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Translational Autoregulation of *ermC* 23S rRNA Methyltransferase Expression in *Bacillus subtilis*

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***ermC* specifies an rRNA methyltransferase that confers resistance to erythromycin. The expression of this determinant is induced by the addition of erythromycin. The induction mechanism has been shown to operate posttranscriptionally, and its mechanism has been elucidated. We now show that synthesis of the *ermC* gene product in *Bacillus subtilis* is also autoregulated by a mechanism operating on the level of translation. The synthesis of methyltransferase was shown to be gene dosage compensated by Western blot analysis. Several mutants were analyzed that specify altered *ermC* gene products and are deregulated. Analysis of mutants and of the wild-type strain by Northern blotting demonstrated that autoregulation is posttranscriptional. We suggest a translational repression model in which the *ermC* methyltransferase binds to its own mRNA, at a region that resembles the methylation target site on 23S rRNA. The overall control of *ermC* expression is discussed in light of these multiple regulatory mechanisms.**

ermC confers resistance to erythromycin in gram-positive bacteria by specifying a 23S rRNA methylase that N^6,N^6-dimethylates an adenine residue at position 2058 on 23S rRNA, thereby decreasing ribosomal affinity for erythromycin (31, 36). *ermC* expression is induced by erythromycin, and erythromycin binding to the ribosome is required for induction to occur. The induction mechanism, termed translational attenuation, has been intensively studied (reviewed in references 5 and 35).

The *ermC* mRNA exists in an inactive conformation in which the ribosome-binding site (Shine Dalgarno sequence 2 [SD2]) for methylase synthesis is sequestered by base pairing. Stalling of a ribosome under the influence of erythromycin binding during translation of a 19-amino acid leader peptide causes the inactive mRNA structure to open, permitting initiation of methylase synthesis. Translational induction may be further regulated by a methylation-mediated feedback loop. Upon induction the intracellular level of methylase increases, as do the number of methylated ribosomes, with a concomitant decrease of the ribosomal affinity for erythromycin and prevention of further induction. Several lines of evidence support this model. *ole-2* is a mutation of the *Bacillus subtilis* ribosomal protein L17 that decreases erythromycin binding to ribosomes. In an *ole-2* background, methylase could not be induced (13, 29). Similar results were obtained when preinduced minicells were used to analyze methylase expression, suggesting that perturbation of erythromycin binding to the ribosome, either by mutation of the L17 protein or by prior methylation, prevents further induction.

Similarly, Gryczan et al. (13) have shown the absence of induction of β-galactosidase activity from an *ermC-lacZ* translational fusion which coexists in the same host with a constitutively expressed wild-type *ermC* determinant. Kirsch and Lai (20) have published similar results. This is to be expected, since the ribosomes in such strains are fully methylated.

Consistent with the existence of this methylation-mediated feedback loop is the observation that most of the erythromycin-sensitive *ermC* mutations analyzed so far cause the overproduction of structurally altered methylase in vivo. However, as we have pointed out (11, 29), this hyperinducibility is also consistent with another interpretation: feedback may be exerted by a direct interaction of the methylase protein with its own mRNA or with DNA. This possibility was also raised by Horinouchi and Weisblum (16), who pointed out that the *ermC* mRNA contains an AAAG sequence. The latter sequence is also found at the target for methylation of 23S rRNA by macrolides-lincosamides-streptogramin B methylase. Autoregulation through direct protein-nucleic acid interaction has been described for a number of systems (3, 8, 19, 33). In some cases, such as ribosomal proteins acting as translational repressors, the usual function of these proteins involves nucleic acid binding. In this sense the 29-kilodalton (kDa) product of *ermC* gene presents some particularly interesting similarities to the ribosomal proteins. The methylase is a basic protein with an intracellular function involving a direct interaction with rRNA (27, 30) and may be associated with ribosomes intracellularly (27; C. Denoya and D. Dubnau, unpublished data). With these considerations in mind, we decided to explore further the regulatory mechanism involved in *ermC* gene expression.

This paper presents in vivo data that support the existence of a direct autoregulatory mechanism operating at the posttranscriptional level and independently of the *ermC* methylase-mediated methylation of ribosomes. The structural similarities between the site of action of the methylase on the 23S rRNA and the *ermC* mRNA region around the methylase translation initiation site suggest that this autoregulation operates through a direct methylase-mRNA interaction.

MATERIALS AND METHODS

Strains and plasmids. All the *B. subtilis* strains used are derivatives of strain 168. They are described in Table 1. *Escherichia coli* JM109 was used as host for cloning in bacteriophage M13 (22). Plasmid pE194, which carries *ermC*, was originally isolated from *Staphylococcus aureus* by Iordanescu (18). Two high-copy-number pE194 deriva-

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TABLE 1. *B. subtilis* strains and plasmids

Strains and plasmids	Characters	Source
Strains		
IS75	*his leu met*	
YB886	*trp met*	37
Cu403	*thyA thyB metB divIVB1*	25
BD170	*trpC2 thr-5*	
Plasmids		
pE194	Em^r	18
pBD142	Em^r Cm^r	
pIM13	Em^r	23

tives carrying the *cop-6* and *cop-1* mutations were used in some experiments (12; R. Villafane, Ph.D. thesis, New York University School of Medicine, New York, 1985). Plasmid pBD142 consists of pE194 *cop-6* plus the chloramphenicol resistance determinant of pC194 (18) on a 1,668-base-pair *ClaI* fragment. Plasmids pE194 or pBD142 carrying the wild-type or a point mutant *ermC* determinant (see Fig. 1) were integrated on the *B. subtilis* chromosome by homologous recombination with a chromosomal pE194 element (15). *ermC* deletion mutants were generated by exonuclease digestion at a unique *HpaI* site (29) and by BAL 31 digestion at the *BclI* site (R. Villafane, unpublished data).

DNA preparation and sequencing. Isolation of plasmid DNA, transformation, and preparation of *B. subtilis* competent cells were done as described previously (10). Restriction fragments containing the deleted or mutated regions were cloned into M13mp10 and M13mp11 (22), and the resulting DNA preparations were used for sequencing by the dideoxynucleotide method of Sanger et al. (26) as described in the Amersham Corp. protocol. Thin (0.35-mm) 6% polyacrylamide-urea gels were used.

Induction of methylase protein. Cultures were grown in VY medium (7) at 32°C overnight with shaking. A 0.2-ml sample of this seed culture was inoculated into the same medium (10 ml) in a 125-ml Erlenmeyer flask and incubated at 32°C with vigorous shaking to a density of 40 to 50 Klett units. Erythromycin (0.02 μg/ml) was added if required, and incubation continued.

[^{35}S]methionine labeling was performed under similar conditions, except that minimal medium (1) was used, supplemented with 5% VY medium, 0.5% glucose, trace elements, 0.001% yeast extract, 0.005% casein hydrolysate, 0.02 mg of each required metabolite (except methionine) per ml, and 0.002 mg of methionine per ml. Induction was with erythromycin (0.05 μg/ml). At the indicated times, 1-ml samples were removed and transferred to 25-mm tubes, and cells were labeled with 20 μCi of [^{35}S]methionine (1,250 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) per ml by further incubation for 30 min at 32°C with shaking. Growth was measured by following viable counts or Klett units or by protein determination.

Assay of RNA methylase activity. RNA methylation was performed *in vitro* with purified *ermC* methylase essentially as described previously (27), with the following modifications. A 2-pmol portion of purified 23S rRNA was used per assay, and incubation was for 90 min at 37°C. The methylase used was provided by Sai L. Su.

Minicell procedures. Minicells were purified by the procedure of Reeve et al. (25), as modified by Shivakumar et al. (28). Purified minicells were stored at −70°C for up to 6 months without losing amino acid-incorporating activity. Minicells were thawed at 37°C and pelleted by centrifugation for 3 min at 4°C, and the minicell pellet was suspended in growth medium GM-1 (1) containing 20 μg each of valine and isoleucine (0.1 ml) per ml to a density of 5×10^9 minicells per ml (28). Minicells were labeled by the addition of 150 μCi of [^{3}H]leucine (58 Ci/mmol; ICN Pharmaceuticals, Inc., Irvine, Calif.) per ml and incubation at 32°C with shaking during variable periods of time in the absence or presence of erythromycin (0.02 μg/ml).

Sample preparation and protein gel electrophoresis. Minicell or whole-cell suspensions were brought to 1.5 ml with lysis medium (0.1 M EDTA, 0.1 mg of L-leucine per ml, 0.05 M NaCl, 1 mM phenylmethylsulfonyl fluoride, pH 7.0) and placed on ice, and the chilled samples were centrifuged. The pelleted cells were suspended in 50 μl of lysozyme solution (1.7 mg/ml) containing RNase A (0.2 mg/ml), DNase I (0.02 mg/ml), and phenylmethylsulfonyl fluoride (1 mM) in 30 mM Tris hydrochloride (pH 7.5)–50 mM NaCl–5 mM EDTA, and samples were incubated at 37°C for 20 min. A 25-μl portion of 3× cracking buffer (3% sodium dodecyl sulfate [SDS], 3% β-mercaptoethanol, 0.15 M hydrochloride Tris [pH 6.8], 0.006 M EDTA, 0.05% bromophenol blue, 30% glycerol) was then added. The samples were placed in a boiling-water bath for 3 min and were loaded onto 15% polyacrylamide-SDS gels as described previously (21). After electrophoresis, gels were either processed for fluorography or stained with Coomassie brilliant blue. Fluorograms or stained gels were traced by densitometry with a Shimadzu dual wavelength chromatogram scanner, model CS-910. Alternatively, proteins were transferred to nitrocellulose (see below).

Western blotting. Proteins were electrophoretically transferred to nitrocellulose (BA 85; Schleicher & Schuell, Inc., Keene, N.H.) by the method of Towbin et al. (32), using an electroblot apparatus (Transphor TE50; Hoeffer Scientific Instruments) with the transfer buffer described in the Hoeffer catalog and with a transfer time of 1 h at 0.9 A. After transfer, the method for detection of alkaline phosphatase-conjugated antirabbit antibody described by Blake et al. (2) was followed. Primary antibodies were raised in a rabbit by using highly purified *ermC* methylase (27) as the immunogen (with the assistance of J. Oppenheim). The quantitation of the reaction was performed by scanning the reflectance of the blots in a Shimadzu dual wavelength chromatogram scanner, model CS-910, at 520 nm. The areas under the peaks were determined and related to the amount of pure methylase loaded onto parallel lanes as standards. A linear relationship was observed in the range of 0 to 75 ng. The protein concentration of the methylase standard was determined by the microassay procedure with the Bio-Rad protein reagent.

RNA isolation. To isolate RNA for blotting, cells were grown as described for protein isolation. At 2 h postinduction 1-ml samples were removed, and nucleic acids were extracted as described by Ulmanen et al. (34). After ethanol precipitation, nucleic acids were treated with RQ1 DNase as recommended by the supplier (Promega Biotech, Madison, Wis). The typical yield from 1 ml of cells was 30 μg of RNA. Total RNA was extracted from *B. subtilis* BD170 ribosomes with phenol-chloroform. 23S rRNA was fractionated by ultracentrifugation through a sucrose gradient.

Analysis of RNA by slot-blot hybridization. The slot blotting technique was performed as described by the manufacturer of the blotting apparatus (Schleicher & Schuell). About 500 ng of RNA was loaded per slot for hybridization with the *ermC* probe. As a control for RNA concentration, parallel

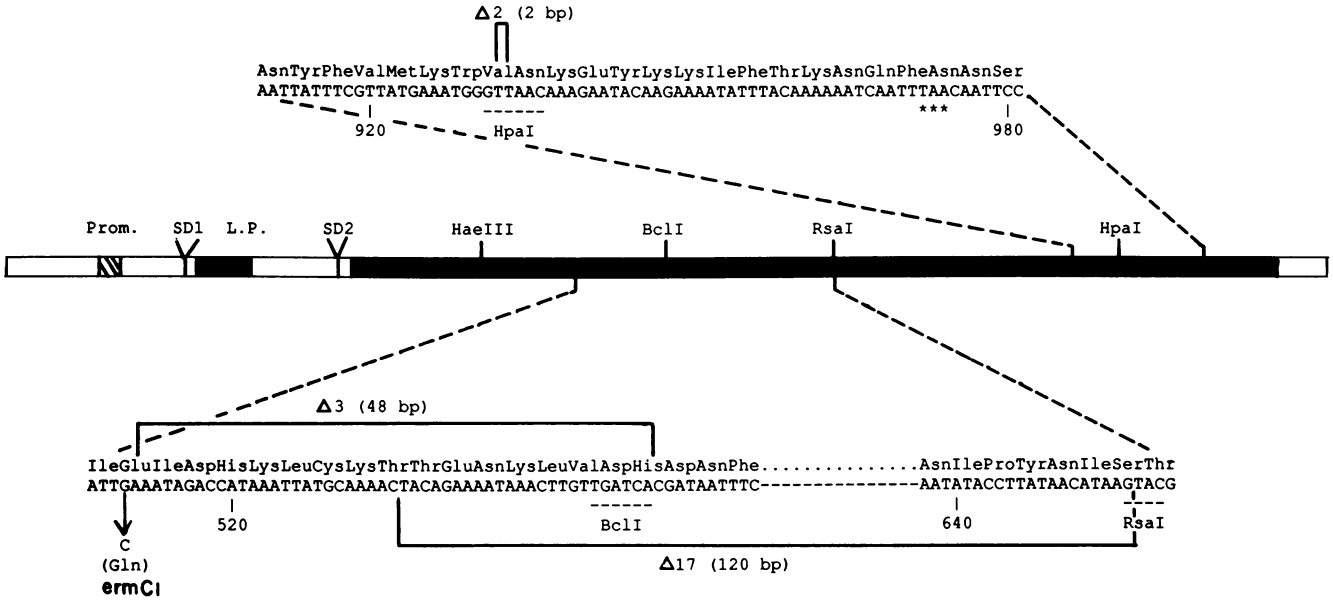

FIG. 1. Sequence analysis of *ermC* mutations. The sequence alterations of three deletion and one point mutation affecting the structure of the *ermC* gene product were determined by M13-dideoxy nucleotide sequencing. The locations of the *ermC* promoter, the leader peptide (L.P.), and Shine-Dalgarno sequences 1 and 2 (SD1 and SD2) are shown. Prom., Promoter; bp, base pairs.

filters with 50 ng of RNA per slot were probed with an rRNA probe. To construct an *ermC* probe the *ermC*-containing *Taq*I fragment of plasmid pE194 was cloned into the *Acc*I site of M13mp11. Several clones which were examined proved to have the wrong orientation for RNA probing, so an *Sst*I-*Bam*HI subfragment was subcloned in the correct orientation into M13mp11. The *ermC* sequences contained in the single-stranded phage DNA from the selected clone were homologous to *ermC* mRNA. We also constructed an rRNA probe. A 530-base-pair *Bam*HI-*Eco*RI fragment, which is contained within the *B. subtilis* 23S rRNA-coding region (9), was cloned into M13mp18 from the plasmid p14B8 (9). The orientation was such that the inserted single-stranded ribosomal DNA is homologous to rRNA. Labeled *ermC* or rRNA probes were generated as described previously (17) with the following modifications: M13 hybridization primer and template were heated to 90°C for 3 min, kept at 65°C for 10 min, and cooled to room temperature; NaCl was omitted from the extension reaction; the probe was passed through a Sephadex G-50 column to separate unincorporated nucleotides. Prehybridization of the slot-blot filters was in 5× Denhardt solution (4)–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS–100 μg of denatured salmon sperm DNA per ml–50% formamide for 2 h at 45°C. Hybridization was overnight at 37°C in the same solution except with 1× Denhardt solution and 5% dextran sulfate. The filters were then washed twice with 2× SSC–0.1% SDS and twice with 0.1× SSC–0.1% SDS at 50°C. Radioactive slots were cut out, and radioactivity was determined by scintillation spectrometry.

RESULTS

Minicell analysis of *ermC* mutants. Previous analysis of several erythromycin-sensitive *ermC* deletion mutants showed that all of them exhibited a hyperinducible phenotype (29). The overproduction of the mutant proteins was consistent with the postulated methylation-mediated induction feedback loop and with the notion that erythromycin must bind to ribosomes for induction to occur, since these altered proteins were unable to methylate 23S rRNA, and the feedback loop was thus broken. However, as pointed out above, hyperinduction is also consistent with autoregulation of synthesis as distinct from feedback inhibition of induction. To further analyze the hyperinducibility phenomenon, we compared a number of *ermC* structural mutants available in the laboratory. Figure 1 shows the sequence alterations owing to the mutations included in this study. *ermCΔ3* and *-Δ17* are BAL 31-generated deletion mutations. The *ermCΔ3* and *-Δ17* mutations remove 48 and 120-base pairs, respectively, preserving the wild-type reading frame. *ermCΔ2* is a mutation that removes two bases at the *Hpa*I site, changing the reading frame and introducing a termination codon 39 nucleotides downstream. The *ermCΔ2* methylase lacks the C-terminal 32 amino acid residues of the wild-type enzyme. Finally, a point mutation (*ermC1*) (6) consisting of a G-to-C change in codon 59 (Glu to Gln) was analyzed (Fig. 1).

In accordance with previous analyses of *ermC* deletion mutants, both *ermCΔ3* and *-Δ17* exhibited hyperinducible phenotypes in minicells (Fig. 2A). *ermCΔ3* expressed a 24.5-kDa truncated methylase, and *ermCΔ17* expressed a 22.5-kDa product, in both cases corresponding to the sizes expected from the sequence analyses. The *ermCΔ2* strain was previously shown to overproduce a truncated protein of approximately 23 kDa in minicells (29). The *ermC1* determinant, however, presented a phenotype resembling the wild type when analyzed in minicells (Fig. 2A). The kinetics of accumulation of the 29-kDa point mutant and wild-type ^{3}H-labeled *ermC* proteins are similar (Fig. 2B). These findings suggest that the syntheses of the wild-type and *ermC1* proteins are autoregulated, whereas that of the deleted truncated proteins is not. The point mutation appears to distinguish the catalytic from the regulatory functions of the *ermC* protein, suggesting that autoregulation occurs independently of rRNA methylation. However, it is possible that autoregulation of the point mutant protein is due to residual methylating activity.

Analysis of residual methylase activity in the point mutant

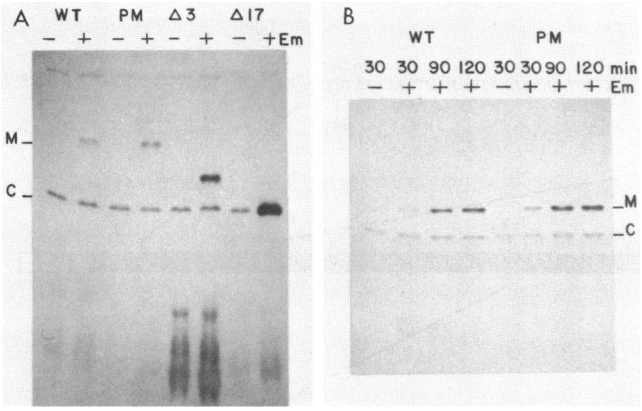

FIG. 2. Minicell analysis of wild-type and mutationally altered *ermC* gene products. (A) Synthesis of protein in the presence and absence of erythromycin (Em) was visualized by fluorography in extracts of *B. subtilis* minicells carrying *ermC*, the point mutant (PM), *ermCΔ3*, and *ermCΔ17*. The extracts were resolved by SDS-polyacrylamide gel electrophoresis. (B) The appearance of wild-type and point mutant gene products is shown at several times after the addition of inducing erythromycin. The positions of the wild-type methylase (M) and of chloramphenicol acetyltransferase (C) are indicated.

strain. The point mutant *ermC1* strain exhibited an erythromycin-sensitive phenotype. The possibility that the *ermC1* gene product possessed some residual methylase activity was further analyzed by measuring the availability of methylatable sites in 23S rRNA isolated from the *ermC1* strain grown with and without erythromycin induction, compared with a strain containing the wild-type determinant grown under similar conditions.

23 rRNA isolated from each strain was subjected to in vitro methylation with a purified *ermC* methylase preparation under conditions in which maximum methylation occurs (Table 2). It was possible to incorporate about two methyl groups into each *B. subtilis* 23S rRNA molecule extracted from a strain lacking an *ermC* determinant. When the RNA originated in a fully induced strain carrying the *ermC* wild-type gene in a high-copy-number replicon (pE194 *cop-6*), this 23S rRNA was almost completely methylated in vivo, and it was possible to further incorporate only about 0.2 methyl groups per molecule in vitro. 23S rRNA extracted from the point mutant strain, either with or without erythromycin induction, incorporated about two methyl groups per molecule, clearly demonstrating that the point mutant *ermC* protein lacks detectable methylase activity in vivo. Since the point mutant strain contains unmethylated ribosomes, the failure to hyperproduce the mutant protein (Fig. 2) cannot be due to residual methylation activity causing an interruption of induction. These results suggest that the mutant (and presumably the wild type) protein are directly autoregulated

TABLE 2. In vitro methylation of 23S rRNA

ermC allele[a]	Erythromycin induction	pmol of methyl groups/pmol of RNA
None	−	2.02 ± 0.08
ermC$^+$	−	0.72 ± 0.10
	+	0.19 ± 0.04
ermC1	−	1.98 ± 0.10
	+	2.05 ± 0.06

[a] A high-copy (*cop-6*) replicon was used.

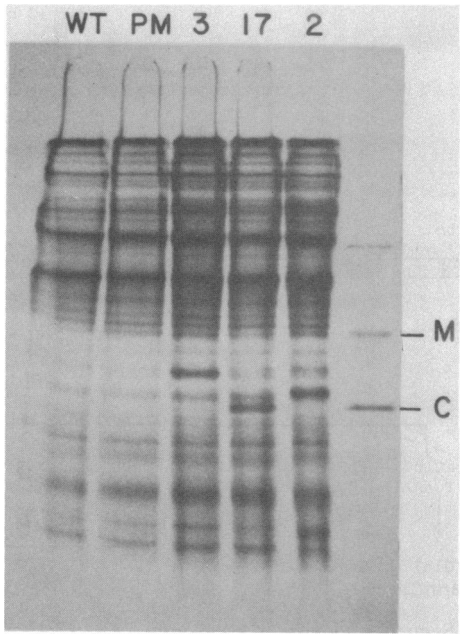

FIG. 3. Synthesis of methylase proteins in whole cells. Extracts of [^{35}S]methionine uniformly labeled cells carrying the wild type (WT), *ermC1* (PM), *ermCΔ3*, *ermCΔ17*, and *ermCΔ2* alleles were resolved on an SDS-polyacrylamide gel and exposed by fluorography. The positions of wild-type methylase (M) and of the chloramphenicol acetyltransferase (C) proteins are indicated.

in minicells by a mechanism that is independent of the *ermC* catalytic activity.

***ermC* expression in actively growing whole cells.** Since minicells may constitute an artificial system, we extended the preceding analysis to exponentially growing cells. The wild-type and point mutant methylase proteins were not detectable even after 4 h of induction when ^{35}S-labeled crude extracts were subjected to SDS-polyacrylamide gel analysis and fluorography (Fig. 3). No differences between induced and uninduced extracts could be seen under these experimental conditions (data not shown), indicating that methylase synthesis represents a minor portion of total protein synthesis. On the contrary, all the deleted derivatives (*ermCΔ3*, *-Δ17* and *-Δ2*) produced clearly visible bands with the expected mobilities in SDS-polyacrylamide gels, representing more than 2 to 3% of the total ^{35}S label incorporated (see Materials and Methods). Similar results were obtained after visualization by Coomassie blue staining (data not shown). These results indicate that the hyperinducible phenotype and the apparently regulated synthesis of the wild-type and mutant proteins were not artifacts of the minicell system and that *ermC* has a high expression capacity.

Effect of gene dosage on levels of wild-type and point mutant methylase production. The effect of *ermC* gene dosage on methylase concentration was analyzed in the wild-type and in the point mutant strains by quantitative immunoblotting. Rabbit antiserum raised against purified methylase reacted specifically with both wild-type and *ermC1* methylases in crude extracts (Fig. 4). The color reaction used to detect the antibody-antigen complexes (see Materials and Methods) was linear in the range of 0 to 75 ng. The results of such measurements as a function of gene dosage are presented in Table 3. The data demonstrate that an increase of at least 100-fold in the number of methylase genes per cell is

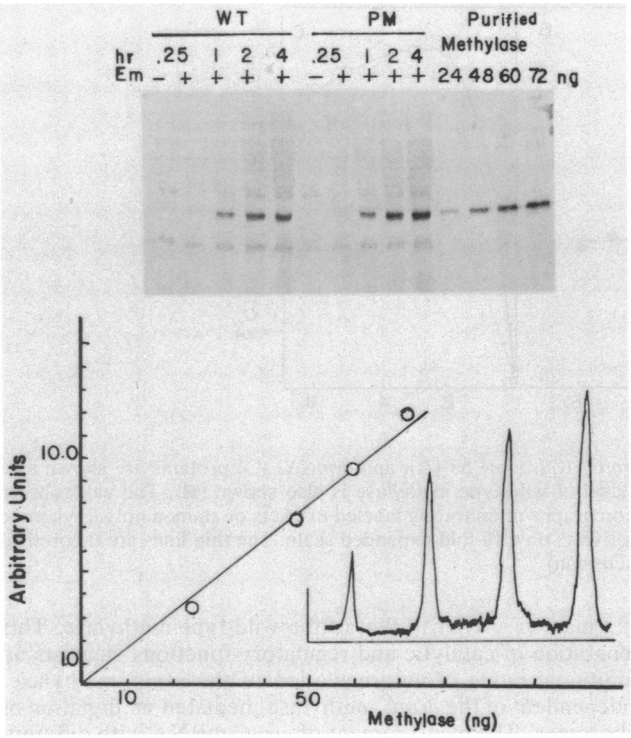

FIG. 4. Analysis of regulation in whole cells by immunoblotting. The indicated amounts of purified methylase were resolved on an SDS-polyacrylamide gel and detected by Western blotting as described in Materials and Methods. Tracings of the bands and the resulting calibration curve are displayed in the lower portion of the figure. The Western blot also shows the results obtained with extracts of growing cells carrying *ermC* (WT) or the point mutant *ermC1* (PM) allele, sampled at various times after the addition of inducer (erythromycin [Em]).

accompanied by only an approximately twofold increase in methylase concentration. Table 3 further reveals that synthesis of the point mutant protein is also gene dosage compensated. An increase of more than 100-fold in gene dosage again produces only an increase of two- to threefold in altered methylase protein. These results are consistent with the autoregulated expression of methylase protein and reveal that the presence of methylated ribosomes is not needed for this regulation.

Intracellular concentration of *ermC* methylase mutant proteins during exponential growth. The amounts of wild-type, *ermC1*, *ermCΔ2*, and *ermCΔ3* methylase proteins, as percentages of total cellular protein, were monitored during the logarithmic phase of growth, through a 6-h period after induction with a subinhibitory concentration of erythromycin (Fig. 5). The concentrations of *ermCΔ2* and *-Δ3* proteins increased constantly upon induction, reaching levels 10- to 20-fold higher than that of the wild-type methylase protein as the cultures approached the beginning of the stationary phase. The concentrations of the wild-type and point mutant proteins appeared to reach a new steady state about 2 to 4 h postinduction.

These results are consistent with an autoregulatory mechanism that controls the rate of synthesis of methylase to maintain a fixed maximum intracellular concentration of this protein. The higher concentration achieved by the point mutant protein when compared with the wild-type methylase might be due to a lower affinity of this mutant protein toward its regulatory target site or to the absence of a minor contribution of methylation-mediated feedback to overall regulation.

Methylase protein stability in vivo. The stability of both the wild-type and the point mutant protein was analyzed in minicells. Under our experimental conditions, after 2 h of incubation, the protein synthetic capacity of minicells diminished to less than 10% of the initial capacity. When compared by gel fluorography, the bands of wild-type and point mutant methylases detected in a sample of minicells incubated for 2 h with [^{35}S]methionine were similar in amounts to those demonstrated in a similar sample incubated for 3 h (data not shown). These results suggest that the point mutant and wild-type proteins have similar stabilities and refute the possibility that a grossly higher degradation rate of the point mutant methylase explains its lower accumulation rate when compared with the deleted variants. In fact, the values obtained for the *ermCΔ3* protein represent underestimates, since this truncated protein is clearly subject to degradation (Fig. 2A).

Measurement of in vivo levels of *ermC* mRNAs. Finally, we determined whether methylase autoregulation occurs on the level of transcription by determining the relative amounts of *ermC* mRNA present under various conditions. Total RNA was extracted 2 h after the addition of erythromycin, and the amount of *ermC* mRNA was determined by slot-blot hybridizations. The results (Table 4) show that the amount of *ermC* mRNA isolated from the pE194 culture was about one-fifth

TABLE 3. Variation of methylase concentration (%) with gene dosage[a]

ermC allele	Replicon[b]	Copy no.	Methylase/total protein (%)	
			2 h	4 h
ermC$^+$	Chromosome	1	0.18 ± 0.03	0.16 ± 0.01
	pE194	6 ± 2	0.24 ± 0.01	
	pE194 *cop-6*	80 ± 10	0.37 ± 0.06	
	pE194 *cop-1*	125 ± 25	0.46 ± 0.07	
	pBD142 *cop-6*	52 ± 7	0.25 ± 0.02	0.20 ± 0.02
ermC1	Chromosome	1	0.14 ± 0.02	0.12 (1 expt.)
	pBD142	3 ± 1	0.06 ± 0.02	
	pBD142 *cop-6*	51 ± 14	0.43 ± 0.03	0.41 ± 0.04
	pBD142 *cop-1*	70 ± 10	0.38 ± 0.02	0.36 ± 0.02
	pIM13	160 ± 15	0.30 ± 0.01	0.25 ± 0.03

[a] Average plus or minus standard deviation of three experiments.
[b] The pBD142 replicon is derived from pE194 but carries a chloramphenicol resistance determinant and hence has a slightly lower copy number. pIM13 is a high-copy-number *B. subtilis* plasmid.

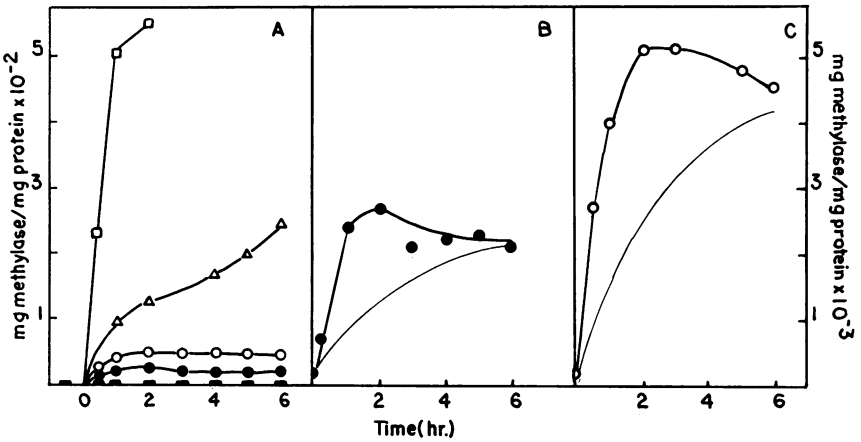

FIG. 5. Kinetics of regulation. (A) Concentrations of wild-type (●), *ermC1* (○), *ermCΔ3* (△), and *ermCΔ2* (□) proteins are shown as a function of time after the addition of inducer (erythromycin). The basal level of wild-type methylase is also shown (■). The values were obtained by immunoblotting the *ermC* and *ermC1* extracts or by tracing fluorographs of uniformly labeled extracts or stained polyacrylamide gels (*ermCΔ2* and *ermCΔ3*). (B and C) Results for *ermC* and *ermC1*, respectively, on a 10-fold-expanded scale. The thin lines are theoretical curves drawn from the assumption that no autoregulation occurs (see Discussion).

that of the *ermC* mRNA from the *cop-6* strain. This is roughly proportional to the difference in copy number; pE194 exists at 6 to 10 copies per chromosome, and *cop-6* has 60 to 80 copies per chromosome. The amount of methylase in these two strains, determined by immunoblotting, differed by less than twofold. Table 4 also shows that strains carrying the wild-type and *ermCΔ3* determinants on identical replicons contain nearly identical amounts of mRNA, whereas the rate of truncated protein synthesis is dramatically higher than that of the wild type 2 h after induction (Fig. 2, 3, and 5). Thus, the autoregulation of *ermC* appears to be exerted posttranscriptionally.

DISCUSSION

Our results show that the expression of *ermC* methyltransferase is probably autoregulated at the level of its own translation. Three of the structural mutants analyzed here (*ermCΔ2*, *-Δ3*, and *-Δ17*) overproduce truncated forms of inactive methylase. The overproduction of inactive methylase in *ermC* mutants with lesions in the structural gene suggests that the synthesis of an active methylase is necessary for the autogenous control of *ermC* gene expression and is consistent with the already suggested methylation-mediated feedback loop. Since unmethylated ribosomes able to bind erythromycin are required to induce the translation of the *ermC* methylase message, the synthesis of an active methylase in a host carrying the wild-type *ermC* determinant will eventually produce a population of methylated ribosomes, shutting down further induction. However, we showed that the synthesis of one inactive methylase protein, the product of the *ermC1* mutant gene, is regulated in a manner similar to that of the wild-type methylase. This separation of catalytic and regulatory functions suggests an additional mode of autoregulation of the *ermC* methylase, independent of the *ermC* methylase-mediated methylation of ribosomes. The measurement of *ermC* mRNA with different conditions of gene dosage and different *ermC* alleles supports the idea that this autoregulation is posttranscriptional.

TABLE 4. *ermC* mRNA accumulation 2-h postinduction

ermC allele	Replicon	Copy no.	mRNA (normalized)	Methylase (normalized)
ermC$^+$	pE194	6 ± 2	1	1
ermC$^+$	pE194 *cop-6*	80 ± 10	5.3	1.5
ermC$^+$	pBD142 *cop-6*	51 ± 14	1	—[a]
ermCΔ3	pBD142 *cop-6*	51 ± 14	1.2	—

[a] The relative levels and rates of methylase synthesis with the *ermCΔ3* and wild-type alleles at 2-h postinduction can be inferred from Fig. 5.

The existence of an autoregulatory mechanism can also be inferred from a kinetic analysis of the data in Fig. 5. We assume that upon induction the rate of enzyme synthesis switches from zero (ignoring the low basal synthesis) to a new value R (in milligrams of enzyme per milligram of total protein per minute) and that the value R does not change during exponential growth in the presence of inducer. These assumptions are equivalent to the absence of autoregulation. The enzyme concentration C (in milligrams of enzyme per milligram of total protein) will then increase toward a new asymptotic limit that depends on R and on the exponential growth constant k. The rate of change of E, the total enzyme, can be expressed by: $dE/dT = RP = RP_0e^{kt}$, where P is total protein and P_0 is initial protein. Integrating, we obtain: $E = (RP_0/k)(e^{kt} - 1)$. Dividing by $P = P_0e^{kt}$ gives: $C = E/P = (R/k)(1 - e^{-kt})$.

k was determined for the experiment shown in Fig. 5. The doubling time T was 113 min, and k (= ln 2/T) was therefore 6.134×10^{-3} min^{-1}. The final steady-state values of C for the wild-type and *ermC1* strains were estimated from the data in Fig. 5 as 0.0022 an 0.0045 mg of enzyme per mg of protein, respectively. These should be equal to R/k for large values of t. Inserting these values into the equation permitted the calculation of the theoretical curves for the absence of autoregulation, given the observed asymptotic limits. These curves are shown in Fig. 5. They clearly do not fit the data, suggesting that the initial values of R are greater than those estimated above and that R is then regulated downward to reach the new steady state. That the true initial value of R is quite high can be inferred from the kinetics obtained with *ermCΔ2*, which reveal an elevated and unregulated synthetic capacity (Fig. 5). The overshoot exhibited by the wild-type and *ermC1* data is also consistent with the operation of a feedback regulatory mechanism.

The results presented in Fig. 5 show that after about 2 to 3 h of induction the concentration of wild-type methylase

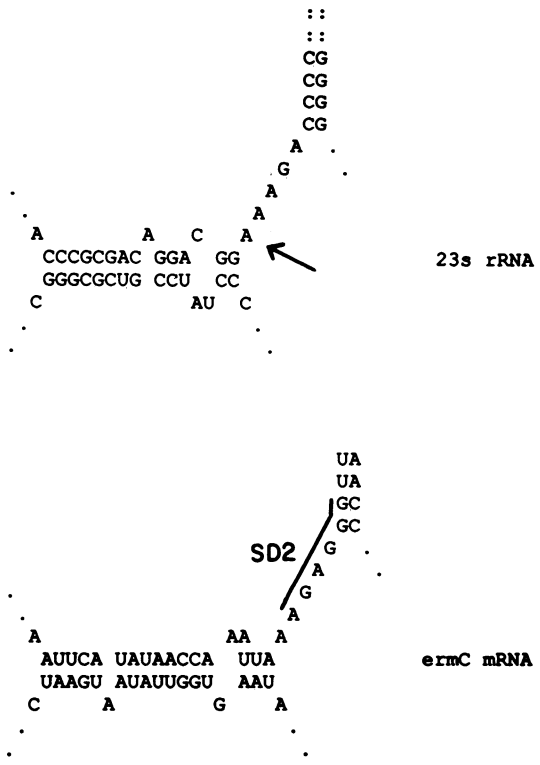

FIG. 6. *ermC* methyltransferase target sites. The target site of N^6,N^6-dimethylation on 23S rRNA (position 2058) is shown by an arrow (24, 30). The proposed autoregulatory target on the *ermC* mRNA is also shown.

reaches a maximum intracellular level, which is then maintained during exponential growth. We calculated that after reaching this steady state, the number of methylase molecules per cell is about 2×10^4 to 3×10^4, which is approximately equal to the number of ribosomes per cell. We also observed, using purified enzyme, that the *ermC* product binds tightly to ribosomes. These findings suggest the existence of an intracellular compartment for methylase, formed by interaction with ribosomes in vivo. It may be that free enzyme, in excess of that required to saturate the ribosomal pool, is available to interact with the regulatory target site.

The *ermC* transcript is highly stable after induction (29; D. Bechhofer and D. Dubnau, unpublished data). This induction-specific stabilization extends the half-life of *ermC* mRNA from 2 to 3 min to about 45 min. Enhanced mRNA stability presumably increases the rate of the induction response. It may also explain the need for a fast and specific mechanism of synthesis shutdown, possibly acting directly on the mRNA ribosomal complex, which could operate in addition to the methylation-mediated feedback loop already proposed. The methylation-mediated mechanism would prevent further induction, but would allow the translation of already induced message to proceed at a significant rate even after the pool of ribosomes is completely methylated. The direct autoregulatory mechanism acts at the level of synthesis, when sufficient free enzyme has been produced. Results presented in this paper demonstrate that the *ermC* gene is highly expressed when autoregulatory mechanisms are disrupted. The hyperinducible derivatives are able to accumulate altered methylases to a level greater than 2% of the total protein content. This observation suggests that the acquisition of a shutoff mechanism is highly advantageous.

The promoter-proximal portion of the *ermC* mRNA presents structural similarities with the site of action of the methylase on 23S rRNA (Fig. 6). In the case of 23S rRNA, the single-stranded sequence AAAGA is located 3′ to a base-paired structure. A similar single-stranded sequence within the Shine-Dalgarno sequence of the methylase structural gene (SD2) is also located 3′ to a base-paired structure. This base-paired stem is probably present both before and after induction (11). In addition, SD2 may have evolved to resemble the sequence found at the site of action of the methylase on the 23S rRNA (Fig. 7). A typical SD sequence of gram-positive origin contains the sequence GGAGG (14). Moreover, the first GG doublet is conserved in more than 80% of the 41 SD sites compared (14). A sequence complementary to the 3′ end of 16S rRNA (AAGGAGG) found in SD1 of *ermC* and in many other gram-positive genes is replaced by AAGAGGG in the SD2 of the *ermC* determinant. If the inversion of the GA doublet increases the resemblance of SD2 to the rRNA target site, this has been at the expense of complementarity to the 3′ end of 16S rRNA. Interestingly, three additional differences between SD2 and SD1 may serve to compensate for this loss (Fig. 7). The primary and secondary structural similarities noted above suggest that the translational autoregulation of methylase synthesis operates through a direct interaction of the methylase protein with the regulatory moiety of its own mRNA, thereby occluding the ribosome-binding site.

The features of *ermC* gene expression discussed above and the results presented in this paper allow us to propose a model for the regulation of methylase synthesis (Fig. 8). In the absence of erythromycin, a pool of inactive *ermC* mRNA is available that turns over with a half-life of 2 to 3 min. Upon induction, the *ermC* mRNA structure changes to a translationally active form, and the half-life of the mRNA increases. These changes permit the enhanced synthesis of methylase protein. As methylase accumulates, an increasing proportion of ribosomes are specifically methylated, and the concentration of free intracellular methylase increases, possibly after the pool of ribosomes is saturated with bound enzyme. As the methylatable sites on the 23S rRNA molecules are saturated, further induction must cease. We do not know the kinetics of this process after induction. When the concentration of free methylase exceeds a critical level, binding to *ermC* mRNA occurs at a site resembling the

```
3'end Bacillus 16s rRNA    3' UCUUUCCUCCACUAGGUCGG 5'

Complementary sequence     5' AGAAAGGAGGUGAUCCAGCC 3'

ermC SD1                      UAUAAGGAGGAAAAAAUAUG
                                 -------  -
                                 ***

ermC SD2                      UUAAAGAGGGUUAUAAUGAA
                                 ----  --- --
                                 *****

23s rRNA methylation          GGAAAGACCCCGUGGAGCUU
site
```

FIG. 7. Comparison of the 23S rRNA methylation site with SD2. The sequence of the 3′ terminus of *B. subtilis* 16S rRNA and its complement are shown, together with the sequences of SD1 and SD2 and the methylation target site on 23S rRNA. Matches with the latter site are indicated by asterisks. Matches with the complement of the 16S rRNA sequence are shown by dashes.

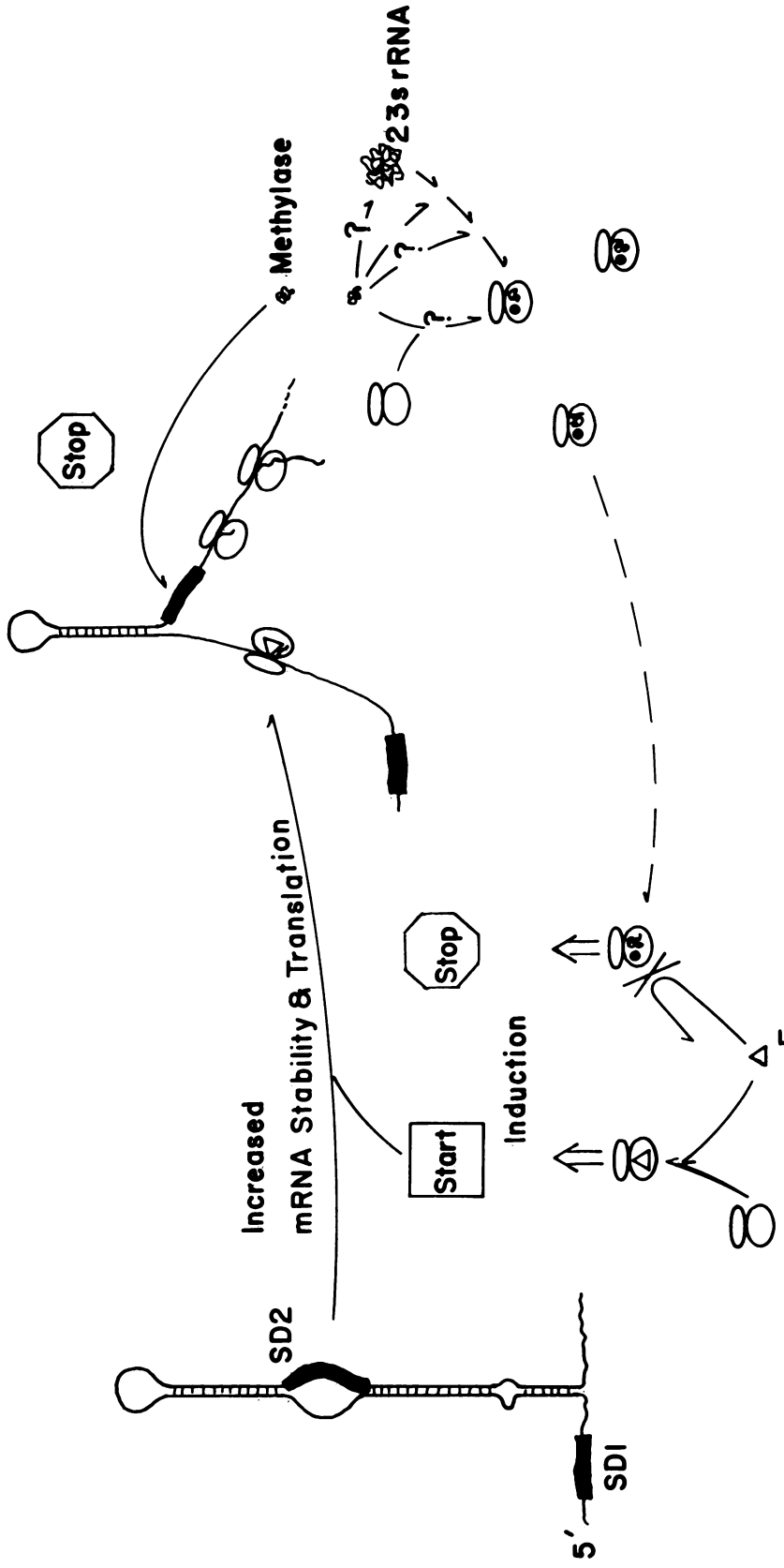

FIG. 8. Model for the overall regulation of *ermC* expression. The presence of methyl groups on 23S rRNA is indicated by black dots, and erythromycin (Em) is shown by a triangle. Binding of methylase to the ribosomes is also shown. The uncertainty concerning the in vivo substrate for methylation (i.e., free 23S rRNA, mature ribosomes, or an intermediate in ribosome assembly) is reflected in the figure. Further details are provided in the text.

methylation site on 23S rRNA, repressing the synthesis of additional enzyme.

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