Resistance to the macrolide-lincosamide-streptogramin B group of antibiotics in bacteria is mediated through the methylation of 23S rRNA at a specific adenine residue (A-2085 in \textit{Bacillus subtilis}; A-2058 in \textit{Escherichia coli}). The gene for ErmC' was cloned and expressed to a high level in \textit{E. coli}, and the protein was purified to virtual homogeneity. Studies of substrate requirements of ErmC' have shown that a 262-nucleotide RNA fragment within domain V of \textit{B. subtilis} 23S rRNA can be utilized efficiently as a substrate for methylation at A-2085. Kinetic studies of the monomethylation reaction showed that the apparent $K_m$ of this 262-nucleotide RNA oligonucleotide was 26-fold greater than the value determined for full-size and domain V 23S rRNA. In addition, the $V_{max}$ for this fragment also rose sevenfold. A model of RNA-ErmC' interaction involving multiple binding sites is proposed from the data generated.

**Materials and Methods**

**Bacterial strains, plasmids, and growth conditions.** \textit{E. coli} DH5$\alpha$ (F$^-$/endA1 hsdR17 supE44 thi-1 mcrA rpsL150 gyrA96 relA1 (8089dutZAM15) ) was obtained from the supplier with the following modification: an additional half amount of RNA was used.\textit{B. subtilis} 168 (19) was obtained from D. Dubnau. Plasmid pJO200 is a derivative of pTB210 (1) that contains multiple cloning sites.

**DNA manipulations.** Procedures for plasmid isolation (minipreparations), restriction digestion, ligation, agarose gel electrophoresis, recovery of DNA from agarose gels, and DNA hybridization were performed as described previously. All DNA sequencing employed double-stranded templates with Sequenase 2 (U.S. Biochemicals). All restriction digestion, ligation, agarose gel electrophoresis, recovery of DNA from agarose gels, and DNA hybridization were performed as described previously.

**In vitro RNA preparations.** The 23S rRNA gene was purified from RNA isolated from \textit{B. subtilis} BD170 chromosomal preparation and cloned into \textit{E. coli} DH5$\alpha$ (F$^-$/endA1 hsdR17 supE44 thi-1 mcrA rpsL150 gyrA96 relA1 (8089dutZAM15)). The 23S rRNA gene was transcribed in vitro and purified as described previously.

**In vitro methylation reactions.** Reaction mixtures contained 5 pmol of [3H]SAM (84 Ci/mmol; Amersham, Arlington, TX) with 20 pmol of template DNA. Pyridine was added to the reaction mixture to achieve a final concentration of 10 mM. The reaction mixture was incubated at 37°C for 1 h, and then the reaction was terminated by the addition of 20 μl of formamide. The reaction mixture was heated at 95°C for 5 min, and then the samples were loaded onto a 5% denaturing polyacrylamide gel. The gel was dried and exposed to X-ray film.
ton Heights, Ill.), 50 mM Tris-HCl (pH 7.5), 40 mM KCl, 4 mM MgCl₂, 10 mM dithiothreitol, 50 μM of bovine serum albumin, 10 U of RNase inhibitor (5 Prime to 3 Prime, Inc., Boulder, Colo.) per ml, and 0.25 mM dithiothreitol, 50 μM -D-thiogalactopyranoside (IPTG) when an 860 of 0.8 to 1.0 was reached, and growth was continued for an additional 14 to 15 h. The cells were harvested, and the pH was adjusted to 7.0. Methylation synthesis was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when an A₅₉₀ of 0.8 to 1.0 was reached, and growth was continued for an additional 14 to 15 h. The cells were harvested, and ErmC was purified essentially as described previously (19), with two modifications. The phosphocellulose chromatographic step was replaced by S-Sepharose with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.6, as the column buffer, and the DEAE-Sephadex step was omitted. The final preparation was essentially pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The modified purification method described here was simpler and faster compared with the previously reported one and yielded material of equal or greater purity.

RESULTS

Cloning and expression of ermC in E. coli. PCR primers were designed with SalI and EcoRI restriction sites for subcloning the ermC sequence from pLM13 into plasmid pJO200. To provide translational coupling of ermC to kdsB, the sense PCR primer contained a ribosome binding site for the ermC cistron and a stop codon for the upstream kdsB gene 5' to the start codon for ermC (Table 1). The upstream cistron encodes the first 14 amino acids of the kdsB gene followed by a 3-ami-no-acid linker. The dicistronic vector carrying ermC was designated pERM-1. The integrity of ermC was confirmed by nucleotide sequencing. Expression of the gene was examined in E. coli XL-1 Blue. As can be seen in Fig. 2, growth at 37°C yielded high-level expression but most of the ErmC produced was found as an insoluble aggregate in the cellular pellet (lanes 5 and 6). Proportionately more soluble material was found when the cells were grown at 30°C, and virtually all of the ErmC produced at 25°C was soluble, although the total amount of ErmC produced was somewhat reduced. Purification of ErmC (see Materials and Methods) from a 10-liter culture grown at 25°C in a fermentor resulted in the recovery of >200 mg of highly purified, soluble enzyme.

Kinetics of ErmC methylation of in vitro-produced 23S rRNA. Although B. subtilis 23S rRNA has been shown to be the optimum substrate for ErmC and ErmC (3), a commercially available, unpurified preparation of rRNA extracted from E. coli has generally been used as a substrate for ErmC and many other Erm methylases. We have found that the RNA produced from the in vitro transcription of the cloned B. subtilis 23S rRNA gene could serve as a methylation substrate for ErmC at least as well as the commercial E. coli rRNA preparation (data not shown). A very low concentration of SAM (0.08 pmol/50 μl) relative to that of RNA (5 pmol/50 μl) was employed so that most of the reacted RNA was found in the monomethylated form (Fig. 3). In all experiments reported here, therefore, the ErmC concentration was chosen from a lower point within the linear range to give a high degree of reproducibility of the reaction rate. Under our assay conditions, the reaction was linear for up to 1 h (data not shown). The relative kinetics of the various RNA substrates used in the reactions shown here did not change when the SAM concentration was raised to allow dimethylation to occur. In methylation studies, in vitro-produced RNAs treated at 75°C for 10 min in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer and then slowly cooled to room temperature showed as substrates no difference from those without the hybridization treatment, indicating that the proper secondary and tertiary structures had been formed during the in vitro RNA synthesis reactions.

Substrate specificity. The DV segment of 23S rRNA (nt 2065 to 2687) was predicted to include a central loop and five regions, A to E, containing secondary structure (Fig. 1). This 623-nt segment was produced by in vitro transcription with T7 RNA polymerase by employing a DV DNA fragment generated by PCR that used a 5' primer containing the T7 promoter. When this RNA was used in kinetic experiments with ErmC, it was found that the apparent Kₘ and Vₘₐₓ matched the values determined for the full-length substrate (Table 2 and Fig. 4A). In addition, the site of methylation on DV RNA was shown by TLC to have adenine as the only base to have been methylated (Fig. 3). The specificity of methylation on A-2085 was confirmed by site-directed mutagenesis. All three mutated DV fragments, A-2085 to T (A2085T), A2085G, and A2085C, were methylated to less than 10% of the level observed with the correct DV RNA fragment (Table 3). In addition, the fragment designated DV (A'), designed to have the central loop structure of DV broken (Fig. 1), was also found to have very low substrate activity toward ErmC (Table 3).

To test whether the primary sequence of DV is essential for enzyme recognition, mutations at the nucleotides neighboring A-2085 were generated and tested for their activities as sub-
strates of ErmC’. The G2084A change reduced the methyl-
ation of the resultant DV fragment to ca. 12% of the level of the wild-type DV fragment under the same assay conditions (Table 3). In a preliminary test, DV RNA carrying the A2086T change was methylated to ca. 50% of the level of wild-type DV RNA (Table 3). Kinetic studies with this substrate indicated very little change in the \( V_{\text{max}} \) but an about fivefold increase in the \( K_m \) (Table 2; Fig. 4A). Thus, a change in the sequence surrounding A-2085 can affect the binding of the RNA to the enzyme but may not change the overall rate of methylation.

A small fragment of DV was constructed by combining sequences from nt 2065 to 2097 and nt 2464 to 2687 of the 23S rRNA gene. RNA transcribed from this fragment was expected to form a central loop-like structure on the basis of the computer model shown in Fig. 1 but would be missing the 466-nt segment (nt 2098 to 2463) that was predicted to contain a great amount of secondary structure of segment B. The apparent \( K_m \) of this DV (B\(^-\)) 262-nt RNA oligonucleotide was 26-fold greater than the value determined for DV RNA. In addition, the \( V_{\text{max}} \) for this fragment also increased sevenfold (Fig. 4B; Table 2).

We have also tested a number of RNA oligonucleotides ranging in size from 10 to 50 nt. The oligonucleotides were designed as either a simple single strand with A-2085 in the center or with the segment A-2085AAAGA either on the end of segment A or preceding segment B. None of the oligonucleotides made displayed detectable substrate activity. Thus, we have not yet determined the minimal size of RNA required for methylation by ErmC’.

**DISCUSSION**

Translational coupling in a dicistronic vector that employed \( kdsB \) as the upstream gene was used previously for the high-level expression of human 12-kDa FK506 binding protein in \( E. coli \) (15). We have shown here that this expression system is also useful for obtaining high levels of ErmC’. The finding that the production of soluble, enzymatically active protein required cultivation of the cells at 25°C was unexpected, however. Previous work has shown that active ErmE methylase could be obtained from \( E. coli \) grown at 37°C or higher (7, 22) but that the enzyme is found associated with ribosomes. We
have not examined whether all of the active ErmC recovered from E. coli grown at either 25 or 37°C was also ribosome associated. In addition, we have not attempted to resolubilize the insoluble ErmC aggregates produced at the high temperatures.

We have shown here that in vitro-generated RNA containing the sequence of the 23S rRNA from B. subtilis can be used as a substrate for methylation by the ErmC methylase and that methylation takes place very likely at A-2085 (according to our site-directed mutagenesis data), the naturally occurring site of methylation in vivo. We have also shown that in vitro-generated RNA corresponding to DV of B. subtilis 23S rRNA can be methylated at A-2085 by ErmC equally as well as the full-length rRNA sequence. This finding is in agreement with the recent results of Kovalic et al. (8) and Vester and Douthwaite (22), who employed ErmSF and ErmE, respectively, to methylate A-2085 of in vitro-produced DV from B. subtilis 23S rRNA and A-2058 from E. coli 23S rRNA. Both the K_m and V_max of the methylation reactions do not change when the DV fragment is used in place of the full-length RNA, suggesting that the local structure of the RNA that binds to ErmC and undergoes methylation is the same in the two different-size substrates. It should be noted that the RNA substrates used in our kinetic measurements were produced by in vitro transcription and thus were totally free of posttranscription modification, whereas the RNA used by Denoya and Dubnau was mature 23S rRNA isolated from B. subtilis (4). The differences in RNA sources may explain the discrepancy of K_m values between the two reports. In addition, the measurement of apparent K_m of RNA reported here was achieved under extremely low concentrations of SAM, well below the reported K_m. Since only monomethylation was observed in our assays, the kinetic data observed here can reflect a simple one-step reaction instead of a mixture of two consecutive reactions as discussed by Denoya and Dubnau (4). Under these extreme conditions, the apparent K_m could differ from true K_m values.

The proposed structure of DV has the methylation site A-2085 in a single-stranded region that is bordered immediately upstream and 5 nt downstream by regions containing secondary structure, segments A and B, respectively (Fig. 1). Chemical footprinting analysis has shown that ErmC methylase covers an extensive region on 23S rRNA surrounding A-2085 including regions in segment A and B (21). Kinetic experiments presented here are consistent with the notion that both segments are involved in the binding of the substrate to the enzyme. The near total loss of substrate activity in both the DV fragment missing the 3′ region of segment A (A−) and in the mutated substrate carrying the G2084A change suggests that both G-2084 and the region of segment A containing the G-2084–C-2639 base pair make important contacts with the enzyme to position A-2085 correctly at the active site for efficient methylation. Because of the proximity of segment A to the methylation site, the removal of the segment or a change in its structure would be expected to reduce the overall rate of methylation. The large increase in the K_m of methylation for the DV (B−) RNA fragment, which is missing most of segment B, implicates this segment, as well, in the binding of the substrate to the enzyme, but the sevenfold increase in the V_max of the DV (B−) RNA fragment indicates higher turnover of the substrate. Thus, although the presence of segment B in the RNA enhances its binding to ErmC, it is apparent that the binding of segment B has an unfavorable effect on the rate at which the enzyme produces methylated RNA. Because of its potentially large size—up to almost 400 nt—and its 5-nt dis-

![FIG. 2. Expression of ermC at various temperatures: SDS–10% PAGE of E. coli XL-1 Blue expressing ermC from pERM-1. Cells were grown in shaker flasks in Superbroth medium containing 50 μg of ampicillin per ml and 15 μg of tetracycline per ml. The cultures were induced with 1 mM IPTG upon reaching an optical density at 600 nm of 2.0 and grown for a further 18 h before being harvested. The cells were then resuspended in 50 mM Tris (pH 8.0)–50 mM NaCl–1 mM EDTA and lysed by sonication. All lanes contain the supernatant or pellet from centrifugation at 10,000 × g for 1 min. Lanes 1 and 2, supernatant and pellet, respectively, from 25°C expression; lanes 3 and 4, supernatant and pellet, respectively, from 30°C expression; lanes 5 and 6, supernatant and pellet, respectively, from 37°C expression; lanes 7 and 8, supernatant and pellet, respectively, of XL-1 Blue (no plasmid) grown at either 25 or 37°C was also ribosome associated.](http://jb.asm.org/)

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![FIG. 3. TLC of hydrolysates from in vitro-methylated B. subtilis 23S rRNA (●) and DV (B−) (□). Methylated RNA labeled with 3H was hydrolyzed with 1 N HCl and the resulting hydrolysates were separated by cellulose TLC (see Materials and Methods). DV gave the same TLC pattern.](http://jb.asm.org/)

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action. (A) Plots of the reciprocal of initial velocity versus the reciprocal of highernet turnover of methylated RNA. Rapid release from the enzyme after methylation results in lacking segment B may bind less tightly to the enzyme, its more enzyme after methylation occurs. Thus, though the substrate bisadecreased rate at which the RNA is released from the tation of A-2085 in the activesite or on the chemistry of the site and that such binding has little effect either on the orien-
tance from the site of methylation, we propose that segment B binds at one or more sites on the enzyme away from the active site and that such binding has little effect either on the orientation of A-2085 in the active site or on the chemistry of the methylation event. Rather, the effect of the binding of segment B is a decreased rate at which the RNA is released from the enzyme after methylation occurs. Thus, though the substrate lacking segment B may bind less tightly to the enzyme, its more rapid release from the enzyme after methylation results in higher net turnover of methylated RNA.

The possibility that the increase in $V_{\text{max}}$ through the loss of segment B of DV is the result of a change at the reaction center cannot be ruled out. For example, the binding of segment B may cause an allosteric effect on the enzyme resulting in either destabilization of the enzyme-substrate reaction center (although the overall enzyme-substrate complex is stabilized) or a change in the chemistry of the reaction, possibly through changes in enzyme-substrate linkages at the reaction center. Though possible, it is difficult to imagine that the enzyme, which has been selected to confer upon the host the ability to survive, would also be selected to be underutilized by its natural substrate. It is more likely that the substrate, the mass of which exceeds that of the enzyme about 30-fold, binds the enzyme independently at several sites and that the rate of breakage of one of these ErmC-RNA interactions is the rate-determining step of the reaction.

TABLE 3. Methylation of various rRNA fragments by ErmC methylase in vitro

<table>
<thead>
<tr>
<th>RNA fragment</th>
<th>RNA size (nt)</th>
<th>% Substrate activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-size 23S rRNA</td>
<td>2,927</td>
<td>100</td>
</tr>
<tr>
<td>DV (A&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>554</td>
<td>&lt;10</td>
</tr>
<tr>
<td>DV A2085T</td>
<td>623</td>
<td>&lt;10</td>
</tr>
<tr>
<td>DV A2085C</td>
<td>623</td>
<td>&lt;10</td>
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<tr>
<td>DV A2085G</td>
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</tr>
<tr>
<td>DV A2086T</td>
<td>623</td>
<td>53</td>
</tr>
<tr>
<td>DV G2084A</td>
<td>623</td>
<td>12</td>
</tr>
</tbody>
</table>

* Determined by measuring $^3$H incorporation under standard assay conditions (see Materials and Methods) and comparing counts per minute with the value from full-size 23S RNA. The absolute numerical value that corresponds to 100% is 4,934 ± 432 cpm.

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REFERENCES


