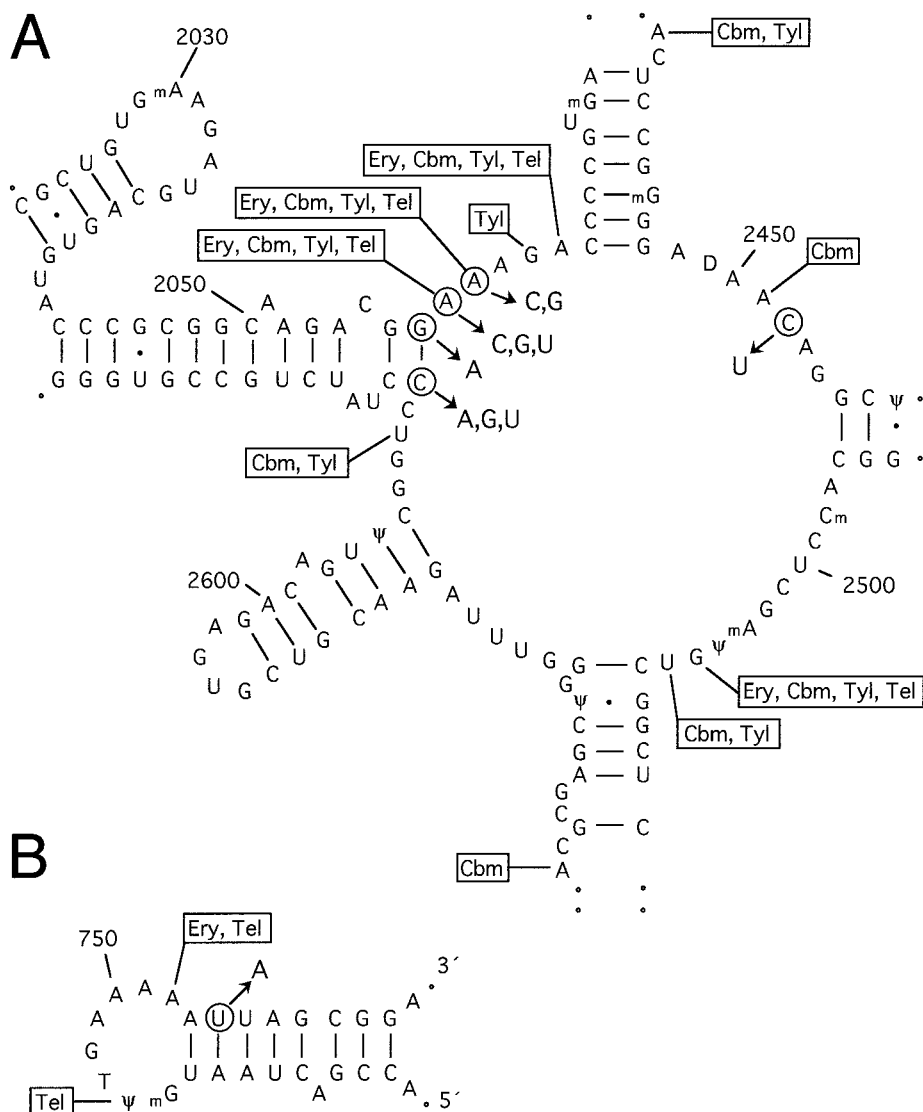


FIG. 2. Secondary-structure models of the peptidyl transferase center in domain V of 23S rRNA (A) and hairpin 35 in domain II (B) (60). Nucleotides at which macrolide drugs interact (as defined by chemical footprinting experiments) are indicated (62, 87, 107, 156). The circled nucleotides indicate the positions of mutations that confer macrolide drug resistance in bacterial pathogens and laboratory strains (details and references are given in Table 2). These data are depicted here on the secondary structure of the *E. coli* rRNA; the rRNA secondary structures of all other organisms are believed to be the same (60, 93). The single-stranded nucleotides involved in macrolide interaction and resistance are conserved in all of the wild-type bacterial rRNAs discussed in this review. However, the identities of the base-paired nucleotides (at positions 754, 2057, and 2611) can vary between different bacteria (see text). Drug abbreviations and classifications are given in Table 1. Erythromycin and clarithromycin interaction sites on the rRNA are identical.

site of peptide bond formation. The elucidation of an exact model of the tertiary folding and spatial orientation of these 23S rRNA components is beyond the scope of biochemical and molecular genetic approaches and is now in the hands of the X-ray crystallographers. However, the data that are presently available do enable us to go quite some way towards understanding the mechanisms of macrolide binding and resistance and make it possible to predict what new resistant strains might emerge and how these could best be combated therapeutically.

rRNA mutations confer resistance. The rRNA mutations reported for laboratory and clinical strains that have relevance for macrolide binding and resistance are listed in Table 2. Pertinent information on cross-resistance to other MLS_B drugs

is included. Mutations at A2058, or at A2059 for certain macrolides, confer the highest levels of resistance. All of the mutations in Table 2 presumably, to greater or lesser degrees, perturb the structure of the drug binding pocket and thereby reduce the ability of the drug to interact with and inhibit ribosomes (41, 94). Methylation of the rRNA at A2058 by Erm methyltransferases is thought to confer resistance by a similar mechanism (56). Lower-level drug resistance is provided by mutations at positions 2057, 2452, and 2611 (Fig. 2), which are close by in the secondary structure although slightly outside the focal point of macrolide interaction. Low-level macrolide resistance is conferred in an *E. coli* laboratory strain by a mutation at position 754 in hairpin 35 (156), which provides addi-

tional support for the proximity of this hairpin and the peptidyl transferase loop in the rRNA tertiary structure.

It can be seen from the data in Table 2 that while all of the mutations discovered in clinical strains have also been observed in laboratory strains, the converse is not the case. The distinction is that rRNA resistance mutations in a clinical pathogen often first become apparent after a drug therapy program has failed to eradicate the pathogen. Drug therapies are generally as aggressive as is expedient, and thus strains containing mutations that confer the highest resistance will be selected. In contrast, rRNA mutations created under laboratory conditions have been done so intentionally to increase our understanding of drug interaction mechanisms. Under the controlled conditions of the laboratory, a range of less effective resistance phenotypes can be nurtured. Such rRNA mutations are useful in helping us to delineate the macrolide interaction site on the ribosome, but, unless they segregate with another resistance mechanism, it is not expected that they will be observed in clinical isolates. Clinical pathogens in which rRNA mutations have been shown to confer macrolide resistance are considered below.

(i) Resistance in *H. pylori*. *H. pylori* colonizes the stomach in over 30% of the adult population. Although the majority of infections are asymptomatic, *H. pylori* is nevertheless the main etiological agent in most duodenal and many gastric ulcers; *H. pylori* has also been linked with the development of some types of gastric cancer (30). The preferred treatment for aggressive infections is a drug combination including the erythromycin derivative clarithromycin (Fig. 1), which has improved acid stability and uptake properties compared to erythromycin (57). *H. pylori* is susceptible to many antibiotics in vitro, although treatment in vivo is less trivial as the stomach is a difficult environment in which to carrying out successful antimicrobial therapy (58). Clinical treatment often entails multiple drug therapy, consisting of two antimicrobial agents in addition to a proton pump inhibitor, with bismuth as an extra option (reference 97 and references therein).

Recently, clarithromycin resistance was shown to arise during drug therapy and was traced to mutations at positions A2058 or A2059 in the 23S rRNA (142). A number of similar reports have subsequently been made (Table 2). No *erm* genes or macrolide efflux systems have yet been found in *H. pylori* despite searches for them (35, 64), and resistance mechanisms thus seem to be confined to rRNA mutations. The presence of a gastric *H. pylori* infection can be rapidly ascertained by any of several methods (see, e.g., references 5, 26 and 136), although more-involved procedures are required to establish whether the infecting strain has 23S rRNA mutations that confer macrolide resistance. *H. pylori* is slow to culture in vitro, and thus microbiological approaches to determine a resistance profile are often inappropriate in the case of an acute infection. A solution to this problem is offered by techniques based on PCR that facilitate rapid analysis of a relatively small number of *H. pylori* cells in a gastric juice or gastric biopsy sample. The *H. pylori* 23S rRNA gene region around nucleotide A2058 has been amplified and analyzed for altered restriction enzyme patterns (82, 94, 119) and by hybridization to oligonucleotide probes (83, 137). Such methods are potentially valuable tools for optimizing drug therapy and avoiding relapse, and it is

envisaged that they will also be used to identify resistance in other slow-growing bacteria with few RNA operons.

(ii) Resistance in other pathogens. Erythromycin-resistant isolates of *Mycoplasma pneumoniae* with A2058G and A2059G mutations display phenotypes similar to those of *H. pylori* mutants (79). The same mutations were found in resistant clinical isolates of propionibacteria, although in some isolates resistance was conferred by a G2057-to-A mutation (111). Pathogenic species of mycobacteria also develop resistance during clarithromycin treatment (references 90 and 113 and references therein). In *Mycobacterium intracellulare* and *Mycobacterium avium* all three possible base substitutions have been observed at position 2058 (84), whereas substitution at position 2059 is more restrictive (Table 2). *Brachyspira hyodysenteriae*, the etiological agent of swine dysentery, possesses a single *rm* operon. Isolates of *B. hyodysenteriae* that are resistant to tylosin (which is commonly used both as a therapeutic agent and as a growth promoter in swine production) exhibited G or U substitutions at position 2058 (69). The resistance phenotypes conferred by the various base substitutions are considered in greater detail below.

PHENOTYPIC CONSEQUENCES OF TARGET SITE MUTATIONS

Phylogenetic conservation of rRNA. Change in the structure of rRNA has been subject to severe limitations during the course of evolution. The overall shape of rRNA, determined by secondary and tertiary structural folding, is remarkably similar in all organisms (60, 93). The base sequences within the paired stems of the rRNA can vary a great deal between species, because the size and shape of stems can be maintained by a variety of different Watson-Crick and other base-pairing interactions. However, within certain single-stranded loop regions of the rRNA, such as those depicted in Fig. 2, sequences tend to be highly conserved. Nucleotide 2058 is conserved as an adenosine in all (wild-type) bacteria, whereas this position is a guanosine in most archaeal ribosomes and in all eukaryal cytoplasmic ribosomes (which are refractory to macrolides). Nucleotide 2059 is conserved as an adenosine in all organisms. The identities of the bases at positions 2057 and 2611, which form the base pair closing the neighboring stem structure (Fig. 2), are not conserved, although a Watson-Crick pair is generally found here in all organisms. A priori it might be expected that the higher the level of phylogenetic conservation of a base the more drastic would be the phenotypic consequence of changing it. Surprisingly, this is not always the case.

Genetic stability of rRNA mutations. Depending on a nucleotide's position and functional importance in the rRNA, its substitution either can be phenotypically silent, can be deleterious, or can confer an advantage such as drug resistance. It might then be asked why a substitution such as A2058G, which obviously confers a clear advantage to the cell, has not been consolidated as the "wild-type" sequence in all bacteria. This probably reflects the fact that the phenotypic effect of a mutation may vary according to the environmental conditions. Competitive growth experiments with low levels of clarithromycin show that *H. pylori* with an A2058G or an A2059G mutation has a clear advantage compared to the wild-type strain or to strains with any of the other bases at these positions

(147). However, in stationary-phase cultures of *E. coli* that are maintained in the absence of drug, A2058G mutant ribosomes are distinctly less stable than wild-type ribosomes (2). The advantage conferred by A2058G in the presence of macrolides must be weighed against any disadvantage of having this substitution in the absence of drug and whether the disadvantage can be ameliorated by other factors. The biological cost of maintaining such mutations will determine how stable they are in pathogen rRNA, which in turn is important for determining subsequent drug therapy.

H. pylori, when grown in vitro in the absence of antibiotic selective pressure, stably maintained the A2058G and A2059G mutations through 21 (34) and 50 (64) passages, whereas A2058U and A2059C mutations were less stable (34). It should be noted, however, that another study showed a considerable loss of resistance over only five generations (155), although here the genetic basis for the resistance was not known. In a clinical setting, *H. pylori* with resistant rRNA mutations persisted in patients 3 months after completion of an unsuccessfully therapy with clarithromycin (64). In other drug resistance systems it has been shown that the biological cost of maintaining a resistance mutation can be alleviated by a second mutation at another site (6). Possibly a second-site mutation in the rRNA or in another ribosomal component allows the mutations at positions 2058 and 2059 to be maintained at no extra cost to the bacterium. Whether such second-site mutations exist and whether they compensate for the initial mutation under all growth conditions are not presently known.

Clinically important rRNA mutations. Given the conservation in structure and function of ribosomes, it is tempting to predict that identical mutations will give the same phenotype in different bacterial species. This seems to be generally the case, although a few disparities exist. The sites of rRNA mutations conferring macrolide resistance in clinical pathogens are considered in detail below.

(i) Position 2057. The occurrence of mutations at position 2057 in clinical isolates is presently limited to a group of erythromycin-resistant propionibacteria (111) and to a clarithromycin-resistant, double mutant strain of *H. pylori* (Table 2). The latter strain contained a mutation at position 2032 in addition to the 2057 substitution (64), although the 2057 substitution most likely determines the macrolide-resistant phenotype (39). The 2057 substitutions disrupt the 2057-2611 base pair at the end of the stem adjacent to the drug interaction site (Fig. 2). This confers low-level resistance to 14-member-ring macrolides and no resistance to 16-member-ring macrolides (47, 111). Substitution of position 2611 results in a similar disruption in the rRNA structure and confers a similar phenotype (139, 144). Resistant 2611 mutant isolates of *S. pneumoniae* have been noted after extensive in vitro selection with the macrolide derivative azithromycin (Table 1) (129).

(ii) Position A2058. Many independent lines of evidence indicate that adenosine 2058 is the key nucleotide involved in macrolide interaction on the ribosome. A2058 to G was the first rRNA mutation shown to confer erythromycin resistance and is presently the most frequent clinically isolated substitution (38, 94, 141). Relative to other rRNA mutations, A2058G gives the highest level of resistance to 14-member-ring macrolides (34, 126, 148). The A2058G mutation does not seem to influence growth rate adversely in the absence of drug, al-

though as mentioned above, A2058G mutant rRNA is preferentially degraded in *E. coli* (2).

C and U mutations at nucleotide 2058 also confer resistance (Table 2), but the phenotype apparently varies according to the organism. A2058 to C seems to be lethal in *E. coli* (S. Gregory, personal communication), whereas in *H. pylori*, A2058C confers a resistance level similar to that conferred by the G substitution (34, 94, 148). Another species discrepancy is seen with the A2058-to-U mutation, which in *E. coli* confers resistance to MLS_B antibiotics (120), and this mutant *rm* allele can be stably maintained on a plasmid without affecting growth rates in the absence of drug (our unpublished observations); however, in *H. pylori*, A2058 to U gives lower resistance, strongly decreases growth, and is easily lost in the absence of drug selection (34, 148). No A2058-to-U mutation has yet been identified in clinical *H. pylori* isolates. *B. hyodysenteriae* isolates selected for tylosin resistance were shown to possess either G or U at position 2058 (69). All three possible base substitutions at position 2058 have been found in two different species of *Mycobacterium*, where they all seem to be functional and to confer resistance (84, 90). Considering the high phylogenetic conservation of this rRNA region, it appears to be counterintuitive that a particular substitution can confer such varied phenotypes in different bacterial groups. This variation may yet be shown to be caused either by differences in the sequences of rRNA regions that interact with A2058 or by peculiarities in other ribosomal components in the individual species.

Mutations at position 2058 are the only substitutions to confer "true" MLS_B resistance, defined as high resistance to all the drugs in this group. This should be viewed with the caveat that the term MLS_B resistance has been assigned in a number of different ways, often without due reference to a comprehensive set of 14- and 16-member-ring macrolide, lincosamide, and streptogramin B antibiotics. Mutations that have been conclusively demonstrated to exhibit the MLS_B phenotype are A2058U in *E. coli* (120), A2058C/G/U in *H. pylori* (148), A2058G in *Propionibacterium* spp. (111), and A2058G in *Streptomyces ambofaciens* (98). However, the present indications make it judicious to assume that the 2058G mutation would confer true MLS_B resistance in any bacterium with a low *rm* copy number. In addition, an *S. pneumoniae* strain with A2058G in two of its four *rm* alleles exhibits the MLS_B-resistant phenotype (P. Appelbaum, personal communication).

(iii) Position A2059. As shown in Table 2, A2059-to-C or -G mutations have been found in vivo in mycobacteria, propionibacteria, *H. pylori*, and, most recently, *S. pneumoniae*. Mutations at position 2059 have also arisen under in vitro selection in *M. pneumoniae*, and *S. pneumoniae*. *H. pylori* 2059 mutants have lower levels of clarithromycin resistance than 2058 mutants in growth experiments in vitro (34, 148). A2059 to C in *H. pylori* is not very stable, and the U substitution cannot be maintained (34). The *H. pylori* A2059-to-G and -C mutations give moderate resistance to clarithromycin and clindamycin (a lincosamide) but no resistance to quinupristin (a streptogramin B) (148). During treatment for *H. pylori* infection, there seems to be variation in the relative frequency with which the 2058 and 2059 mutations occur (38, 82, 94, 141). This is probably dependent on a number of factors, including the therapeutic regimes employed (which are not always stipulated). A clinical macrolide-resistant isolate of *S. pneumoniae* was

recently reported to contain A2059G substitutions in three of its four *rm* operons (A. Tait-Kamradt, T. Davies, L. Brennan, F. Depardieu, P. Courvalin, J. Duignan, J. Petitpas, A. Walker, L. Wondrack, M. Jacobs, P. Appelbaum, and J. Sutcliffe, Abstr. 5th Int. Conf. Macrolides, Azalides, Streptogramins, Ketolides, Oxazolidinones, abstr. 2.22, 2000). It is presently unclear whether this is an exceptional case or whether this form of resistance is prevalent in pneumococci and has previously escaped detection.

In propionibacteria, A2059G confers resistance to both 14- and 16-member-ring macrolides but gives significantly higher resistance to the 16-member-ring macrolide josamycin than that seen for A2058G (111). This is consistent with the same mutation in *M. pneumoniae*, which confers higher resistance than the A2058G mutation to 16-member-ring macrolides such as tylosin and spiramycin (79). This could reflect subtly different modes of interaction of 14- and 16-member-ring macrolides with 23S rRNA. Both types of macrolides protect positions 2058 and 2059 from modification by dimethyl sulfate (Fig. 2), but the focus of the interaction of the bulkier 16-member-ring macrolides is possibly shifted towards position 2059.

Phenotypic variability. Recently, reports of different *H. pylori* phenotypes arising from the same rRNA mutation have been made: strains with A-to-G mutations at position 2059 exhibited high resistance to erythromycin but variable levels of resistance to clarithromycin (52, 83). An explanation for these observations is not immediately clear, although to avoid conflict with a basic premise of microbial genetics (that isogenic strains will display the same phenotype under the same growth conditions), one must assume that these strains were not isogenic. Unexpectedly high diversity in the genetic footprints of *H. pylori* strains has been established (30) and is possibly one of the causal factors in the aberrant phenotypes. In addition, *H. pylori* has two *rm* operons (17, 68, 133), and although both operons often contain the same mutation (34, 148), heterozygous strains, which exhibit intermediate or high levels of drug resistance have been found (64, 126, 142). Paradoxes about resistance phenotypes are best resolved using strains engineered by in vitro site-directed mutagenesis (34, 148), where the specific effect of a single substitution can be ascertained unambiguously.

FUTURE PERSPECTIVES

Predicted resistance in other pathogens. After exposure to macrolide antibiotics, the types of rRNA mutations described above can rapidly dominate bacterial populations in which the individual cells possess only one or two *rm* operons. Table 3 summarizes the relationship between the number of *rm* operons in a pathogen and the mechanism by which resistance occurs. A general pattern emerges indicating that the fewer *rm* operons a bacterium possesses, the greater the likelihood that macrolide resistance, if and when it arises, will be conferred by rRNA mutations. These spontaneous mutations are constantly arising at a low frequency in any bacterial population, and the drugs merely exert a selective pressure towards their proliferation. In this context, the potential influence of adaptive mutation mechanisms, which can come into play in residual populations of nondividing or slowly dividing cells (103), should also be noted.

TABLE 4. Copy numbers of rRNA operons in pathogens for which macrolide resistance mechanisms have not been reported^a

Organism	No. of rRNA operons	Reference(s)
<i>Chlamydia pneumoniae</i>	1	50
<i>Coxiella burnetii</i>	1	3
<i>Mycobacterium leprae</i>	1	118
<i>Mycobacterium tuberculosis</i>	1	13
<i>Mycoplasma genitalium</i>	1	48
<i>Mycoplasma hyopneumoniae</i>	1	130
<i>Rickettsia prowazekii</i>	1	8
<i>Borrelia burgdorferi</i>	2 ^b	33, 115
<i>Chlamydia trachomatis</i>	2	46
<i>Leptospira interrogans</i>	2	49
<i>Mycobacterium callatum</i>	2	102
<i>Mycoplasma gallisepticum</i>	2 ^c	25
<i>Bordetella pertussis</i>	3	89
<i>Campylobacter jejuni</i> - <i>C. coli</i>	3	71, 131
<i>Moraxella catarrhalis</i>	4	91
<i>Neisseria meningitidis</i>	4	153
<i>Pseudomonas aeruginosa</i>	4	108
<i>Bacillus cereus</i> group	6–10	101
<i>Haemophilus influenzae</i>	6	77
<i>Listeria monocytogenes</i>	6	86
<i>Salmonella</i> spp.	7	78
<i>Vibrio cholerae</i>	9	75

^a Most of these bacteria are sensitive to macrolide antibiotics, at least in vitro (see, e.g., references 12, 15, 24, 65, 72, 81, 100, 114, 127, and 135). For *M. tuberculosis*, some controversy exist about the effect of macrolides in vivo (81, 135). It is expected that macrolide resistance conferred by rRNA mutations is more likely to arise in the bacteria in the upper portion of the table (see text).

^b One 16S RNA gene and two 23S RNA genes.

^c One operon of rRNA genes plus a separate set of 16S and 23S RNA genes.

In bacteria with multiple *rm* operons, the effect of a beneficial mutation in one operon is likely to be diluted out so that it offers no significant phenotypic advantage. However, amplification of a mutant allele, so that it occupies the majority of the bacterium's *rm* operons, could confer a resistant phenotype, as has been observed in *S. pneumoniae* (Table 2). In general, however, in bacteria with multiple *rm* copies resistance is mediated by an *erm*-encoded methyltransferase, which can potentially modify all ribosomes, or by an efflux system such as that encoded by *msrA* in *Staphylococcus* (110). While implementation of both of these latter systems requires the acquisition of exogenous genetic material, moderate levels of macrolide resistance have been observed in *Neisseria gonorrhoeae* upon overexpression of an endogenous membrane transport system (61). Probably many pathogens have inherent efflux mechanisms that provide some tolerance to macrolides and other antimicrobial agents, e.g., the *mmr* gene in *Mycobacterium tuberculosis* (36) and the *acrAB* homolog in *Haemophilus influenzae* (112).

The occurrence of macrolide resistance in many bacterial pathogens remains largely undocumented. Examples of pathogens where this is the case are listed together with their *rm* copy numbers in Table 4. It is predicted that there is a high potential for macrolide resistance to occur by mutations in the 23S rRNAs of the bacteria in the upper portion of the table. The probability of resistance developing would of course depend on the types and quantities of drug to which these organisms are exposed. Development of macrolide resistance in any of the remaining bacteria in the lower portion of Table 4 would be most likely linked to rRNA methylation or drug

efflux. The potential risks of resistance developing by modification of endogenous efflux systems such as *mtrRCDE* of *N. gonorrhoeae* (61) or by drug inactivation remain to be assessed. So far there have only been a few reports of resistance conferred by macrolide inactivation, which include strains of enterobacteria (9, 95), an isolate of *S. aureus* (154), and a drug-producing actinomycete (66).

Drug development to overcome resistance. Naturally occurring macrolides have been derivatized in most conceivable ways to improve their acid stability, uptake, resilience to modification and efflux, and improve ribosome binding, not least to MLS_B-resistant ribosomes. The latest generation of macrolides, the ketolides, include telithromycin (HMR 3647) and ABT-773 (20), which possess a 3-keto group instead of cladinose and a carbamate at C-11–C-12 (Fig. 1). Telithromycin is presently nearing the end of clinical trials and is showing considerable promise against bacterial pathogens (see, e.g., references 44, 45, 106, and 109). Telithromycin binds to ribosomes with up to 10-fold-higher affinity than erythromycin (62), and this appears to be a direct consequence of improved contact between an alkyl-aryl extension from the C-11–C-12 carbamate of the drug and the loop of hairpin 35 in domain II of the rRNA (62, 156).

Telithromycin binding is appreciably reduced by the A2058G mutation in *E. coli* ribosomes, although its binding remains over 20-fold higher than that of erythromycin and clarithromycin (42). It appears that the improved domain II interaction enables the ketolide to maintain a precarious, but possibly crucial, foothold on resistant ribosomes. The drug-domain II interaction is only just beginning to be understood and is undoubtedly capable of further improvement. As discussed above, structural models of the ribosome will soon become available at a resolution that is high enough to disclose additional sites for potential drug contact. This will not only enable further macrolide and ketolide development but should reveal new ribosome targets against which novel drugs can be designed.

Future therapies against infections caused by pathogens with a low *rmn* copy number have the potential to be improved in several ways. A rapid pretreatment analysis of the infecting strain to ascertain the *rmn* genotype would facilitate an optimal choice of drugs. Prescription of a tailor-made drug cocktail, leading to quick and complete eradication of an infection, would minimize the occurrence of resistance mutations in rRNA. Previous experience has shown, however, that the best that can be hoped for is a delay in the development of bacterial resistance, which can be expected to continue to evolve and spread in step with drug development and use. It is therefore important to base therapeutic strategies upon an accurate and detailed understanding of antibiotic action and resistance mechanisms and hopefully in this way to stay one step ahead of intractable bacterial infections.

ADDENDUM IN PROOF

The most recent high-resolution crystallographic structure of the 50S subunit (N. Ban, P. Nissen, J. Hansen, P. B. Moore, and T. A. Steitz, *Science* **289**:905–920, 2000) clearly reveals the positions of all the 23S rRNA nucleotides and shows how position G748 in domain II lies close to (within 10 Å of) A2058 in domain V. The site of peptide bond formation is

close by and is catalyzed by domain V of the rRNA (P. Nissen, J. Hansen, N. Ban, P. B. Moore, and T. A. Steitz, *Science* **289**:920–930, 2000). Model of comparable resolution are also available for the 30S subunit (F. Schluenzen, A. Tocilj, R. Zarivach, J. Harms, M. Gluehmann, D. Janell, A. Bashan, H. Bartels, I. Agmon, F. Franceschi, and A. Yonath, *Cell* **102**:615–623, 2000; B. T. Wimberly, D. E. Brodersen, W. M. Clemmons, R. J. Morgan-Warren, A. P. Carter, C. Vornrhein, T. Hartsch, and V. Ramakrishnan, *Nature* **407**:327–339, 2000). The rRNA mutations described, as well as a comprehensive list of other rRNA mutations, can be found in the rRNA database (<http://ribosome.fandm.edu>) that is maintained by Kathleen Trimman, Franklin and Marshall College, Lancaster, Pa.

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