Clavulanic acid is for Penicillins, What is for Erythromycin?

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Introduction

Erythromycin inhibits protein synthesis by its effect on ribosome function(1,2,3). The metabolic modifications that enale cells to cope with the inhibitory action of erythromycin fall under general major antibiotic resistance mechanisms that include (i) target site alteration, (ii) antibiotic modification, and (iii) altered antibiotic transport. 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 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. Therefore, this type of resistance has also been referred to as MLS(macrolide-lincosamide-streptogramin B) 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 A2085 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(4,5,6,7,8,9); 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(10,11); 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(12,13,14,15,16, 17,18,)(Fig. 1), and (iii) footprinting experiments show that the nucleotides in 23S rRNA

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

erm allele	Organism
ermA	*Staphylococcus aureus*
ermAM	*Streptococcus sanguis*
ermB	*Staphylococcus aureus*
ermBC	*Escherichia coli*
ermB-like	*Streptococcus faecalis*
ermC	*Staphylococcus aureus*
ermCD	*Corynebacterium diphtheriae*
ermD	*Bacillus licheniformis*
ermE	*Streptomyces erythraeus*
ermF	*Bacteroides fragilis*
ermFS	*Bacteroides fragilis*
ermFU	*Bacteroides fragilis*
ermG	*Bacillus sphaericus*
ermGT	*Lactobacillus reuteri*
ermIM	*Bacillus subtilis*
ermJ	*Bacillus anthracis*
ermK	*Bacillus licheniformis*
ermM	*Staphylococcus epidermidis*
ermP	*Clostridium perfringens*
ermQ	*Clostridium perfringens*
ermR	*Arthrobacter luteus*
ermSF	*Streptomyces fradiae*
ermZ	*Clostridium difficile*
carAB	*Streptomyces thermotolerans*
clr	*Streptomyces caelestis*
lmrAB	*Streptomyces lincolnensis*
lrm	*Streptomyces lividans*
mdmA	*Streptomyces mycarofaciens*
myrAB	*Micromonospora griseorubida*
srmABCD	*Streptomyces ambofaciens*
tlrABCD	*Streptomyces fradiae*

[a]Most *erm* methylases have been reported as alleles of *erm*. (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, whereas *carB* specifies an *erm* methylases; (iii) *lmrA* specifies a transport ATPase, and *lmrB* specifies an *erm* methylase; 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*. (v) *srmA*, *srmB*, *srmC*, and *srmD* were characterized as cloned DNA fragments that conferred resistance to spiramycin. *srmB* specifies a transport ATPase. 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. *tlrA* (synonym *ermSF*) and *tlrD* specify *erm* methylases. *tlrC* specifies a transport ATPase, and the activity associated with *rlrB* has not yet been reported.

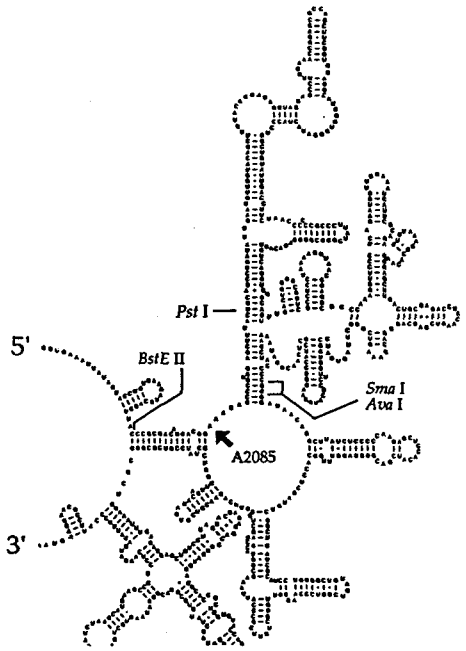

Fig. 1. 23S rRNA domain V rRNA from *B. subtilis*

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(19, 20)(Fig. 2).

The *erm* family of genes is not alone in conferring clinical resistance to macrolide

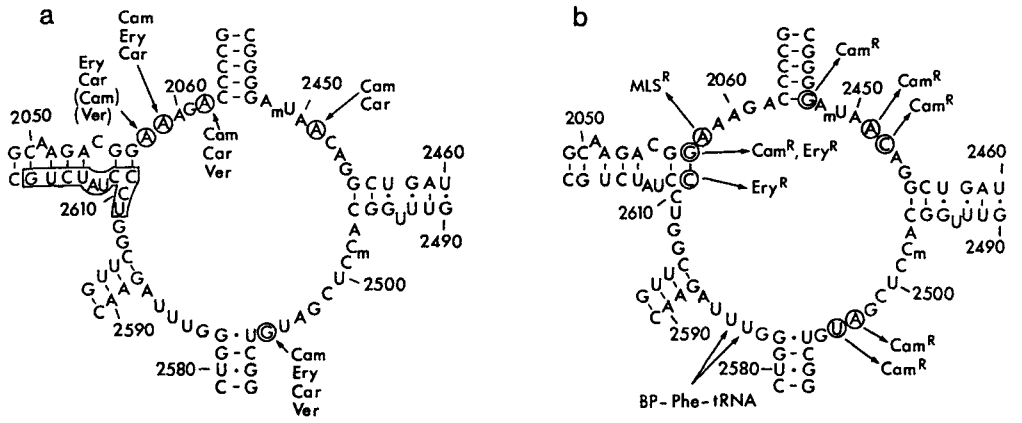

Fig. 2. Schematic diagrams of the central loop of domain V of 23S rRNA from *E. coli*. In **a**, the sites of protection by antibiotics are circled, and the protecting antibiotics indicated. Antibiotics causing enhancement of reactivity are shown between parentheses. In **b**, bases whose mutaion gives resistance to antibiotics are circled.

antibiotics. A notable early exception to the established MLS resistance pattern was the MS pattern reported by Janosy and coworkers(21, 22), who described clinical isolates that were coresistance 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.(23) to involve the active efflux of erythromycin and streptogrmin 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(24), glycosylation(25), and lactone ring cleavage by erythromycin esterase(26, 27), have been added to the list.

Resistance Mechanisms

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, 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. 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, consistent picture emerges pointing to the interaction between MLS antibiotics and nuceotides in the RNA sequence that comprises the peptidyl transferase center of the ribosome(28).

(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(29, 30). In the first phase of work, it was noted that 23S rRNA contained N6-dimethyladenine if cells were induced with erythromycin during growth in 14C- or 3H-methyl-labeled methionine. Afterward, the location of the methylated adenine was reported as equivalent to *E. coli* coordinate 2058(A2058), which corresponds to coordinate 2085 in *B. subtilis* 23S rRNA. 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 form susceptible cells were found to be resistant when they were tested in vitro for their abiilities to support protein synthesis(31). Stern et al. (32) 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(33) 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.

(ii) Protection of A2058 and neighboring nucleotides in the peptidyltransferase circle by bound antibiotics. The RNA protection studies described by Stern et al. (32) 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. Thus, Moazed and Noller (20) 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 (34), 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(Fig. 2).

(iii) **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 (35,36) 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(37,38) that confer susceptibility to kasugamycin(Fig. 3).

Erythromycin as a regulator of *ermC* expression: Conformational isomerization of the *ermC* leader sequence. The 141-nucleotide *ermC* mRNA leader can assume at least three alternative conformations, as shown in Fig. 4. In its nascent form, the leader would be expected to assume the conformation shown in Fig. 4A. The pattern of association shown was assigned on the basis of the temporal order of synthesis of complementary segments. Thus, in the uninduced "ground state" shown in Fig. 4A, segment 2 associates with segment 1, and segment 4 associates with segment 3. With the *ermC* message in this conformation, translation of the ErmC protein is initiated with a low efficiency because the first two codons of ErmC, AUG and AAU (fMet Asn), as well as the *ermC* ribosome binding site, are sequestered by secondary structure. Induction provides conditions that favor a translationally acitive conformation of the *ermC* message shown in Fig.4B. Induction of *ermC* starts with the

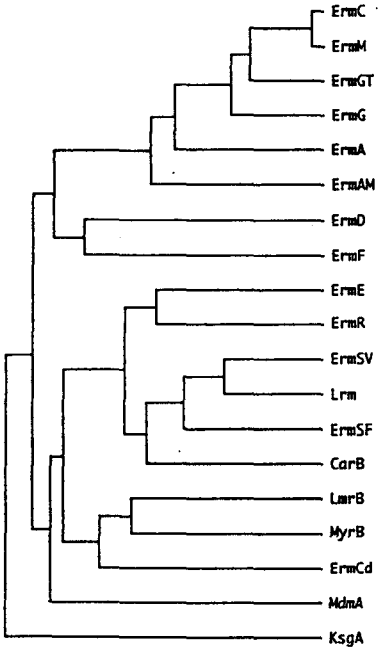

Fig. 3. Erm dendrogram. Erm sequences were analyzed by the program PILEUP, which quantifies the amino acid sequence similarities of a set of proteins. The output includes a dendrogram (shown here) and an ordere gapped listing of sequences (not shown). The sources of the protein sequence information used 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.

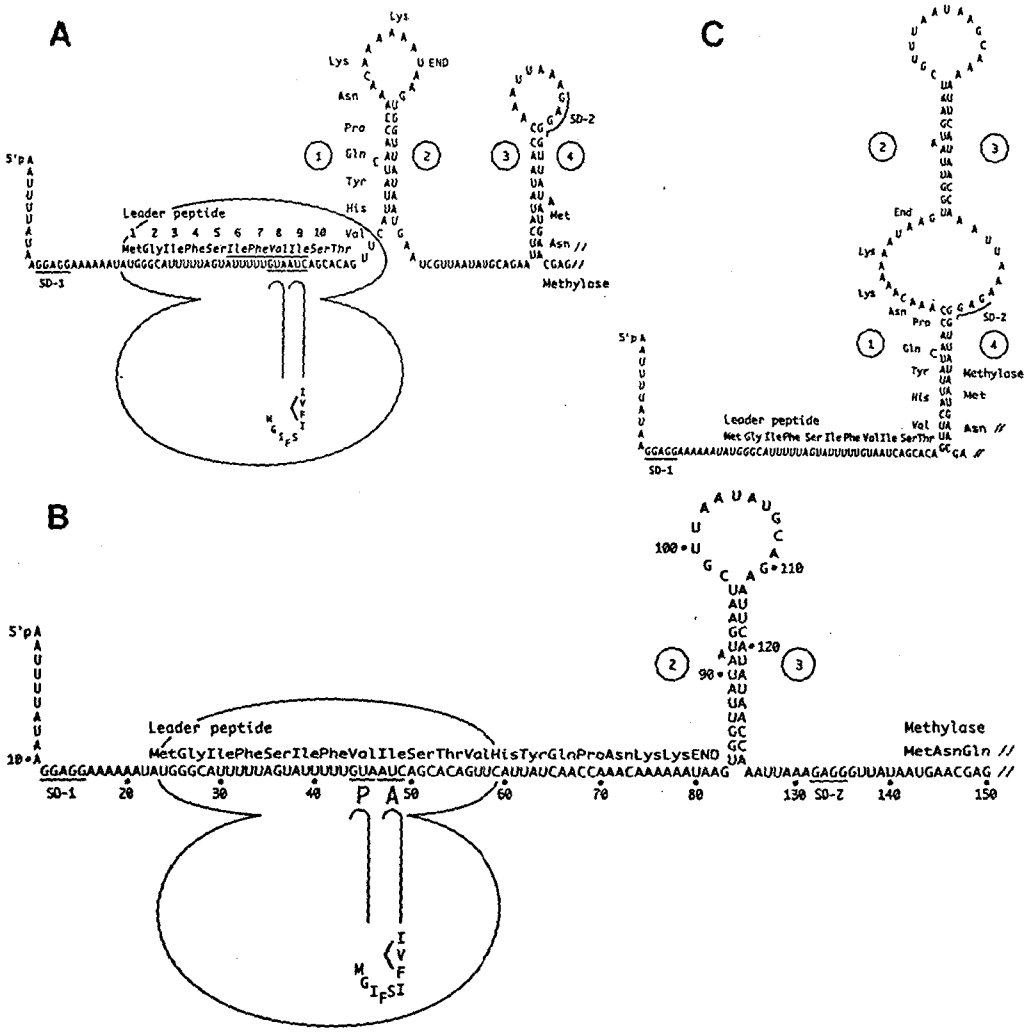

Fig. 4. Proposed conformational trasitions of the *ermC* leader sequence during induction. (A) Conformation of *ermC* mRNA leader sequence early during induction by erythromycin. (B) Conformation of the *ermC* mRNA leader sequence in its fully induced state. As a consequence of stable complex formation, the erythromycin-ribosome complex, and the *ermC* message, the association between segment 1 and segment 2 is prevented. (C) Inactive conformation of the *ermC* mRNA leader sequence resulting from either removal of erythromycin or another inducer or from maximal methylation of 23S rRNA and a maximum concentration of resistant ribosomes.

binding of erythromycin to ribosomes that synthesize a 19-amino-acid peptide, MGIFSIFVISTVHYQPNKK, encoded by the *ermC* mRNA leader upstream of the ErmC open reading frame, and culminates with an increased efficiency of ErmC translation owing to the conformational isomerization of its message to the acive form shown in Fig. 4B. Thus, for erythromycin to act as an inducer it must inhibit synthesis of a peptide whose composition and location as an open reading frame are critically defined and located in relation to the

conformation of the *ermC* leader sequence. Because the ribosome must pause while it is translating the leader peptide, the process of induction based on this principle is generally called "attenuation," and because the regulatory signal directly affects translation, the mechanism of regulation is therefore referenced as "translational attenuation." If the condition is satisfied of a reduction in the original inductive stimulus, i.e., erythromycin concentration, or saturation of the cell with the products of induction, i.e., methylated ribosome, down regulation(negative feed back regulation) is achieved through the refolding of the leader region to assume an inactive conformation, shown in Fig. 4C. The conformational transition 4B to 4A would also repress *ermC*, but energy would first be required to dissociate sterm 2:3. In contrast, the conformational transition 1B to 1C would not require additional energy; it would be expected to be exothermic, and would therefore be favored. The translational attenuation model predicts that the synthesis of methylase should eventually become self-limiting since a critical concentration of susceptible ribosomes is needed to sustain induction.

Substrate Specificity of Erm Methylase

It has long been suspected that the nascent 23S rRNA can act as methylase substrate. If this is so, which sequence characteristics of 23S rRNA confer the specificity that enables its recognition by Erm methylase? To address this question, one of the *erm* family methylase should be obtained in large quantity. Most probable candidate is ErmC from *Bacillus subtilis*, but overexpressed ErmC in *E. coli* makes the inclusion body and cannot be renatured to its active conformation. *ermSF*(synonym *tlrA*) from *Streptomyces fradiae* NRRL 2702 confers resistance to the MLS superfamily of antibiotics. Furthermore, ErmSF specifically methylates *B. subtilis* 23S rRNA in vitro at A2085. The *ermSF* gene was cloned, overexpressed in *E. coli* and renatured(Fig. 5). PCR cloned and in vitro transcribed domain V in peptidyltransferase of 23S rRNA was successfully methylated by overexpressed ErmSF. This methylation was

Fig. 5. Purification of recombinant ErmSF. *E. coli* HJJ103 cells were treated with lysozyme, dissolved in a buffer containing 6 M urea, and fracionated by immobilized metal ion affinity chromatography with a Ni^{2+} affinity column. Lane 1, crude cell extract; lane 2, immobilized metal ion affinity chromatography column run-through; lane 3, immobilized metal ion affinity chromatography column 300mM imidazole eluate. The fastest-moving protein band, 14.3kDa, corresponds to the egg white lysozyme that was used to disrupt the cells; the 43- and 29-kDa standards are ovalbumin and carbonic anhydrase, respectively.

monitored by paper chromatography and reverse transcription(Fig. 6, 7). Before these observations, Noller et. al. (40) have reported that 23S rRNA preparations obtained by proteolytic digestion followed by phenol extraction can catalyze the synthesis of peptide bonds. Actually, this preparation contained from two to as many as eight intact proteins which can be detected by two dimensional gel electrophoresis.

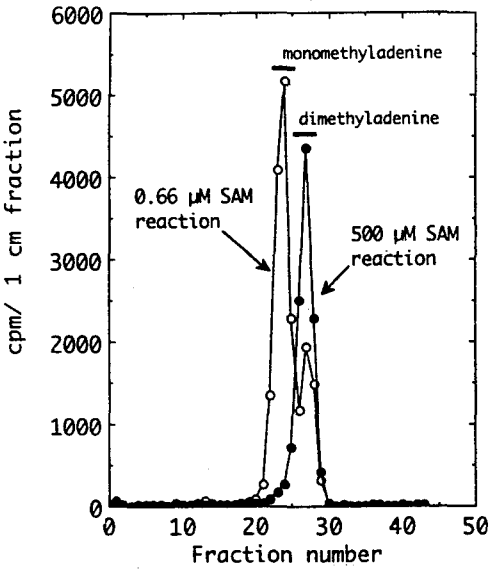

Fig. 6. Paper chromatography analysis of bases in domain V RNA methylated in vitro. A 400 μl methylation reaction mixture was assembled in vitro with domain V RNA, 0.66 μM[^{3}H]- *S*-adenosyl-methionine, and ErmSF methylase. Reaction mixtures were incubated at 37℃, and the reactions were terminated by phenol extraction and ethanol precipitation. The resultant precipitates were digested with HCl and were fractionated, togerher with standards, by descending paper chromatography on Whatman 3MM paper. The paper chromatogram was dried and cut into 1-cm segments; counts were determined in a liquid scintillation spectrometer.

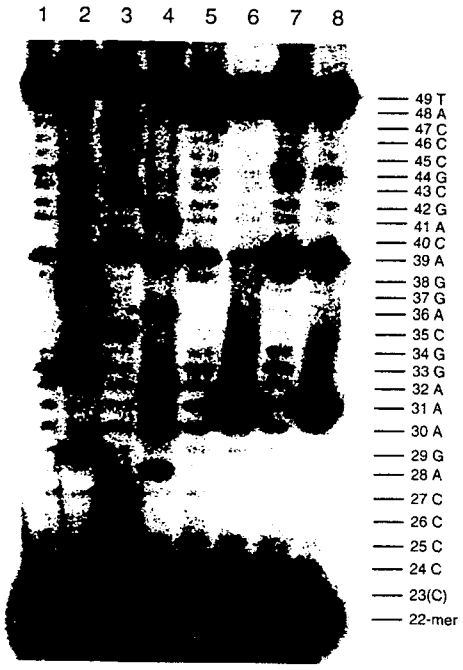

Fig. 7. Sequencing gel analysis of bases in domain V RNA methylated in vitro. For all samples, reverse transcriptase mapping was primed with oligonucleotide 9147. Lanes 1 to 4, sequencing of domain V by primer extension with reverse transcriptase, erythromycin-sensitive BR151 rRNA *B. subtilis* as a template, and termination with ddA, ddC, ddG, or ddT, respectively. Lane 5, negative control, reverse transcriptase extension product obtained with total rRNA from erythromycin-sinsitive *B. subtilis* BR151 as a template, unmethylated at A2085. Lane 6, positive control in which total rRNA from erythromycin-resistant *B. subtilis* BR151(pE194 *cop-6*), methylated at A2085, was used. Lane 7, reverse transcriptase product obtained with domain V RNA, unmethylated by ErmSF methylase, as a template. Lane 8, same as lane 7, except that domain V RNA, methylated in vitro by ErmSF methylase, was used as a template. The reactions in lanes 5 to 8 were strongly terminated by the combined omission of dATP and inclusion of ddATP in the reaction mixture. The bracketed C at coordinate 23 was expected but not seen because of the intense primer band.

In this study, following facts were found. i) rRNA fragment transcripts, synthesized in vitro, can serve as a substrate for methylation by the *ermSF* methyltransferase; ii) overexpressed ErmSF in *E. coli* methylates only adenine. Since this methylase preparation was nearly homogeneous and lacked demonstrable interfering activities, these observations suggest that rRNA alone, to the exclusion of rRNA complexed with other ribosomal components or rRNA posttranscriptionally modified can serve as a methylase substrate, since the transcripts that were used have not been previously assembled into ribosomes. It will be interesting to determine how many more nucleotides can be deleted from domain V while allowing retention of its activity as a substrate for methylation. Domain V of *B. subtilis* 23S rRNA consists of around 660 nucleotides(nts). To investigate the above question, successively smaller domain V constructs containing 337, 113, 53, 46, and 41 nts(Fig. 8, 9) were prepared by PCR cloning and in vitro transcription. These in vitro transcribed rRNA products were recognized and successfully dimethylated at A2085 by ErmSF. Minimalist substrate, 41 nts contains 23S rRNA stem 73(41), flanking a tetraloop-like(UUCG), and the unpaired sequence A*AAGA, at the 3′ end containing A2085(A*). A set of systematic alterations introduced into the sequence suggested that the four unpaired nucleotides in stem 73 are necessary for methyl-acceptor activity, whereas inversion of 11 out of 13 paired bases in stem 73 conferred no significant reduction in methyl-acceptor activity(Fig. 9).

Perspectives

For several years, medical microbiologists have been tracking an alarming trend. With

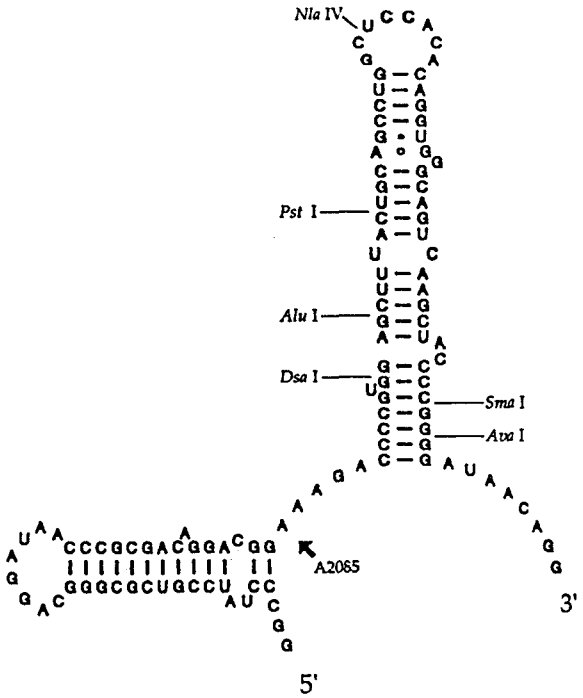

Fig. 8. pDK107 full length (113- nucleotide) transcript showing single occurrence restriction sites. Template DNA was prepared and digested with either *Bst*UI, *Dsa*I, *Alu*I, *Pst*I, *Nla*I, *Sma*I, or *Ava*I restriction endonuclease, and runoff transcripts were prepared.

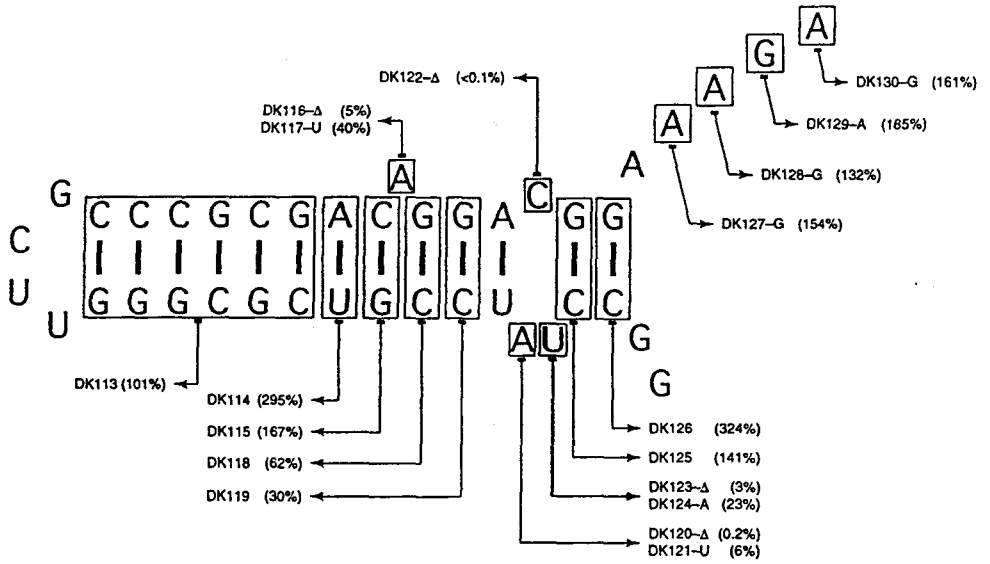

Fig. 9. Minimalist 41-nucleotide methylase substrate consisting of stem 73 and adjacent peptidyltransferase circle nucleotides are shown. The constructs in which bases or base pairs have been systematically altered are also boxed. The sequence change at each altered position is indicated as either the nucleotide substitution or △ for deletion. No specification indicates base pair inversion(s). Percent incorporation in th standard in vitro methylation assay is indicated for each construct, in parentheses.

increasing frequency, they've been finding that antibiotics that formerly killed bacterial pathogens with ease were becoming ineffective-the result of resistance mechanisms develped by the ever-crafty bacteria. This raise the nightmare of what one researcher calls the "post-microbial era"-a return to the bad old days when a simple infection could turn life-threatening for lack of effective treatment. Among the mechanisms identified, enzymic inactivation of antibiotics is one of the most common biochemical processes that engenders resistance to a wide variety of antibiotic structural types in bacteria(42). In MLS superfamily of antibiotics, several examples of transferable resistance mechanisms that involve the covalent modification of antibiotics could be found in clinical bacterial isolates. For example, O-phosphorylation of erythromycin has been identified in a number of bacterial isolates(43), and hydrolytic cleavage of the lactone ring of this class of antibiotics has also been described. The lincosamides(lincomycin and clindamycin) have been shown to be inactivated by enzymic O-nucleotidylation in Gram-positive bacteria. For the MLS group, such forms of antibiotic inactivation seem (at least for the moment) to be a relatively minor mechanism of resistance. In clinical isolates, enzymic modification of rRNA is the most prevalent mechanism of resistance. One of the most effective tactic to combat the surge in antibiotic-resistant microbes is to develope the effective inhibitors of resistant factor. At present, around 30 *erm* genes have been characterized from clinical pathogens to actinomycetes that produce antibiotics. The higher degree of amino acid identity in these proteins allows their sequences to be aligned easily and suggests that they are related to a common progenitor.

Furthermore, *erm* methylase as a antibiotic factor can cross-talk over the genera, that is methylase from different genera can methylate exact position(adenine residue) of 23S rRNA. From the substrate specificity of ErmSF, minimal substrate of 41 nucleotides is determined and some base substitution can enhance the methylation activity of ErmSF. Relatively small around 35 kd protein is not thought to recognize the whole sequence or its structural characteristics of minimalist substrate(The fact that base substitution can enhance the methyl acceptor activity should support the this reasonings.). Probably other sequences other than real recognition of ErmSF is necessary to maintain the three dimensional structure to be recognized by methylase. The truth can be uncovered by ErmSF structural analysis and its three dimensional interaction with minimalist substrate of 41 bp.

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