Methylation of Minimalist 23S rRNA Sequences *in Vitro* by ErmSF (TlrA) *N*-Methyltransferase†

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ABSTRACT: *ermSF* (synonym *tlrA*) from *Streptomyces fradiae* NRRL 2702 confers resistance to the macrolide−lincosamide−streptogramin type B (MLS) superfamily of antibiotics. ErmSF specifically methylates *Bacillus subtilis* 23S rRNA *in vitro* at A2085 (*B. subtilis* coordinate, which is equivalent to the *Escherichia coli* coordinate A2058). In the present studies, partial *B. subtilis* 23S rRNA sequences containing portions of the peptidyltransferase circle which include A2085 were constructed in order to identify structural requirements needed for RNA to function as substrate of ErmSF. A model methylase substrate based on the 41-nucelotide construct DK111, ggCCUAUCCGUCGCGGGUUCGCCCGC-GACAGGACGGA*AAGA, had methyl-acceptor activity. This sequence contains 23S rRNA stem 73 [Stade, K., et al. (1994) *Nucleic Acids Res. 22,* 1394−1399] underlined, flanking a tetraloop-like (UUCG), and the unpaired sequence AAAGA, 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.

The Erm *N*-methyltransferases comprise a group of enzymes that confer resistance to the macrolide−lincosamide−streptogramin type B (MLS) superfamily of antibiotics by means of the post-transcriptional modification of a specific adenine residue of 23S rRNA to form N^6,N^6-dimethyladenine (Lai & Weisblum, 1971). The methylated adenine occurs within the sequence GAAAG (Lai et al., 1973), and its precise location within the 23S rRNA is at *Escherichia coli* coordinate A2058 (Skinner et al., 1983), which, in turn, corresponds to A2085 in *Bacillus subtilis* 23S rRNA (Gutell & Fox, 1988). Details of biochemical and genetic aspects of inducible resistance associated with Erm proteins have been reviewed (Leclercq & Courvalin, 1991; Weisblum, 1995a,b). Previous experiments (Shivakumar & Dubnau, 1981) have shown that partially purified ErmC could methylate phenol-extracted rRNA from *B. subtilis*. Recently, Vester and Douthwaite (1994) and Kovalic et al. (1994) have shown, respectively, that the actinomycete enzymes ErmE and ErmSF are able to methylate correctly *in vitro*-transcribed rRNA corresponding to *B. subtilis* 23S rRNA domain V [ca. 660 nucleotides (nts)]. We next constructed a series of RNA fragments to test for methyl-acceptor activity as part of a study leading to an understanding of how the Erm methylases selectively recognize their 23S rRNA substrate. Here we show the ability of successively smaller domain V constructs containing 337, 113, 53, 46, and 41 nts, respectively, to function *in vitro* as substrates for purified ErmSF, with resultant formation of N^6,N^6-dimethyladenine at the expected location within the substrate. Individual nts or base pairs within the smallest active substrate were modified to test their role in determination of methyl-acceptor activity.

Table 1: Bacterial Strains and Plasmids

bacterial strain or plasmid	description	reference or source
bacterial strains		
E. coli ATCC 77406	strain JM109 haboring pDK101	Kovalic et al., 1994
E. coli BDK105	strain JM109 harboring pDK105	Kovalic et al., 1991
E. coli BDK106	strain JM109 harboring pDK106	this work
E. coli BDK107	strain JM109 harboring pDK107	this work
E. coli JM109	host strain for cloning	Messing, 1983
plasmids		
pDK101	T vector for direct cloning of PCR products purified from ATCC 77406	Kovalic et al., 1991
pDK105	*B. subtilis* 23S domain V rDNA cloned into pGem3Z (Promega)	Kovalic et al., 1994
pDK106	*Pst*I-*Sma*I deletion of pDK105 as shown in Figure 1	this work
pDK104	PDK101 cloned with annealed oligos 5875 and 5878	this work
pDK107	minimalist construct consisting of stems 73, 74, and Δ75 as shown in Figure 2	this work

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids Primers, and Restriction Endonucleases. The strains and plasmids used in this study are listed in Table 1. Oligonucleotide primers are listed in Table 2. Restriction endonucleases were purchased from New England Biolabs (Tozer, MD), except for *Dsa*I, which

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Table 2: DNA Oligonucleotide Primers

oligo number	sequence and description
5524	5′-GGG GTA GCT TGA CTG CGC ACC TGT GTG GAG CCA GGC TGC A 40-mer PCR primer 1 for construction of pDK106
5525	5′-GCC TGG CTC CAC ACA GGT GCG CAG TCA AGC TAC CCC 36-mer PCR primer 2 for construction of pDK106
5875	5′-CCT ATC CGT CGC GGG CAG GCC TA 23-mer PCR primer 1 for construction of pDK107
5878	5′-AGG CCT GCC CGC GAC GGA TAG GA 23-mer PCR primer 2 for construction of pDK107
5879	5′-ATA ACC CGC GAC AGG AC 17-mer PCR primer 3 for construction of pDK107
20351	5′-CGG TCC TCT CGT ACT AAG GAC AGC T 25-mer PCR primer 4 for construction of pDK107, downstream PCR primer for construction of templates for *in vitro* transcription (pDK105 and pDK106)
5882	5′-CCT GTT ATC CCC GGG GT 17-mer downstream PCR primer for construction of templates for *in vitro* transcription (pDK107)
22759	5′-TAA TAC GAC TCA CTA TAG GGT GAA ATT ATA GTA CCT GTG AAG 42-mer containing T7 promoter sequence (17 nts) in tandem with upstream sequences of *B. subtilis* 23S rDNA (25 nts) contained in pDK105 and pDK106
22620	5′-TAA TAC GAC TCA CTA TAG GCC CTA TCC GTC GCG GGC AGG 39-mer containing T7 promoter sequence (17 nts) in tandem with 22 nts upstream sequences of insert of pDK107
1849	5′-GCA CCT GTG TGG AGC CAG GCT 21-mer reverse transcriptase primer used to map position of methylated bases
2919	5′-TAA TAC GAC TCA CTA TAG GAA AGA CCC CGT GGA GC 35-mer upstream PCR primer for construction of DK108 DNA template
2690	5′-TCT TTC CGT CCT GTC GC 17-mer downstream PCR primer for construction of DK109 DNA template
2769	5′-CCG TCC TGT CGC GGG TT 17-mer downstream PCR primer for construction of DK110 DNA template
2878	5′-TAA TAC GAC TCA CTA TAG G 19-mer top strand for all oligonucleotide-based *in vitro* transcription reactions, consists of consensus type III T7 RNA polymerase promoter −17 to +2
2879	5′-TCT TTC CGT CCT GTC GCG GGC GAA CCC GCG ACG GAT AGG CCT ATA GTG AGT CGT ATT A 58-mer bottom strand for oliginucleotide-based *in vitro* transcription reaction of construct DK111
2880	5′-CCG TCC TGT CGC GGG CGA ACC CGC GAC GGA TAG GCC TAT AG GAG TCG TAT TA 53-mer bottom strand for oliginucleotide-based *in vitro* transcription reaction of construct DK112

was obtained from Boehringer-Mannheim (Indianapolis, IN).

Cloning, Overexpression, and Purification of ErmSF. ErmSF was prepared as previously described (Kovalic et al., 1994).

Templates for in Vitro Transcription Reactions. Templates for transcription reactions consisted of DNA with consensus type III T7 RNA polymerase promoter sequence placed directly upstream of the rDNA sequence to be transcribed. Templates were made either by polymerase chain reaction (Mullis & Faloona, 1990) (PCR) or by the annealing of chemically synthesized T7 promoter-containing oligonucleotide primers (Milligan, 1987).

Products DK105−DK110 were generated by PCR. Templates for the PCR reactions were plasmid DNAs described below. T7 promoter sequences were incorporated at the 5′ ends of the upstream PCR primers (primers 22759, 22620, or 2919). Plasmid constructs were verified by DNA sequencing.

(a) pDK105 contains the entire sequence of 23S rRNA domain V and was obtained by the use of primers 20350 and 20351 (Kovalic et al., 1994) (see Figure 1).

(b) pDK106 was constructed by removal of the *Pst*I site in the pDK105 polylinker (cutting the flanking *Sph*I and *Acc*I sites, blunting with mung bean nuclease, and religating) and then insertion of a chemically synthesized linker (annealed primers 5524 and 5525) between the *Pst*I and *Sma*I sites of the resultant plasmid. The predicted extent of *in vitro* transcription is shown in Figure 1.

(c) pDK107 was constructed by first cloning a chemically synthesized linker (annealed primers 5875 and 5878, necessary for transcription of the loop and lower strand of stem 73) into the *Xcm*I-digested T vector pDK101 (Kovalic et al., 1991) and selecting for inserted plasmid with the correct orientation, yielding pDK104. This was followed by cloning a blunt-ended PCR product (mung bean nuclease-treated product generated by using primers 5879 and 20351, and pDK106 as template) into the *Stu*I site of pDK104 and selecting for inserts with the correct orientation and unit size (see Figure 2).

DNA templates for *in vitro* transcription reactions were generated by PCR amplification, with primer pairs 22759 and 20351 (DK105 and DK106 constructs), 22629 and 5882 (DK107 construct), 22620 and 2690 or 2769 (DK109, and DK110 constructs), or 2919 and 5882 (DK108 construct).

To synthesize products DK111−DK130, 1 nmol each of top strand (oligo 2878) and bottom strand (oligo 2879 or 2880 or modified as described in Figure 6 below) oligonucleotides were annealed as described by Milligan et al. (1987). Twenty picomoles per in vitro transcription reaction was used.

In Vitro Transcription of RNA. Purified PCR products, as described above, were either used directly or digested to completion by restriction enzymes, purified, and transcribed *in vitro* with T7 RNA polymerase. Annealed oligonucleotide templates were transcribed directly. The reactions were performed under conditions recommended by the supplier [Epicenter Technologies (Madison, WI) or Promega (Madison, WI)].

In Vitro Methylation of RNA. In vitro transcripts, 10 pmol, were methylated with 5 μl of ErmSF preparation (150 ng of protein/μL) in a 50 μL reaction mixture containing 50 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 40 mM KCl, 10 mM dithiothreitol (DTT), 0.66 μM methyl[^{3}H]-*S*-adenosylmethionine ([^{3}H]SAM) (specific activity of 85 Ci/mM), and 10 u of RNasin (Promega, Madison, WI). Incubation was at 37 °C for 20 min. Following this incubation, (a) the reaction mixture was phenol-extracted and incorporated radioactivity counted by TCA precipitation and filtering, or (b) the reaction

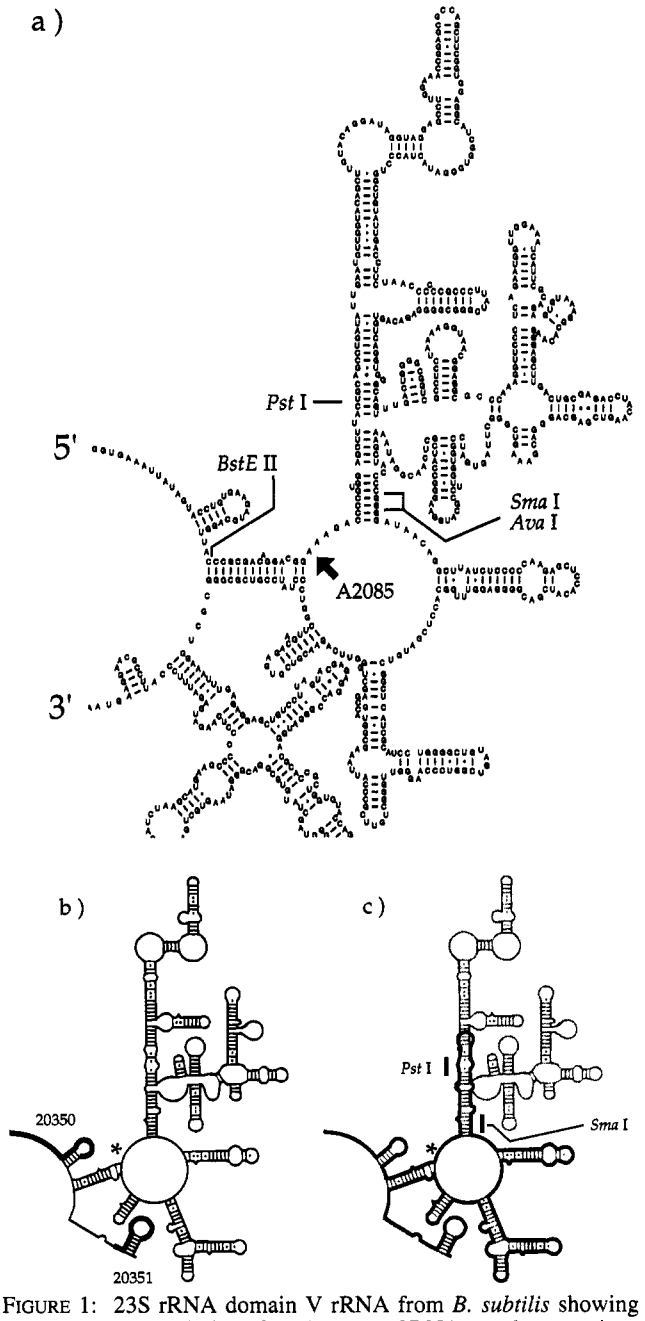

FIGURE 1: 23S rRNA domain V rRNA from *B. subtilis* showing (a) extent of transcription after cleavage of DNA template at unique sites for *Bst*EII, *Pst*I, *Sma*I, and *Ava*I restriction endonucleases, (b) location of primers 20350 and 20351 used to obtain domain V DNA, and (c) extent of the domain V insert present in pDK106, containing 337 nucleotides. See text for details of the construction in which 325 nucleotides between the *Pst*I and *Sma*I sites were deleted from the pDK105 (complete domain V insert).

mixture was supplemented with 500 μM unlabeled SAM and the incubation continued for another 45 min. The latter reaction mixture was extracted twice with phenol, and then chloroform, and the resultant aqueous phase was precipitated with ethanol (twice from ammonium acetate). The products of the reaction were analyzed as described below.

Analysis of Methylated Nts. Bases in domain V (Kovalic et al., 1994) and the minimalist constructs derived from it were analyzed by hydrolysis of the RNA in 1 N HCl at 100 °C for 45 min followed by descending paper chromatography on Whatman 3MM paper, in a solvent system that contained isopropanol:water:ammonia (85/15/1.3), as described previously (Lai & Weisblum, 1971). Using the reaction conditions described above, with 500 μM added SAM, the methylated species cofractionated with N^6,N^6-dimethyladenine; no detectable ^{3}H-labeled methylguanine or other methylated base was observed.

The precise location of the methylated adenine residue within the RNA transcript was determined by primer extension with avian myeloblastosis reverse transcriptase (Promega, Madison, WI) from radioactively end-labeled primer, oligo 1849. The reaction mixture contained equimolar concentrations of dATP, dCTP, dGTP, and dTTP. The resultant product was either a full length product terminated by runoff from the RNA template or a 44-mer terminated by the inability of AMV reverse transcriptase to proceed past N^6,N^6-dimethyladenine at position 2085.

Table 3: Incorporation of [^{3}H]Me from [^{3}H]SAM into Model Test Transcripts[a]

		template (PCR product)	cut with enzyme	ErmSF	cpm
expt 1	complete	DK105	none	+	9839
		DK105	*Ava*I	+	202
		DK105	*Pst*I	+	148
		DK105	*Bst*EII	+	143
		DK106	none	+	13936
		DK106	*Ava*I	+	184
	controls	none	none	−	149
		none	none	+	98
		DK105	none	−	117
		DK105	*Ava*I	−	137
		DK105	*Pst*I	−	141
		DK105	*Bst*EII	−	96
		DK106	none	−	100
		DK106	*Ava*I	−	152
expt 2	complete	DK105	none	+	6513
		DK106	none	+	7691
		DK107	none	+	8513
	controls	none	none	−	52
		none	none	+	55
		DK105	none	−	51
		DK106	none	−	72
		DK107	none	−	69
expt 3	complete	DK107	none	+	6067
		DK107	*Ava*I	+	6903
		DK107	*Sma*I	+	8849
expt 4	complete	DK107	none	+	10124
		DK107	*Nla*IV	+	5944
		DK107	*Pst*I	+	6493
		DK107	*Alu*I	+	4764
		DK107	*Dsa*I	+	4118
	controls	none	none	−	128
		none	none	+	116
		DK107	none	−	247
		DK107	*Nla*IV	−	126
		DK107	*Pst*I	−	110
		DK107	*Alu*I	−	149
		DK107	*Dsa*I	−	151
expt 5	complete	DK107	none	+	12487
		DK108	none	+	122
		DK109	none	+	14239
		DK110	none	+	594
	controls	none	none	−	155
		none	none	+	124
		DK107	none	−	120
		DK108	none	−	163
		DK109	none	−	126
		DK110	none	−	116

[a] Model transcripts were tested for their ability to accept [^{3}H]Me from [^{3}H]SAM. After incubation, samples were phenol-extracted, precipitated, and filtered, and ^{3}H incorperation was determined by liquid scintillation. Multiple transcripts were obtained from the same template construction by cutting the latter with restriction endonucleases, as indicated.

RESULTS AND DISCUSSION

It was previously shown that 23S rRNA domain V alone, ca. 660 nts, contained the necessary information for functioning as substrate for either ErmE methylase (Vester &

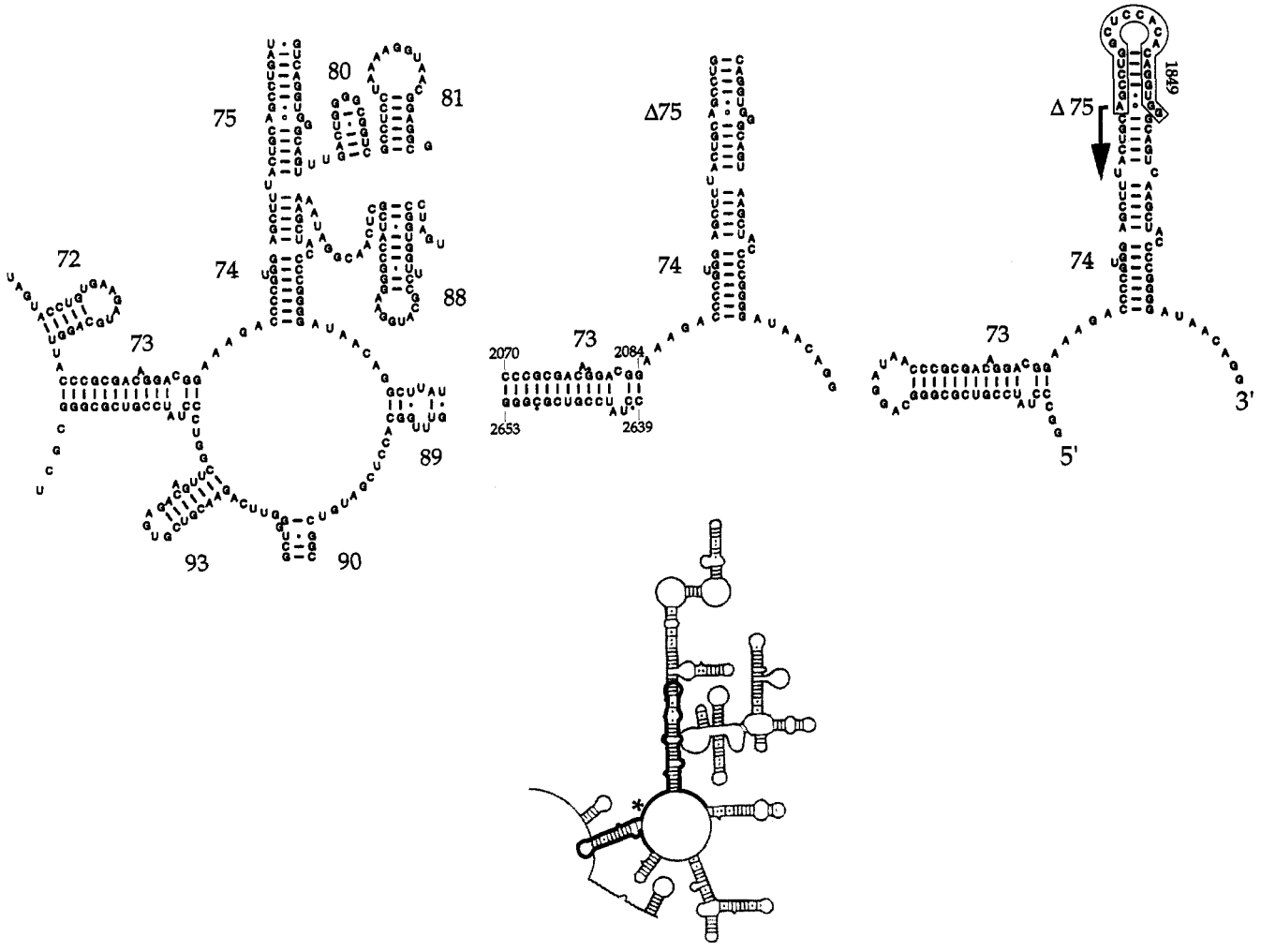

FIGURE 2: Construction of pDK107, showing the peptidyltransferase circle with stem sequences numbered according to Stade et al. (1994). Stem 73 (*B. subtilis* coordinates of this stem are indicated) has been revised by addition of a loop sequence, as shown, which allows synthesis of stem 73 in its entirety before synthesis of stem 74+75. The relation of the resultant 113-nucleotide construction to domain V is also shown. The location of the complementary sequencing primer (1849) is indicated.

Douthwaite, 1994) or the homologous ErmSF methylase (Kovalic et al., 1994). In the present work, we further reduce the size of the minimal 23S rRNA domain V subsequence capable of specific methylation by ErmSF methylase.

Reduction of Domain V from 660 to 337 Nucleotides, pDK106. Domain V, cloned as the insert in pDK105, contains single sites for *Bst*EII, *Pst*I, *Sma*I, and *Ava*I, as shown in Figure 1a. By cutting with either *Bst*EII, *Pst*I, or *Ava*I restriction endonucleases prior to transcription, we obtained truncated transcripts, none of which had activity as methyl acceptor (Table 3, experiment 1).

The intervening ca. 300 nt sequence between *Pst*I and *Sma*I comprising stems 75−88 was removed, and the resultant construction was cloned as the insert in plasmid pDK106. The resultant transcript's predicted structure, shown in Figure 1c, resembles the naturally occurring large subunit domain V found in *Crithidia fasciculata* kinetoplasts or *Caenorhabditis elegans* mitochondria (Gutell et al., 1993). The full length 337 nt transcript prepared from pDK106 DNA was methylated by ErmSF methylase, but the runoff transcripts obtained by cutting the template DNA with *Ava*I were not. The reason for the loss of methyl-acceptor activity following *Ava*I cleavage became clear from the subsequent construction.

Reduction of the 337-Nucleotide Substrate to 113 Nucleotides, pDK107. By inspection of domain V, one would expect that stem 73 is the first started and the last completed. It is completed only after stems 74+75 have been transcribed in their entirety. Therefore, cutting the DK105 and DK106 DNAs at *Pst*I not only shortens the resultant transcript but also might destroy both the 73 and 74+75 stem by leaving only single-stranded members from both systems. Cutting with *Ava*I may retain the double-stranded integrity of only 74+75 but not that of stem 73.

To test the requirement for stem 73 independently of stem 74+75, stem 73 was rerouted, as shown in Figure 2, to allow the sequential transcription of a double-stranded stem 73 in its entirety, followed by AAAGA, and then double-stranded stems 74+75. The loop segment added to stem 73 was designed to retain the secondary structure of stem 73 while making it possible to bypass stems 89−93. The resultant construction was predicted to retain stems 73 and 74 (double-stranded) bracketing AAAGA and was found to have activity as a methyl acceptor, as shown in experiment 2 in Table 3. Moreover, analysis by reverse transcriptase mapping showed that the methyl groups were transferred to the expected adenine, as shown in Figure 3. In contrast to the negative results obtained in testing the pDK105 and pDK106 transcripts, cutting pDK107 with *Pst*I, *Sma*I, or *Ava*I (as well as with *Dsa*I; see Figure 4) gave a product that retained methyl-accepting activity (Table 3, experiment 3). These

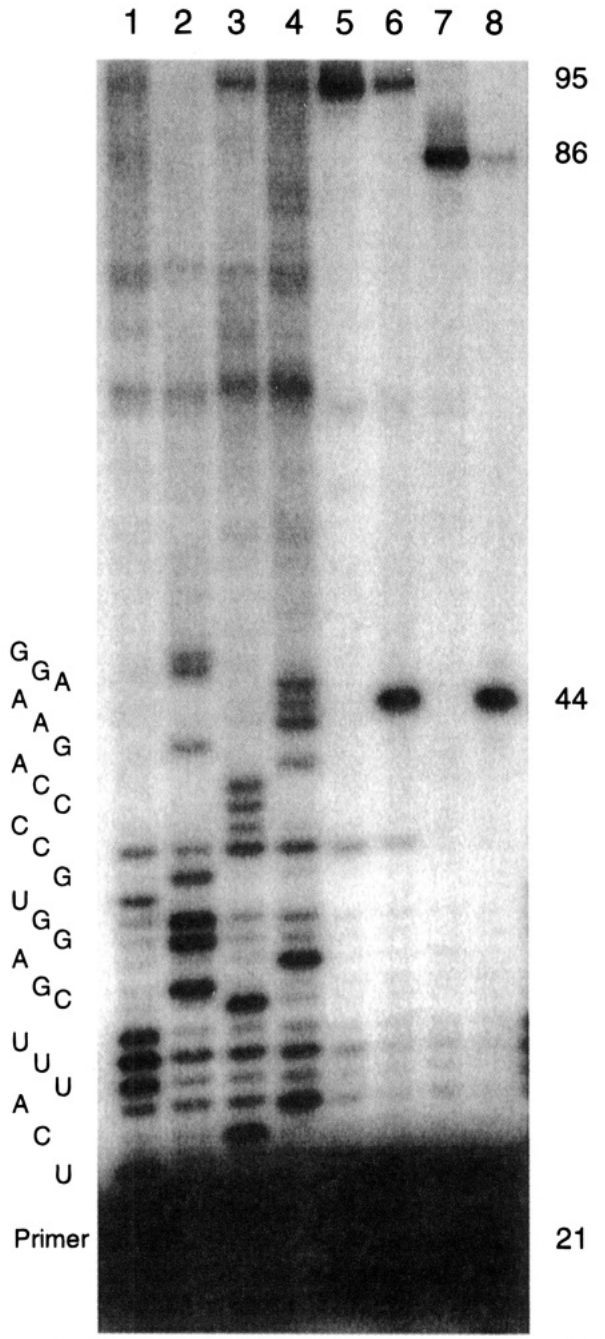

FIGURE 3: Reverse transcriptase analysis of the product obtained by *in vitro* methylation of the 337-nucleotide transcript from pDK106 and the 113-nucleotide transcript from plasmid pDK107. DNA templates derived from pDK106 and pDK107 were transcribed with phage T7 polymerase, and the resultant transcripts were methylated *in vitro* with SAM and ErmSF methyltransferase. The methylated transcript was reverse-transcribed using DNA oligo 1849 as primer and AMV reverse transcriptase. The resultant product was fractionated on an 8% acrylamide sequencing gel along with four sequencing lanes, using the unmethylated pDK106 RNA as template, to locate site(s) of dimethylation: lanes 1−4, sequencing lanes using ddA, ddC, ddG, and ddT, respectively, as terminators; lane 5, unmethylated pDK106 (337 nt) *in vitro* transcript as template; lane 6, methylated pDK106 (337 nt) *in vitro* transcript as template; lane 7, unmethylated pDK107 (113 nt) *in vitro* transcript as template; and lane 8, methylated pDK107 (113 nt) *in vitro* transcript as template. Termination as a 44 nt product represents enzyme stalling at A2085. Full length products shown are assumed to be 95 nt (pDK106) and 86 nt (pDK107) in length.

observations suggested that stem 74+75 was dispensable but that stem 73 was not.

Reduction of the 113-Nucleotide Substrate to Substrates

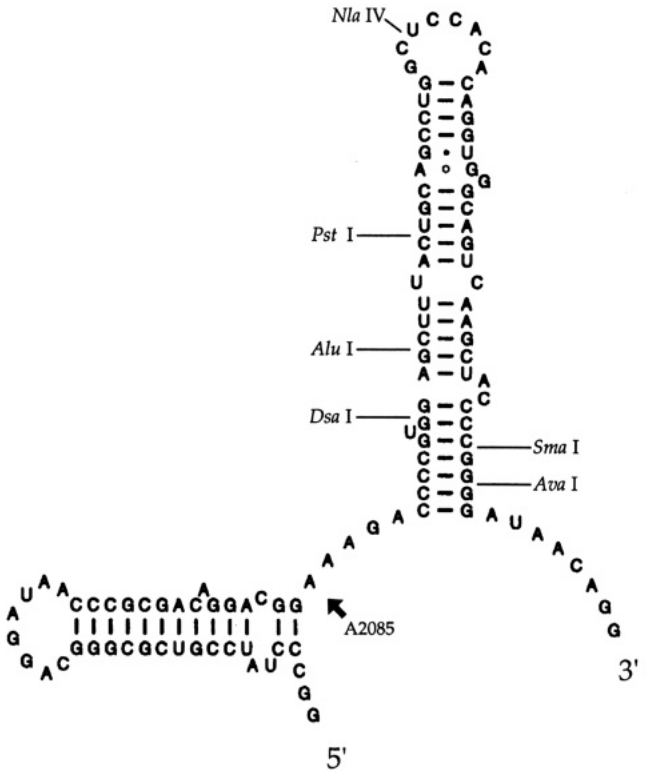

FIGURE 4: pDK107 full length (113-nucleotide) transcript showing single occurrence restriction sites. Template DNA was prepared and digested with either *Bst*UI, *Dsa*I, *Alu*I, *Pst*I, *Nla*I, *Sma*I, or *Ava*I restriction endonuclease, and runoff transcripts were prepared. Methyl acceptor activity of the transcripts is summarized in Table 3 (experiment 4). The 52-nucleotide RNA fragment, obtained as a runoff transcript of pDK107 DNA cut with *Dsa*I, was active, *in vitro*, as methylatable substrate.

53 and 46 Nucleotides in Length. Digestion of pDK107 with *Dsa*I yielded a template whose 52 nt transcript was active as substrate, as did all the enzymes depicted in Figure 4. This suggested that the requirement, if any, that the stem 74+75 sequence have a double-stranded conformation was not valid.

To test this model, we produced transcripts which were subsequences of the pDK107 transcript. Three new transcription templates were constructed by performing PCR reactions using pDK107 as template and primers 22620, 2690, 2769, 2919, and 5882 as indicated below. The predicted transcripts (Genetics Computer Group, 1994; Jaeger et al., 1990) are shown in Figure 5: DK108, 75 nts which comprise stem 74 and Δ75 as represented in the pDK107 construct (primers 2919 and 5882) (Figure 5b); DK109, 46 nts which comprise stem 73 plus five 3′ nts from the peptidyltransferase circle AAAGA (primers 22620 and 2690) (Figure 5c); and DK110, 41 nts which comprise stem 73 only (primers 22620 and 2769) (Figure 5d), as non-methylatable control due to the absence of the sequence AAAGA containing the methylatable adenine.

Results, shown in experiment 5 in Table 3, indicated that no part of stem 74 or 75 was necessary for methylation and that the crucial recognition sequence consists of all or part of stem 73 plus the methylatable adenine as part of an unpaired extension.

Su and Dubnau have studied the interaction between purified ErmC′ (a mutant form of ErmC) and *B. subtilis* 23S rRNA by using the DMS footprinting technique (Su & Dubnau, 1990; Stern et al., 1988). The strongest signals they obtained were approximately 75% reduction of band intensity

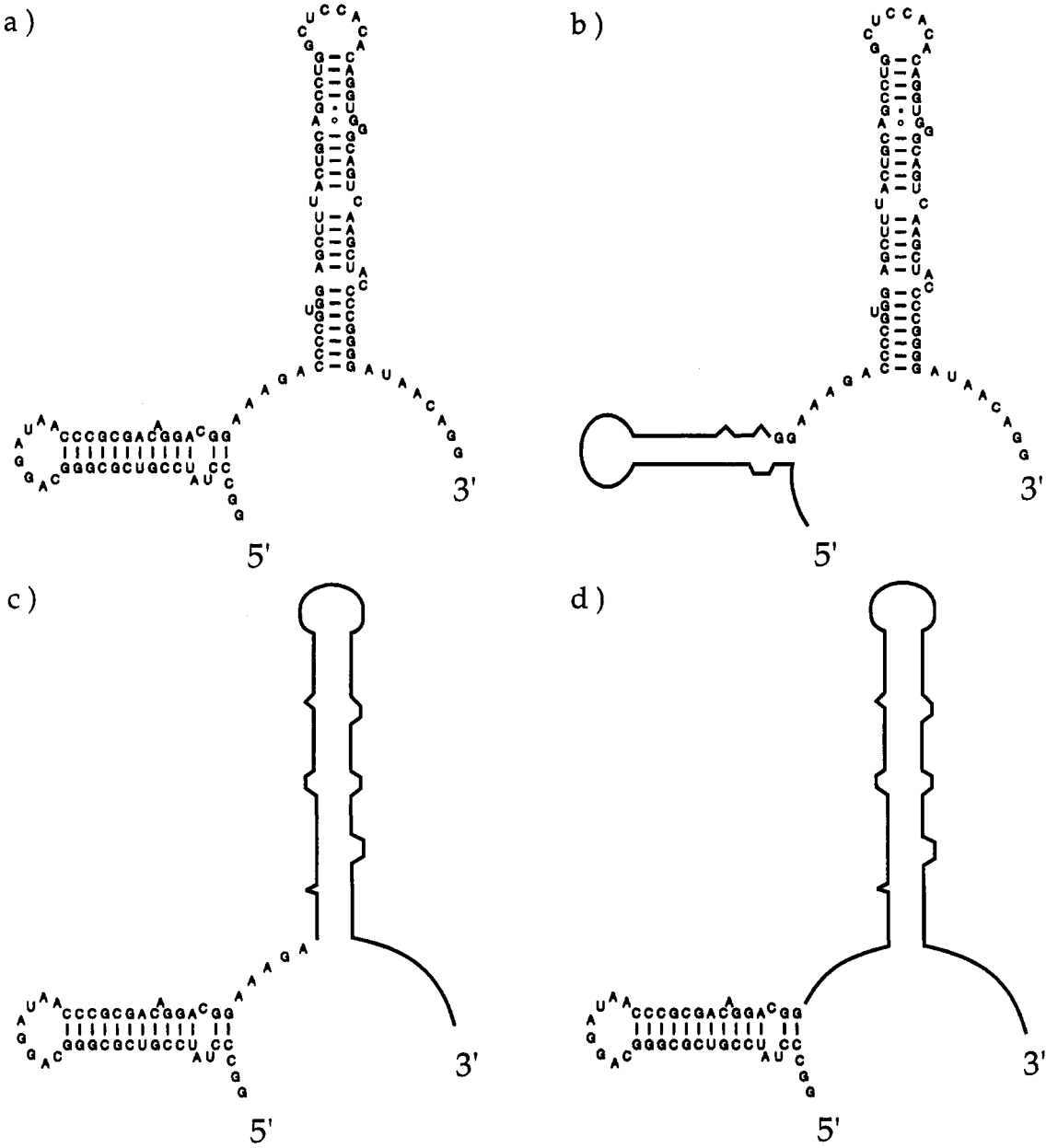

FIGURE 5: Partial sequences of pDK107 that have been tested for methyl acceptor activity. The sequence represented by the nucleotide symbol is included in the respective construct; that shown as black outline is absent. (a) Complete plasmid pDK107 construct, (b) PCR product DK108, (c) PCR product DK109, and (d) PCR product DK110.

due to protection of C2090, G2099, and G2110, all in stem 74 which is immediately adjacent to the peptidyltransferase circle. These three nts are absent from the 46 nt construction described above which is methylated. Our observations suggest that these three bases, despite their protection by purified ErmC′ methylase, are not required by the methylase for substrate recognition or catalytic activity. Their interaction with *ermC* methylase has not been excluded by our observations.

Contribution of Individual Nucleotides. Up to this point, we concentrated on reduction of substrate size. Having found methyl-acceptor activity in a 46 nt model construct, DK109, we next tested the contributions of individual nts in stem 73 and the adjacent peptidyltransferase circle, with results summarized in Figure 6. The parent construct in this series was DK111, 41 nts, ggCCUAUCCGUCGCGGGUUCG-CCCGCGACAGGACGGA*AAGA, in which we replaced the loop sequence CAGGAUAA with the tetraloop sequence UUCG and deleted an unpaired C at the 5′ end. DK111 and its negative control, DK112, ggCCUAUCCGUCGCGGG-UUCGCCCGCGACAGGACGG, i.e., stem 73 alone, were tested along with a set of constructs in which individual bases or base pairs in DK111 were systematically altered. Results summarized in Figure 6 point to unpaired nucelotides in stem 73, i.e., A2078 (constructs DK116 and DK117), C2082 (construct DK122), U2641 (constructs DK120 and DK121), and A2642 (constructs DK123 and DK124), as requirements for substrate activity. Constructs DK113, -114, -115, -125, and -126 in which base pairs were inverted retained activity and even showed enhanced methylation in some cases. Substrates DK118 and -119 showed reduction.

No reduction and even stimulation of methylation were seen in DK127−130, constructs which contained transition mutations in peptidyltransferase circle nts at *E. coli* coordinates A2059, A2060, G2061, and A2062 that have been shown to be protected by MLS antibiotics [summarized in Table 3 in Weisblum (1995b)]. Nucleotide alterations in DK111 that reduced methylation were located exclusively

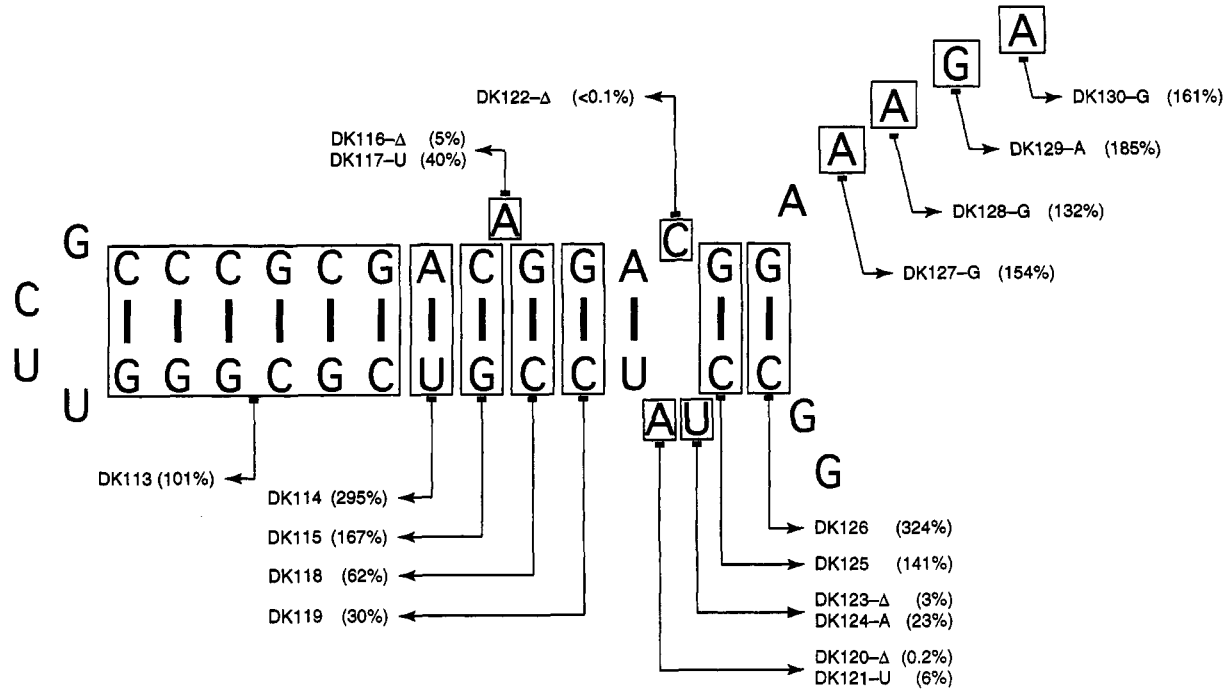

FIGURE 6: Minimalist 41-nucleotide methylase substrate consisting of stem 73 and adjacent peptidyltransferase circle nucleotides present in DK111 are shown. The constructs in which bases or base pairs have been systematically altered are also boxed. The sequence change at each altered position is indicated as either the nucleotide substitution or Δ for deletion. No specification indicates base pair inversion(s). Percent incorporation in the standard *in vitro* methylation assay is indicated for each construct, in parentheses; 100% corresponds to 4580 cpm incorporated using DK111, corrected for 176 pm incorporated with DK112, as blank: DK113, inversion of six base pairs corresponding to coordinates 2648–2653; DK114, inversion of UA base pair coordinate U2647; DK115, inversion of base pair; DK116, deletion of A2078; DK117, transversion of A2078U; DK118, DK119 inversions of base pairs; DK120, deletion of A2642; DK121, transversion of A2642U; DK122, deletion of C2082; DK123, deletion of U2641; DK124, transversion of U2641A; DK125, DK126 inversion of base pairs; DK127, DK128, DK129, DK130, transition mutations A2086G, A2087G, G2088A, and A2089G, respectively.

in stem 73. This may explain why Shivakumar and Dubnau (1981) observed that neither erythromycin nor tylosin was able to inhibit methylation *in vitro* by purified methylase. Both Erm methylases and macrolide antibiotics interact with and therefore must bind to 23S rRNA within the region of peptidyltransferase circle. Our findings suggest that the specificities with which these two classes interact with the peptidyltransferase overlap minimally.

ACKNOWLEDGMENT

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