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MINIREVIEW

Erythromycin Resistance by Ribosome Modification

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INTRODUCTION

Erythromycin inhibits protein synthesis by its effect on ribosome function (14, 118, 119). The metabolic modifications that enable cells to cope with the inhibitory action of erythromycin fall under major headings that include (i) target site alteration, (ii) antibiotic modification, and (iii) altered antibiotic transport. This minireview concentrates on target site alteration, which for erythromycin is the 50S subunit of the ribosome.

The first clinical isolates of macrolide-resistant staphylococci were described in reports from France, England, Japan, and the United States shortly after the introduction of erythromycin into clinical practice in 1953. On the basis of current understanding of the biochemistry of erythromycin's action, resistance in most of the strains that were described in early reports can be ascribed to a posttranscriptional modification of the 23S rRNA by an adenine-specific *N*-methyltransferase (methylase) specified by a class of genes bearing the name *erm* (erythromycin ribosome methylation). The last decade has seen the isolation and characterization of approximately 30 *erm* genes from diverse sources, ranging from clinical pathogens to actinomycetes that produce antibiotics; for many of these genes, both the respective nucleotide sequences that encode the methylases as well as the flanking sequences that control their expression have been determined. A tabulation of the *erm* genes that have been described is presented in Table 1.

Any discussion of mechanisms of resistance to macrolide antibiotics must include the chemically distinct, but functionally overlapping, lincosamide and streptogramin B families as well. This type of resistance has therefore also been referred to as MLS resistance. Members of the MLS antibiotic superfamily include, among the macrolides, carbomycin, clarithromycin, erythromycin, josamycin, midecamycin, mycinamicin, niddamycin, rosaramicin, roxithromycin, spiramycin, and tylosin; among the lincosamides, celesticetin, clindamycin, and lincomycin; and among the streptogramins, staphylomycin S, streptogramin B, and vernamycin B. The streptogramin family is subdivided into A and B groups or alternatively into M and S groups, respectively. Methylation of A2058 confers resistance to the B- and S-group streptogramins but not to the A- and M-group streptogramins. The reason for this grouping was originally based on empirical observations from clinical bacteriology that resistance to one class often involved resistance to the other two classes (11, 16, 35, 39, 41, 135); however, (i) the three classes of antibiotics interact competitively when binding to the 50S subunit, and only one antibiotic molecule can bind per 50S subunit (129, 130); this suggests that the binding sites for these antibiotics overlap or at least functionally interact. (ii) Nucleotide alterations in 23S rRNA, both mutational and posttranscriptional, that confer coresistance to MLS antibiotics appear to cluster in the peptidyltransferase region in 23S rRNA domain V, providing a physical basis and a common location for their sites of action (50, 101–104, 109, 110, 128) (Fig. 1 and Table 2), and (iii) footprinting experiments show that the nucleotides in 23S rRNA domain V are protected by bound MLS antibiotics against modification by agents such as dimethyl sulfate (DMS) and kethoxal that can derivatize purine and pyrimidine bases in single-stranded DNA or RNA (26, 76) (Table 3).

The *erm* family of genes is not alone in conferring clinical resistance to macrolide antibiotics. A notable early exception to the established MLS resistance pattern was the MS pattern reported by Jánosy and coworkers (58, 59), who described clinical isolates that were coresistant to erythromycin and streptogramin B but that remained susceptible to lincosamide antibiotics. The molecular basis for resistance in these strains was subsequently shown by Ross et al. (94) to involve the active efflux of erythromycin and streptogramin B but not clindamycin. Additional mechanisms of macrolide resistance, all associated with the acquisition of new genetic information, including structural modification of erythromycin by phosphorylation (82), glycosylation (60), and lactone ring cleavage by erythromycin esterase (2, 83), have been added to the list.

Mechanisms involving mutational alteration of genes that normally reside in the host and that encode either ribosomal protein or rRNA have also been described and will be discussed below in detail. Reviews of erythromycin resistance that relate to material covered in the present work have been presented previously (4, 18, 20, 21, 28, 29, 133). Recent developments in the synthetic chemistry of semisynthetic macrolides, including the biological and clinical aspects of their actions, have been reviewed by Kirst (65, 66). A forthcoming review covers the inducible nature of MLS resistance and its implications for the mechanism of action of erythromycin (134).

RESISTANCE MECHANISMS

Altered target site: ribosomal protein. The first reports of ribosomal structural changes in erythromycin-resistant mutants of *Escherichia coli* described ribosomal protein alterations, notably of proteins L4 and L22 (1a, 85, 137). These observations, considered together with others involving altered protein S12 in streptomycin-resistant mutants, e.g., *strA* (125), and altered protein S5 in spectinomycin-resistant mutants, e.g., *spc-13* (90), helped to support the prevailing perception of the ribosome as a complex multisubunit protein assembled on an inert RNA scaffold. The perceived relative roles of rRNA and ribosomal proteins in ribosome function have undergone a reversal, in part on the basis of reports relating the state of

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TABLE 1. *ermA* to *ermZ* and beyond[a]

erm allele	Organism	Reference
ermA	*Staphylococcus aureus*	79
ermAM	*Streptococcus sanguis*	53
ermB	*Staphylococcus aureus*	29, 98
ermBC	*Escherichia coli*	13
ermB-like	*Streptococcus faecalis*	98
ermC	*Staphylococcus aureus*	42, 54
ermCD	*Corynebacterium diphtheriae*	52, 97
ermD	*Bacillus licheniformis*	43
ermE	*Streptomyces erythraeus*	6, 123, 126
ermF	*Bacteroides fragilis*	91
ermFS	*Bacteroides fragilis*	107
ermFU	*Bacteroides fragilis*	47
ermG	*Bacillus sphaericus*	78
ermGT	*Lactobacillus reuteri*	117
ermIM	*Bacillus subtilis*	77
ermJ	*Bacillus anthracis*	64
ermK	*Bacillus licheniformis*	67
ermM	*Staphylococcus epidermidis*	72
ermP	*Clostridium perfringens*	5
ermQ	*Clostridium perfringens*	4a
ermR	*Arthrobacter luteus*	93 (also *ermA′*, *AR*)
ermSF	*Streptomyces fradiae*	61
ermZ	*Clostridium difficile*	46
carAB	*Streptomyces thermotolerans*	31, 96
clr	*Streptomyces caelestis*	15
lmrAB	*Streptomyces lincolnensis*	140
lrm	*Streptomyces lividans*	60
mdmA	*Streptomyces mycarofaciens*	48
myrAB	*Micromonospora griseorubida*	56
srmABCD	*Streptomyces ambofaciens*	87, 92, 96
tlrABCD	*Streptomyces fradiae*	7, 138, 139

[a] Most *erm* methylases have been reported as alleles of *erm*. Others, from *Streptomyces* spp., have been given names that emphasize resistance to an antibiotic produced by the *Streptomyces* strain in which they were first found or to an antibiotic other than erythromycin toward which resistance was deemed more pertinent. Some of the *Streptomyces* strains have multiple macrolide resistance determinants, not all of which are methylases, that have been cited for completeness. (i) The DNA sequences reported for the individual listings *clr*, *lrm*, and *mdmA*, specifying resistance to clindamycin, lincomycin, and midecamycin, respectively, show that they encode methylases. (ii) *carA* specifies a transport ATPase (96), whereas *carB* specifies an *erm* methylase (31); (iii) *lmrA* specifies a transport ATPase, and *lmrB* specifies an *erm* methylase (140); (iv) the protein sequence predicted by *myrA* did not resemble that of any previously described protein, whereas the protein predicted by *myrB* showed strong similarity to methylases encoded by *ermE* and *carB* (56). (v) *srmA*, *srmB*, *srmC*, and *srmD* were characterized as cloned DNA fragments that conferred resistance to spiramycin (91). *srmB* specifies a transport ATPase (96). The activities associated with *srmA*, *srmC*, and *srmD* have not been reported. (vi) *tlrA*, *tlrB*, *tlrC*, and *tlrD* were first characterized as cloned DNA fragments that conferred tylosin resistance (7). *tlrA* (synonym *ermSF*) (61) and *tlrD* (138, 139) specify *erm* methylases. *tlrC* specifies a transport ATPase (96), and the activity associated with *tlrB* has not yet been reported.

rRNA methylation to antibiotic resistance. Thus, Helser et al. (51) showed that methylation of adenine in 16S rRNA was required for proficiency to bind to kasugamycin, while Lai and colleagues (69, 70) showed that specific methylation of 23S rRNA in *Staphylococcus aureus* conferred resistance to the MLS family of antibiotics.

The significance of posttranscriptional 23S rRNA alterations is underscored by their occurrence in both antibiotic-producing actinomycetes (34, 40, 122), in which they are presumed to enable the producing strain to avoid suicide, and clinical pathogens, in which they protect the pathogen from the antibiotics produced by the suicide-avoiding actinomycetes.

The most consistent body of biochemical and genetic data relating to the role of ribosomal protein in the action of erythromycin involves protein L22, which was shown to be altered in

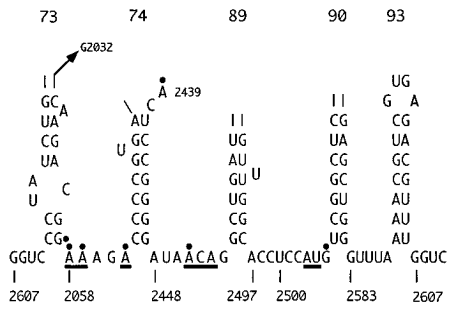

FIG. 1. Peptidyltransferase circle. Linear representation showing the sites (underlined) at which mutations to antibiotic resistance occur, as indicated in Tables 2 and 3. Filled circles indicate nucleotides at which erythromycin leaves a footprint. The numbers at the top enumerate the double-stranded regions in 23S rRNA, as proposed by Brimacombe et al. (12); numbers along the bottom indicate *E. coli* 23S rRNA nucleotide coordinates (44). For precise coordinates of other 23S rRNAs that have been sequenced, see references 44 and 45. For summaries of the mutational changes and footprinting results for these sites, see Tables 2 and 3, respectively.

E. coli mutants selected for resistance (85, 137). Moreover, L22 in ribosomes from both resistant mutants and wild-type cells have been reported to form covalent complexes with erythromycin-based photoaffinity probes (1). In interpreting the results of affinity labeling studies it should be noted that covalent complex formation reflects only the proximity between probe and protein and implies nothing regarding the mechanism.

The more recent report (81) that 23S rRNA preparations, obtained by phenol extraction and proteolytic treatment of ribosomes, can support the synthesis of peptide bonds in vitro and that this process is inhibited by carbomycin raises the question of the extent to which ribosomal proteins play any direct role in either the synthesis of protein or ribosomal resistance to macrolide antibiotics. One might therefore consider whether the amino acid alterations found in L22 mutants play only an indirect role in erythromycin action.

Purified protein L15, in solution, has been reported to bind to erythromycin (121) and, while associated with and part of a functional ribosome, to bind to erythromycin-based photoaffinity probes (1). An affinity constant of 2×10^{-5} liters/mol for the association of L15 with erythromycin was determined by Teraoka and Nierhaus (121). For interaction between 70S ribosomes and erythromycin, an affinity constant of 10^{-7} liters/mol was determined by Fernández-Muñoz and Vazquez (33), and an affinity constant of 10^{-8} liters/m was determined by Pestka et al. (89). Whatever the nature of the binding between erythromycin and protein L15, it apparently does not debilitate ribosomes in a way that cells become resistant to erythromycin by mutations that modify the structure of this protein. In the absence of corroborating genetic findings, the precise role of L15 in the action of erythromycin is even less certain than that of L22.

Additional reports (120) have noted that the 16-membered ring macrolides carbomycin, niddamycin, and tylosin bind selectively to protein L27, whereas erythromycin, a 14-membered ring macrolide, does not. These observations may point to consistency between macrolide ring size and ability to bind to L27 and could reflect the proximity of the larger macrolides to L27. Likewise, binding of rosaramicin to proteins L18 and L19 has been reported (100). On the basis of the example of L22 cited above, even the finding of corroborating mutational data for L15 and L27 would not conclusively link the ribosomal protein directly or functionally to the action of erythromycin. At this time, the direct functional role of ribosomal proteins as major determinants in the actions of macrolide antibiotics seems less certain than it did before.

TABLE 2. Peptidyltransferase circle (and vicinity) mutants and their effects on antibiotic susceptibility[a]

Mutation	Species[b]	Phenotype[c]	Reference
C2611U	*Eco*	Ery^r Lin^r Sgb^r	128
C2611U	*Cre*	Ery^r Cln^r	50
C2611G	*Sce*	Ery^r Spi^r	110
C2611G	*Cmo*	Ery^r	36
C2611G	*Cre*	Ery^r Cln^r	50
G2032A	*Npl*	Lin^r	19
G2032A	*Eco*	Ery^{hs} Cln^r Cam^r	24
G2032U	*Eco*	Ery^{hs} Cln^s Cam^r	24
G2032C	*Eco*	Ery^{hs} Cln^s Cam^s	24
G2057A	*Cre*	Ery^r Lin^s Cln^s	50
G2057A	*Eco*	Ery^r Cam^r	32
A2058G	*Cre*	Ery^r Lin^r Cln^r	50
A2058G	*Eco*	Ery^r	131
A2058G	*Npl*	Lin^r	19
A2058G	*Sce*	Ery^r	109
A2058G	*Min*	Cla^r	75
A2058C	*Min*	Cla^r	75
A2058U	*Eco*	Ery^r	101
A2058U	*Min*	Cla^r	75
A2059G	*Npl*	Lin^r	19
A2062C	*Hha*	Cam^r	74
G2447C	*Hsa*	Ani^r	55
G2447A	*Sce*	Cam^r	30
A2451U	*Mmu*	Cam^r	63
C2452A	*Hsa*	Cam^r	55
C2452A	*Hha*	Cam^r	8
C2452U	*Mmu*	Cam^r	106
C2452U	*Sac*	Cam^r Car^r Cel^r	1
C2452U	*Tth*	Ani^r	115
A2453C	*Hha*	Ani^r	55
A2503C	*Eco*	Cam^r	132
A2503C	*Sce*	Cam^r	30
U2504C	*Hsa*	Cam^r	8
U2504A	*Hsa*	Cam^r	55
U2504C	*Mmu*	Cam^r	9

[a] Mutations in the vicinity of the peptidyltransferase circle that have been associated with antibiotic resistance are tabulated according to the base change and the organism in which it occurs. Nucleotide coordinates are reported as the respective *E. coli* equivalents.

[b] *Cmo*, *Chlamydomonas moewusei* (chloroplast); *Cre*, *Chlamydomonas reinhardtii* (chloroplast); *Eco*, *Escherichia coli*; *Hha*, *Halobacterium halobium*; *Hsa*, *Homo sapiens* (mitochondrial); *Min*, *Mycobacterium intracellulare*; *Mmu*, *Mus musculus* (mitochondrial); *Npl*, *Nicotiana plumbaginafolia* (chloroplast); *Sce*, *Saccharomyces cerevisiae* (mitochondrial); *Sac*, *Sulfolobus acidocaldarius*; *Tth*, *Tetrahymena thermophila* (cytoplasmic).

[c] Ani, anisomycin; Cam, chloramphenicol; Car, carbomycin; Cel, celesticetin; Cla, clarithromycin; Cln, clindamycin; Ery, erythromycin; Lin, lincomycin; Spi, Spiramycin; Sgb, streptogramin type B. hs, hypersusceptible; s, susceptible; r, resistant.

TABLE 3. Peptidyltransferase circle antibiotic footprinting studies[a]

Protected base	Protection by:						Reference
	Cam	Ery	Car	Sgb	Cln	Lin	
A2058	−	++	+	−			76
A2058					++	++	25
A2059		++	+				76
A2059					++	0	25
G2061					+	+	25
A2062		0	++	++			76
A2439		0	0	+			76
A2451	++	0	++	+			76
A2451					++	++	25
G2505	++	++	++	++			76
G2505					++	++	25

[a] Data are summarized from those of Douthwaite (25) and Moazed and Noller (76). Ribosomes with bound antibiotic were reacted with DMS for adenine footprinting or with kethoxal for guanine footprinting. The extracted rRNA was reverse transcribed, and the resultant product was fractionated on a sequencing gel. Protection of a base by antibiotic, generally resulting in loss of a rung(s) in the sequencing ladder, is scored as + or ++, depending on the extent of the intensity loss; no entry indicates no protection; 0 indicates significant absence of protection; − indicates negative protection, i.e., facilitated interaction with derivatizing agent resulting in a rung intensity stronger than that seen in the no-antibiotic control. Not listed is the strong protection of A752 in domain II by sgb (76). For definitions of the abbreviations used, see Table 2.

The genes for the erythromycin-resistant mutant forms of ribosomal proteins L4 and L22 in *E. coli*, *rplD* and *rplV*, respectively, have been cloned and sequenced (17). The deduced amino acid sequences showed alterations from the wild-type sequences found previously. These were, respectively, Lys at position 63 to Glu (Lys-63-Glu) in RplD and deletion of the tripeptide sequence Met-Lys-Arg, corresponding to amino acid residues 82, 83, and 84, respectively, in RplV.

A role for mutants with altered ribosomal proteins in clinical isolates of erythromycin-resistant strains has not yet been found. Pardo et al. (86) noted that their erythromycin-resistant mutant of *E. coli* showed low temperature-sensitive assembly (Sad phenotype) when it was grown at 22°C. This apparent instability of ribosome assembly in the resistant mutant may be too high a price to pay to survive as a resistant mutant in vivo in the absence of antibiotic and might explain why erythromycin-resistant mutants with altered ribosomal proteins have not been found among clinical isolates.

A noteworthy corollary to these studies of ribosomal protein alterations is the observations of Tipper et al. (124) that chromosomal erythromycin resistance mutation involving protein L17 (*Bacillus subtilis*) exhibited temperature-sensitive (47°C) sporulation. Both susceptible cells and resistant mutants grew vegetatively at the same rate at both the permissive temperature (30°C) and the nonpermissive temperature (47°C). These findings suggest an as yet unknown linkage between ribosomal protein integrity and sporulation. That the observed effects were not due to erythromycin resistance per se was shown by Mahler and Halvorson (73), who noted apparently normal sporulation in *B. subtilis* cells carrying *erm* genes specified by either plasmid pBD5 or pIM13.

Altered target site: 23S rRNA. Alterations in rRNA have been found to play an increasingly important role in clinical and other forms of naturally occurring antibiotic resistance. With the exceptions of the examples of altered 50S subunit protein mutants cited above, the general mapping of discrete ribosomal functions onto individual ribosomal proteins that was hoped for never materialized. Concomitantly, a body of growing experimental data has repeatedly pointed to rRNA as the more significant component in this scheme (for reviews, see references 20, 21, and 80). Experimental data pointing to the direct role of rRNA in MLS resistance comes from three sources: (i) posttranscriptional modification of rRNAs that confer resistance, (ii) mutations in rRNAs that confer resistance, and (iii) biochemical footprinting experiments that localize the binding sites of antibiotic probes. In comparing the data obtained independently from each of these areas, a consistent picture emerges pointing to the interaction between

MLS antibiotics and nucleotides in the RNA sequence that comprises the peptidyltransferase center of the ribosome (3) (Fig. 1).

(i) Posttranscriptional methylation of A2058 (23S rRNA domain V) by ErmC *N*-methyltransferase. Altered rRNA rather than protein was shown to be responsible for MLS resistance in clinical isolates of *S. aureus* carrying *ermA* and that were therefore inducibly resistant to erythromycin (69, 70). In the first phase of that work, it was noted that 23S rRNA, which normally lacks any demonstrable N^6-methylated adenine, contained N^6-dimethyladenine if cells were induced with erythromycin during growth in ^{14}C- or ^{3}H-methyl-labeled methionine. The methylated adenine was identified as a part of the 23S sequence GAAAG. This sequence occurs 13 times in *B. subtilis* 23S rRNA, and Skinner et al. (104) determined the precise location of the methylated adenine using *Bacillus stearothermophilus* 23S rRNA and a partially purified *Streptomyces erythreus* methylase preparation. Because of conservation between the 23S rRNA of *B. stearothermophilus*, whose sequence was only partially known, and the 23S rRNA of *E. coli*, whose sequence was completely known, the location of the methylated adenine was reported as equivalent to *E. coli* coordinate 2058 (A2058), which corresponds to coordinate 2086 in *B. stearothermophilus* and coordinate 2085 in *B. subtilis* 23S rRNA (44).

S. aureus was a fortunate choice as an early model system for studying the methylation of 23S rRNA because a survey of five gram-positive strains that were tested showed that they lacked either mono- or dimethyladenine (116). In contrast, a survey of five gram-negative strains showed that they contained mono- but not dimethyladenine in their 23S rRNAs. In a more detailed study to identify and localize modified bases in 23S rRNA, Smith et al. (108) reported three monomethyladenine residues in *E. coli* 23S rRNA, A1618, A2030, and A2503; A2503 is the only adenine residue located in the peptidyltransferase circle (Fig. 1 and Table 2). As shown in Table 2, the mutation of an A to C at coordinate 2503 (A2503C) confers chloramphenicol resistance on both *E. coli* (132) and *Saccharomyces cerevisiae* (30) (mitochondrial) ribosomes. Although *ermC* and *ermE* are capable of phenotypic expression in *E. coli* (13, 49, 62), *B. subtilis* provided a more useful model system than *E. coli* for studying methylation because of the apparent absence of a background of N^6-methylated adenine in 23S rRNAs of MLS-susceptible gram-positive organisms.

To determine whether ribosomal protein alterations are at all necessary for MLS resistance, ribosomes containing reconstituted 50S subunits were tested. The 70S ribosomes that contained 50S subunits reconstituted with 23S rRNA from resistant cells and ribosomal proteins from susceptible cells were found to be resistant when they were tested in vitro for their abilities to support protein synthesis (71). Moreover, the inducibility of resistance provided a useful handle for biochemical studies in *ermA* initially (70) and in *ermC* later (136).

With *B. subtilis* as a transformable, low-methylation-background test organism, the small (3.7-kb) staphylococcal plasmid pE194 (57) provided the model system in which to study the molecular details of the induction of MLS resistance. Indeed, when pE194 was introduced into *B. subtilis* by transformation, it conferred the inducible MLS resistance phenotype (136). The small size of plasmid pE194 allowed it to be sequenced by the DNA sequencing techniques newly available at that time, and its use presented the possibility that the molecular basis for inducible MLS resistance might be determined (42, 53). By using plasmid pE194 to direct minicell protein synthesis and analyzing the products by polyacrylamide gel electrophoresis, Shivakumar and Dubnau (99) showed that this plasmid directed the synthesis of about six proteins. One of these, migrating as a 29-kDa band, was the only protein whose intensity was markedly increased in minicells induced with erythromycin. This band was therefore inferred to represent the *ermC* methylase.

The Erm family comprises a group of homologous methylases that use *S*-adenosylmethionine (SAM) as the methyl donor to modify a single adenine residue in 23S rRNA to form either N^6-mono- or dimethyladenine. Generally, the 23S rRNAs of gram-positive bacteria appear to lack any N^6-methylated adenine unless the cells are resistant to MLS antibiotics by the methylase mechanism (34, 40, 69, 116). There appear to be at least two functionally discernible classes of Erm methylases. The first class includes those that only monomethylate adenine, e.g., Lrm from *Streptomyces lividans* (60), Clr from *Streptomyces caelestis* (15), and TlrD from *Streptomyces fradiae* (139). The second class includes those that predominantly dimethylate adenine, e.g., ErmC from *S. aureus* (22), ErmE from *Saccharopolyspora erythrea* (*Streptomyces erythraeus*) (15), and TlrA (ErmSF) from *S. fradiae* (138). Thus, partially purified Clr was shown only to monomethylate adenine in vitro, even in the presence of an added excess of SAM. The addition of ErmE to the reaction mixture quantitatively converted the monomethyladenine to dimethyladenine, suggesting that Clr possessed the ability only to monomethylate adenine (15). In contrast, TlrA produces monomethyladenine at a low SAM concentration (3 μM) and produces dimethyladenine at a high SAM concentration (500 μM) (138). Associated with monomethylation in vivo, e.g., by Clr, resistance to >1,000 μg/ml was found for lincomycin and clindamycin, whereas MICs of between 50 and 200 μg/ml were found for erythromycin, tylosin, and carbomycin (15). Associated with dimethylation by ErmE in vivo, resistance to >1,000 μg/ml was found for all five antibiotics.

Pulse-chase experiments with labeled adenine fed to growing *S. aureus* cells carrying inducible *ermA* showed that the methylation reaction utilizes nascent 23S rRNA rather than than mature ribosomes (68). Labeled adenine that had been incorporated into mature ribosomes was not detectably converted into methyladenine in vivo if the input radioactivity was diluted by the addition of cold adenine and erythromycin was added to induce the culture subsequently; methyladenine did appear if the radioactive adenine was not diluted by the addition of unlabeled adenine. Shivakumar and Dubnau (99) partially purified ErmC and characterized its in vitro activity, including its interaction with substrates and inhibitors. Their preparation methylated rRNA from susceptible but not from resistant cells and was even reported to methylate RNA in 50S subunit preparations. This latter observation probably has no physiological significance, despite the enhanced survival that would be conferred on an organism under siege that was able to modify all of its ribosomes rapidly. The report that intact 50S ribosome subunits can serve as a substrate for methylase in vitro may reflect the partial disassembly of ribosomes in the reaction mixture to a degree that permits utilization of the RNA as a substrate.

Stern et al. (111) have devised a footprinting method to record antibiotic interactions with rRNA; antibiotics bound to single-stranded rRNA protect it against derivatization by DMS at the N-1 of adenine and cytosine. By reverse transcription from a downstream primer back upstream into the region of interest, it is possible to distinguish regions that were protected against DMS from those that were not. The method can be used to visualize the protection of RNA by bound enzyme as well. Thus, Su and Dubnau (113) reported that the nucleotides present in and around the peptidyltransferase center in domain

V were protected by purified methylase against in vitro methylation by DMS. Additionally, they noted that several bases in domain II as well as in domain VI were protected, albeit to a lower degree than was observed for domain V. Protection of purified 23S rRNAs by bound MLS antibiotics, additionally, is discussed below.

(ii) Mutations in 23S rRNA that confer resistance by altering A2058 and its neighboring nucleotides. There is compelling evidence that the peptidyltransferase loop in domain V of 23S rRNA may contain at least part of the site at which the MLS antibiotics physically bind to the ribosome. Mutants selected for resistance to individual MLS antibiotics showed nucleotide changes in this region, suggesting involvement in the binding of antibiotics. The nucleotides that are involved are shown in Fig. 1, and specific examples are listed in Table 2. The mutants were selected from widely diverse sources, attesting to the fundamental importance of this region in the actions of these antibiotics across species.

At one functional extreme there is the family with a mutation at A2058 in which resistance to only macrolides and lincomycin has been reported. Presumably, these cells are also resistant to streptogramin B, but this has not been tested. The only mutant that was found to be resistant to a member of each subclass of the MLS antibiotics was C2611U. At the other functional extreme there is a group of mutations at coordinates 2447 to 2453, 2503, and 2504 that appear to confer only chloramphenicol resistance and that are included for completeness.

The apparent localization of chloramphenicol resistance mutations over the range of nucleotides between 2062 and 2504 (see Table 2) may in part reflect a bias in the selection of antibiotics that were tested because of the susceptibility of the intact cells in which the respective mutations were selected. The frequency of individual mutations could also contribute to this apparent bias. In a departure from other studies summarized in Table 2, Aagaard and colleagues (1) reported that the previously described mutant C2452U confers resistance to carbomycin and celesticetin, in addition to chloramphenicol, which is expected for this region. These observations extend the range over which the macrolide and lincosamide antibiotics act in the peptidyltransferase circle and suggest that any conclusions concerning the selective localized action of antibiotics in this region be based on a test of members of all relevant classes. For this reason, it would be especially helpful to have a commercial source of streptogramin B for research and clinical testing purposes.

The mutation G2057A that borders this group confers resistance to both erythromycin and chloramphenicol but not to 16-membered ring macrolides or lincomycin. The G2032 family, involving the neighboring stem 72, confers erythromycin hypersusceptibility and, depending on the nucleotide change, resistance or susceptibility to clindamycin and to chloramphenicol. These observations point to the overlapping nature of the binding sites for macrolides, lincosamides, streptogramins, and chloramphenicol and may explain the physical basis for the observation of their competition for binding to ribosomes (129, 130).

(iii) Protection of A2058 and neighboring nucleotides in the peptidyltransferase circle by bound antibiotics. The RNA protection studies described by Stern et al. (111) support the notion that the peptidyltransferase loop plays a direct role in the binding of MLS antibiotics. In those studies, ribosome (or rRNA) preparations were mixed with the test antibiotic and the resultant test complex was allowed to react with DMS or kethoxal. rRNA was purified or repurified and scanned by primer extension with reverse transcriptase from a set of primers spaced throughout the 23S rRNA. Since bases that derivatize at the positions involved in the formation of Watson-Crick base pairs act as a barrier to primer extension by reverse transcriptase, protection by antibiotic shows up as missing rungs in a DNA ladder obtained by electrophoretic fractionation of the reverse transcriptase product. The antibiotic-protected nucleotides demonstrable by this method are listed in Table 3.

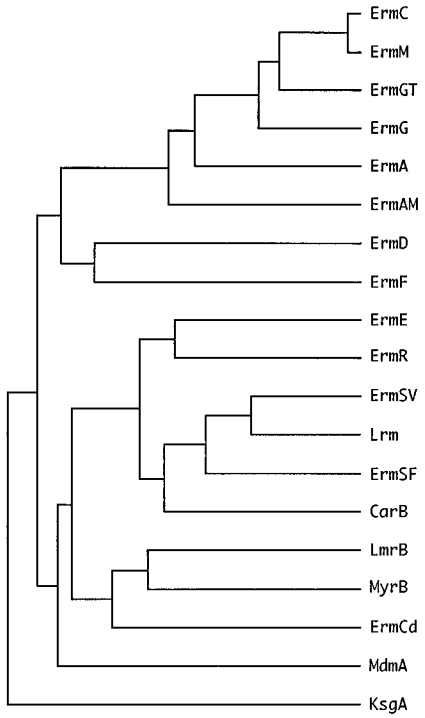

FIG. 2. Erm dendrogram. Erm sequences were analyzed by the program PILEUP (37), which quantifies the amino acid sequence similarities of a set of proteins. The output includes a dendrogram (shown here) and an ordered gapped listing of sequences (not shown). The sources of the protein sequence information used are summarized in Table 1. The amino acid sequence of ErmAM is nearly identical to those of ErmB, ErmB-like, ErmBC, and ErmP proteins; the amino acid sequence of ErmD is nearly identical to those of ErmJ and ErmK.

Thus, Moazed and Noller (76) incubated 70S ribosomes together with antibiotics and showed the direct protection of both A2058 and A2059 by both erythromycin and carbomycin against derivatization by DMS; carbomycin additionally protected A2062. Of these three protectable adenine residues, vernamycin B protected A2062 but not A2058 or A2059. In a comparison of protection by lincomycin and clindamycin reported by Douthwaite (25), it was noted that clindamycin protected both A2058 and A2059, whereas lincomycin protected only A2058. These data are the kind that would be expected if the MLS antibiotics had overlapping but not identical binding sites.

Mutational alterations of the nucleotides C2611, G2057, A2058, A2059, and A2062, all of which are part of the peptidyltransferase circle (Fig. 1), have been found in association with and have been presumed to cause resistance to various combinations of MLS antibiotics, as summarized in Table 2. G2032, which, although it is not part of the peptidyltransferase circle, forms the loop segment of stem 39 and is only 26 nucleotides removed from A2058, has also been strongly implicated in erythromycin resistance (24). Moazed and Noller (76) were unable to obtain evidence that A2058 was protected by vernamycin B (streptogramin B family). This observation raises the question of how posttranscriptional modification of A2058 confers resistance to vernamycin B, and therefore to the rest of the streptogramin B family as well. Of the nucleotide residues whose alteration by mutation appears to confer MLS resis-

tance, so far only posttranscriptional modification of A2058 has been observed as part of a mechanism of naturally occurring resistance. Since mutational alterations at other sites in the peptidyltransferase center can also confer resistance, they could in principle serve as targets for posttranscriptional modifications that have not yet been found.

(iv) Other *erm* genes, from A to Z. The *erm* gene products comprise a group of structurally homologous *N*-methyltransferases (methylases) that specifically methylate a single adenine residue (A2058) located in the peptidyltransferase circle of 23S rRNA. The *E. coli* residue number A2058 is used for uniformity of the nomenclature; however, the precise numerical coordinate of its homolog in other rRNAs from other species varies. Gutell and colleagues (44, 45) have compiled large-subunit rRNA sequences and have presented them in a way that allows a detailed comparison of homologous nucleotide residues. A list of most of the known *erm* methylases is provided in Table 1. The high degree of amino acid sequence identity in these proteins allows their sequences to be aligned easily and suggests that they are related to a common progenitor and, through that progenitor, to the KsgA group of methyltransferases (51, 127) that confer susceptibility to kasugamycin (Fig. 2).

In spite of reports of *Mycoplasma* spp. resistant to macrolide and lincosamide antibiotics, the definitive demonstration of an *erm* methylase from this group of organisms appears to be noticeably absent. The existence of such a methylase was suggested by the report of Stopler and Branski (112), who reported heterogeneous MLS resistance in a strain of *Mycoplasma pneumoniae*. Expression of resistance appeared to be variable in that the level of expression diminished if cells were cultivated in the absence of erythromycin or by treatment of the culture with acridine orange. When resistant cells became susceptible to erythromycin they became susceptible to lincomycin and streptogramin B as well. When susceptible cells were grown in erythromycin they also became resistant to lincomycin and streptogramin B. A second group of cells, described as having homogeneous MLS resistance, expressed resistance stably, even if they were cultivated in the absence of erythromycin. Collectively, these observations suggest a form of MLS resistance in *M. pneumoniae* that is inducible and that is located on an unstable plasmid.

In a biochemically oriented investigation Palu et al. (84) studied a clinical isolate of *Ureaplasma urealyticum* from a patient who had been treated with erythromycin and noted that the strain was resistant both to the macrolides erythromycin, josamycin, and roxithromycin and to the lincosamides lincomycin and clindamycin. The resistant cells showed reduced erythromycin uptake and ribosomes showed reduced erythromycin binding, suggesting that a ribosomal structural alteration was responsible for the observed resistance. On the basis of the information provided, resistance in these cells might be due to either 23S rRNA methylation or a mutation. Technical means are now available to distinguish between these possibilities.

The amino acid sequence similarities of different Erm proteins can be quantified by the program PILEUP that comprises part of the Genetics Computer Group Package (37) and can be displayed as the dendrogram shown in Fig. 2. The major bifurcation within the Erm group separates the eubacteria from actinomycetes (including *Corynebacterium diphtheriae*). KsgA was included in the comparison because it should possess minimal similarity to any of the other Erm proteins. PILEUP failed to make any distinction between the actinomycete Erm proteins that dimethylate adenine, e.g., ErmE (104, 105) and TlrA (ErmSF) (138), and those that exclusively monomethylate adenine, e.g., Clr (15), Lrm (60), and TlrD (139).

(v) Mutations elsewhere in 23S rRNA that confer erythromycin resistance. Mutations that lead to erythromycin resistance have been found in 23S rRNA other than in domain V. To facilitate referencing of these mutations, the system of Brimacombe et al. (12), which assigns numerical labels to each of the double-stranded segments in *E. coli* 23S rRNA, is used. Thus, the mutation G2032A in the loop associated with stem 93 results in chloramphenicol and clindamycin resistance and hypersusceptibility to erythromycin (24). The same alteration was also obtained as a spontaneous mutation in tobacco chloroplast large-subunit RNA, in which it was described as conferring lincomycin resistance (19). G2032 has been found to form an internal *N*-mustard cross-link with A2054 (23). Strains with double mutations that involve G2032 and A2058 show complex phenotypes, suggesting that these nucleotides interact functionally as well. Thus, strains with double mutants G2032A plus G2057A showed resistance to erythromycin, clindamycin, and chloramphenicol, while those with double mutants G2032A plus A2058T showed hypersusceptibility to erythromycin and susceptibility to clindamycin and chloramphenicol (26).

Domain II has also been implicated in the action of erythromycin. Douthwaite et al. (27) have constructed a series of mutant ribosomes with alterations in 23 rRNA domain II. These efforts centered on a helical sequence within domain II that spans nucleotides 1200 through 1250 and that involved various deletions ranging in size from 1 to 23 nucleotides. Two deletions in which three consecutive nucleotides, CAU1231 or AUG1232, were removed were found to confer resistance. Some of the mutant ribosomes bound erythromycin with the same affinity as wild-type ribosomes, as measured by the ability of bound erythromycin to protect A2508 and A2509 against modification by DMS; no protective effect of erythromycin on the domain II sequence was seen.

Douthwaite (24) noted that deletion of selected nucleotides in domain II leads to erythromycin resistance and therefore proposed that the rRNAs in domains II and V interact through a ribosomal protein. Moreover, since the mutant ribosomes that were constructed synthesized protein, it was further proposed that one of the effects of the domain II mutations is to alter the orientation of erythromycin while it is bound to the ribosome rather than the strength with which it is bound to the ribosome and that such an altered orientation might allow the nascent peptide, having reached the critical length of five amino acid residues, physically to circumvent the block that occurs upon the binding of erythromycin by the ribosome. Strains with such mutations have not yet been selected in either the laboratory or the clinic.

CONCLUSIONS

Had there been no model system in which to study the posttranscriptional modification of A2058, the functional significance of this nucleotide residue would have eventually been inferred from its repeated occurrence in mutants resistant to MLS antibiotics as well as to the numerous mutations that occur in its vicinity. The possibility of selecting such recessive mutants directly, i.e., without the benefit of a high-copy-number vector carrying the mutant (resistant) 23S rRNA, was reported by Pernodet et al. (87), who used the 16-membered ring macrolide chalcomycin to select a resistant mutant of *Streptomyces ambofaciens* carrying the mutation A2058G. *S. ambofaciens* has four (88) rather than seven (10) rRNA operons, as are present in *E. coli*. This would suggest that a ratio of three 23S rRNA genes conferring susceptibility to one conferring resistance would allow the functionally useful penetrance of expression of resistance.

The studies reviewed above therefore take an additional

significance in light of the reemergence of resistant strains of mycobacteria. Suzuki et al. (114) found evidence for about one rRNA gene in *Mycobacterium bovis*. If the same low rRNA operon copy number is also present in *Mycobacterium tuberculosis* there is an explanation for the bountiful yield of clinical isolates of streptomycin-resistant mutants with mutational alterations in their 16S rRNAs and, more recently, for clinical isolates of clarithromycin-resistant *Mycobacterium intracellulare* with mutationally altered A2058 (75). The low rRNA gene copy number that enables the selection of resistant mutants that are dominant in *Mycobacterium* spp. but recessive in most of the major pathogens may also contribute to the slow growth of these organisms.

How many other clinically important pathogens will respond to antibiotic pressure with the emergence of dominant mutations that specify an altered rRNA? Gobel et al. (38) reported that *Mycoplasma pneumoniae*, *Mycoplasma hyorhinis*, and *Mycoplasma arthritidis* each contain one rRNA operon, while Sawada et al. (95) counted two sets of rRNA copies in *Mycoplasma capreolum*. To date, neither methylated A2058 nor a *Mycoplasma* methylase have been described. It will be of interest to see whether macrolide-lincosamide-resistant *Mycoplasma* spp. as well as their resistant *Ureaplasma* relatives (84) are resistant on the basis of the mutations A2058G, -C, or -U and how many are resistant by posttranscriptional modification owing to methylase.

A knowledge of the number of rRNA operons is clearly relevant for predicting the organisms in which antibiotics that act on ribosome function are capable of selecting dominant resistant mutants. The development of a new macrolide antibiotic, clarithromycin, with clinically useful activity against atypical mycobacteria provided the most recent example. With hindsight, it would be desirable to have such information on hand in anticipation of the appearance of new antibiotics in clinical practice before the inevitable emergence of the resistant strains that they will select.

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