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Effect of *ermC* Leader Region Mutations on Induced mRNA Stability

KIM K. HUE AND DAVID H. BECHHOFER*

Department of Biochemistry, Mount Sinai School of Medicine, New York, New York 10029-6574

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Induction of translation of the *ermC* gene product in *Bacillus subtilis* occurs upon exposure to erythromycin and is a result of ribosome stalling in the *ermC* leader peptide coding sequence. Another result of ribosome stalling is stabilization of *ermC* mRNA. The effect of leader RNA secondary structure, methylase translation, and leader peptide translation on induced *ermC* mRNA stability was examined by constructing various mutations in the *ermC* leader region. Analysis of deletion mutations showed that ribosome stalling causes induction of *ermC* mRNA stability in the absence of methylase translation and *ermC* leader RNA secondary structure. Furthermore, deletions that removed much of the leader peptide coding sequence had no effect on induced *ermC* mRNA stability. A leader region mutation was constructed such that ribosome stalling occurred in a position upstream of the natural stall site, resulting in induced mRNA stability without induction of translation. This mutation was used to measure the effect of mRNA stabilization on *ermC* gene expression.

The leader region of *ermC* mRNA is a 140-nucleotide RNA sequence that is involved in the posttranscriptional control of expression of the *ermC* structural gene, which encodes an rRNA methyltransferase (methylase) that confers macrolide-lincosamide-streptogramin B (MLS) resistance on a *Bacillus subtilis* host. Methylase expression is induced in the presence of erythromycin. The secondary structure of the *ermC* leader RNA and a 19-amino-acid leader peptide coding sequence are the elements that control expression by translational attenuation (10, 29). In the absence of erythromycin, the stem-loop structure of the *ermC* leader region precludes methylase translation, because the ribosome binding site preceding the methylase coding sequence (SD2 in Fig. 2) is unavailable for translation. In the presence of erythromycin, an erythromycin-bound ribosome stalls while translating the 19-amino-acid leader peptide. This opens the stem-loop structure to allow ribosome binding at SD2 and results in a 20-fold increase in methylase translation. Stalling of an erythromycin-bound ribosome in the leader peptide coding sequence also results in a 15- to 20-fold increase in *ermC* mRNA stability (3, 27). The stalled ribosome protects *ermC* mRNA from attack by an RNase that recognizes the 5′ end of the message (4). Induced mRNA stability of a related MLS resistance gene, *ermA*, occurs by a similar mechanism (26).

Naturally occurring mutations in the *ermC* leader region have been isolated that express elevated levels of methylase in the absence of induction, thereby conferring resistance to noninducing MLS antibiotics, such as tylosin. Analysis of these mutations showed that constitutive expression was due to alterations in the leader region secondary structure that resulted in the unmasking of SD2, thus supporting the translational attenuation model (16, 18). In addition, in vitro-constructed deletions extending from the *ermC* promoter into the leader region resulted in constitutive or noninducible phenotypes, depending on the extent of the deletions (16). More recently, in vitro-constructed point mutations in the *ermC* leader peptide coding sequence demonstrated that only a portion of this coding sequence is required for ribosome stalling in response to erythromycin (20, 22). Specifically, missense mutations in codons 5 to 9, but not in other codons, resulted in reduced induction by erythromycin.

Since both induced methylase translation and induced *ermC* mRNA stability are a result of ribosome stalling in the leader peptide coding sequence, it has not been possible to separate these two effects. In the present study, we constructed deletion, insertion, and point mutations that were designed to examine the requirements for induction of *ermC* mRNA stability and the contribution of mRNA stabilization to *ermC* gene expression.

MATERIALS AND METHODS

Bacterial strains. Strains BE188, BE189, and BE194 were transformants of IS75, which is *hisA1 leu metB5*. All other strains were transformants of BD170, which is *trpC2 thr-5*.

Standard procedures. The preparation of *B. subtilis* growth media and competent *B. subtilis* cultures (11), isolation of plasmid DNA (12), Northern RNA blot analysis and quantitation of mRNA half-lives (4), and β-galactosidase assays (15) were as described previously. The probe used for all Northern blots was the 5′-end probe used in previous experiments (4); this probe represents *ermC* sequences from the *Mbo*I site just upstream of the *ermC* promoter to the *Hae*III site in the methylase coding sequence. Western immunoblot analysis of induced methylase levels was performed as described previously (9).

Plasmids. Plasmid pBD142 is a high-copy-number (*cop-6*) derivative of pE194 (13) that carries the chloramphenicol resistance marker of pC194 on a *Cla*I fragment. The construction of *Bal* 31 deletion derivatives of pBD142 is described in Results. Because analysis of the *ermC* mRNA half-life encoded by *ermC* carried on high-copy-number plasmids requires the addition of erythromycin to a level that is inhibitory to cell growth (unpublished results), we converted high-copy-number derivatives containing *ermC* leader mutations to low-copy-number versions. This was done by digesting the high-copy-number plasmid at its *Sph*I site, which is located in the pE194 *rep* region (Fig. 1). The *Sph*I-digested plasmid DNA was used to transform strain

* Corresponding author.

BE1, which carries wild-type pE194 (low copy number), and chloramphenicol-resistant colonies were selected. Since linear DNA cannot transform *B. subtilis*, chloramphenicol-resistant colonies are the result of homologous recombination between the transforming linear DNA and the resident pE194 plasmid (8). This recombination takes place distal to the *Sph*I site, i.e., outside of the replication region. The resultant plasmid recombinants contained the replication region of pE194 (low copy number) and the mutant *ermC* region of the transforming plasmid.

Oligonucleotides that were used to generate random base changes in the sequence representing the first 11 codons of the leader peptide were purchased from Operon Technologies (San Pablo, Calif.). Three mutagenic oligonucleotide primers were used. The first was a 40-nucleotide (nt) primer with the sequence ATG GGC att ttt agt att ttt gta atc agc aca GTT CAT T. This sequence represents *ermC* leader peptide wild-type codons 1 through 13 plus one nucleotide from codon 14. Nucleotides in uppercase letters are positions that were synthesized without changes, and nucleotides in lowercase letters represent positions that, under the conditions used for oligonucleotide synthesis, should contain random base changes on the average of one per oligonucleotide synthesized. The mutations in clones BE188 and BE245 were derived from this primer. In addition, two 27-nt mutagenic primers were used. Primer 1 (GGC att ttt agt att tTT GTA ATC AGC) represents leader peptide codons 2 through 10, and primer 2 (AGT ATT Ttt gta atc agc aca GTT CAT) represents leader peptide codons 5 through 13. The mutations in clones BE189, BE192, BE246, and BE247 were derived from primer 1, and the mutations in clones BE194 and BE248 were derived from primer 2. Oligonucleotides that were used for directed single-nucleotide changes were purchased from the DNA Core Facility of the Brookdale Molecular Biology Center, Mount Sinai School of Medicine. Oligonucleotide mutagenesis was performed as described previously (19). The single-stranded DNA template for mutagenesis was an M13mp19 clone containing either the entire *ermC* gene, cloned as a *Sac*I-*Bam*HI fragment from a plasmid that had a multiple cloning site DNA fragment inserted into the *Cla*I site located immediately downstream of *ermC* (plasmid pSD85, Fig. 1), or a *Sac*I-*Eco*RI fragment from plasmid pSD101 (Fig. 1), which has an *Eco*RI site in the *ermC* leader region by virtue of an A-to-G change at the position indicated in Fig. 2 (kindly provided by F. Breidt, Public Health Research Institute of New York). After mutagenesis, replicative-form M13 containing the mutagenized DNA was prepared, and fragments were isolated and used to replace the corresponding wild-type fragments in plasmids that were transformed back into *B. subtilis*. To confirm the presence of the desired mutation on the selected transformant, the *ermC* leader region was amplified by using the polymerase chain reaction and sequenced as described previously (2).

The *ermC*::*lacZ* translational fusion in BE259 was constructed as described previously for plasmid pBD246 (15). To make the *ermC*::*lacZ* transcriptional fusion in BE232, the *Eco*RI site in the leader region of pSD101 was used to insert a *lacZ* gene from pTV30 (24), in which the *spoVG* ribosome binding site precedes the *lacZ* coding sequence. This *lacZ* gene had previously been inserted on a *Bam*HI-*Hin*dIII fragment into the multiple cloning site region of pSD85, thus providing an *Eco*RI site to allow ligation to the *ermC* leader region *Eco*RI site in pSD101. (The multiple cloning site region of pSD85 contains the following restriction endonuclease sites: *Eco*RI, *Bam*HI, *Pst*I, *Bgl*II, *Xba*I, and *Hin*dIII.)

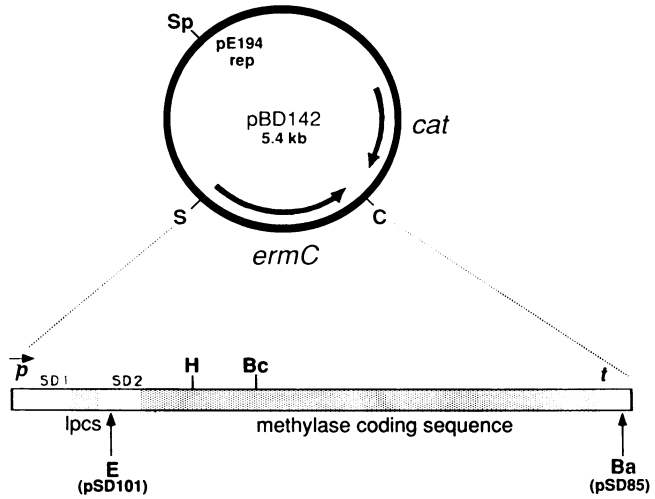

FIG. 1. Structure of plasmid pBD142 and schematic representation of the *ermC* gene. Abbreviations for relevant restriction endonuclease sites: Ba, *Bam*HI; Bc, *Bcl*I; C, *Cla*I; E, *Eco*RI; H, *Hae*III; S, *Sac*I; Sp, *Sph*I. The relative positions of the replication region (pE194 rep), chloramphenicol resistance gene (*cat*), and erythromycin resistance gene (*ermC*) are shown on the circular plasmid map. The portion of the plasmid containing the *ermC* gene is represented linearly. Shaded regions are amino acid coding sequences (lpcs, leader peptide coding sequence). SD1 and SD2 are the ribosome binding sites for the leader peptide and methylase coding sequences, respectively. *p*, *ermC* promoter; *t*, putative transcription terminator sequence. *ermC* mRNA is approximately 920 nt long. The *Eco*RI and *Bam*HI restriction sites are present on plasmids pSD101 and pSD85, respectively.

RESULTS

Deletions extending from the methylase coding sequence into the leader region. Plasmid pBD142 (Fig. 1) consists of pE194 *cop-6* (13) plus the chloramphenicol resistance determinant of plasmid pC194. Plasmid pBD142 DNA was linearized at its unique *Bcl*I site and subjected to *Bal* 31 digestion for various times. After transformation and screening for Em^s colonies, plasmids were selected that were missing the *Hae*III site that is located 100 bp upstream of the *Bcl*I site (Fig. 1). These were characterized further by restriction analysis, and several were chosen for DNA sequencing to determine the extent of the deletion. The induced stability of *ermC* mRNA encoded by the deletion mutations was tested by constructing low-copy-number derivatives (see Materials and Methods) and performing Northern blot analysis of RNA isolated from strains carrying these derivatives. The upstream endpoints of four of these deletion mutations are shown in Fig. 2.

BE97 contained a plasmid that had an in-frame deletion of 223 nt within the *ermC* coding sequence. Northern blot analysis of RNA isolated from BE97 showed that the *ermC* mRNA encoded by this deletion mutation was inducibly stable (Fig. 3), demonstrating that functional methylase protein was not required for induced stability. BE98 contained a plasmid that had a 336-nt deletion whose 5′ deletion endpoint was 6 nt upstream of SD2. *ermC* mRNA encoded by this plasmid was similarly stabilized by erythromycin induction (Fig. 3), proving that translation of the methylase coding sequence was not required for stability. The plasmid carried in strain BE106 had a deletion of 348 nt extending into the *ermC* leader peptide coding sequence, removing the

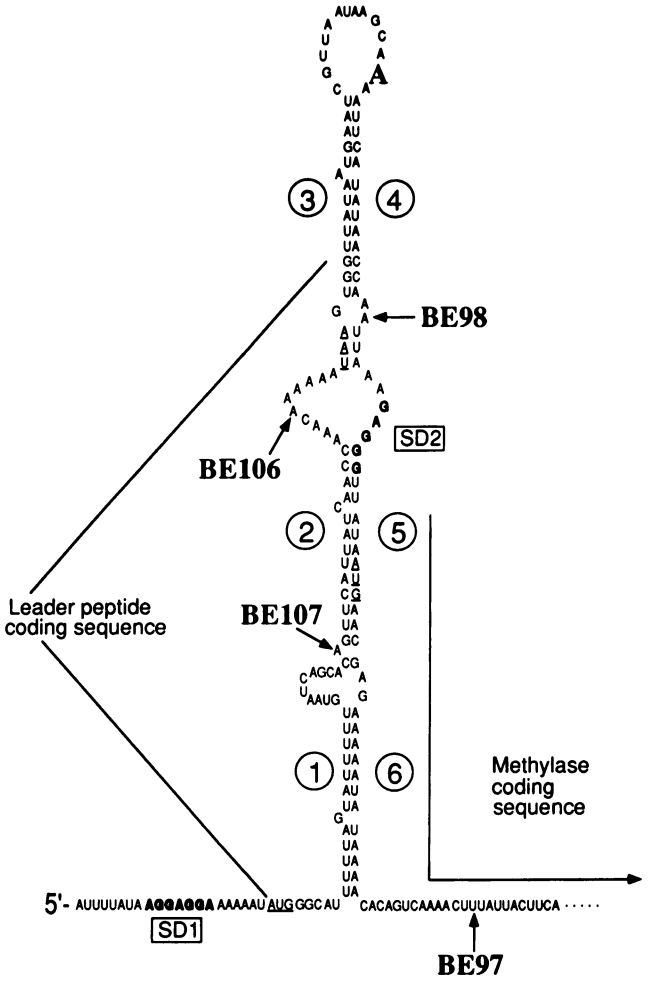

FIG. 2. Secondary structure of the *ermC* leader region in the uninduced state. Initiation and stop codons of the leader peptide coding sequence and the initiation codon of the methylase coding sequence are underlined. Circled numbers are stem segments according to the secondary structure model of Gryczan et al. (14). The upstream deletion endpoints of the *Bal* 31 deletion mutations in strains BE97, BE98, BE106, and BE107 are shown by arrows that point to the last nucleotide deleted. The boldface A nucleotide near the top of the stem-loop structure was changed to a G to create an *Eco*RI restriction site (GAATTC) in plasmid pSD101 (6).

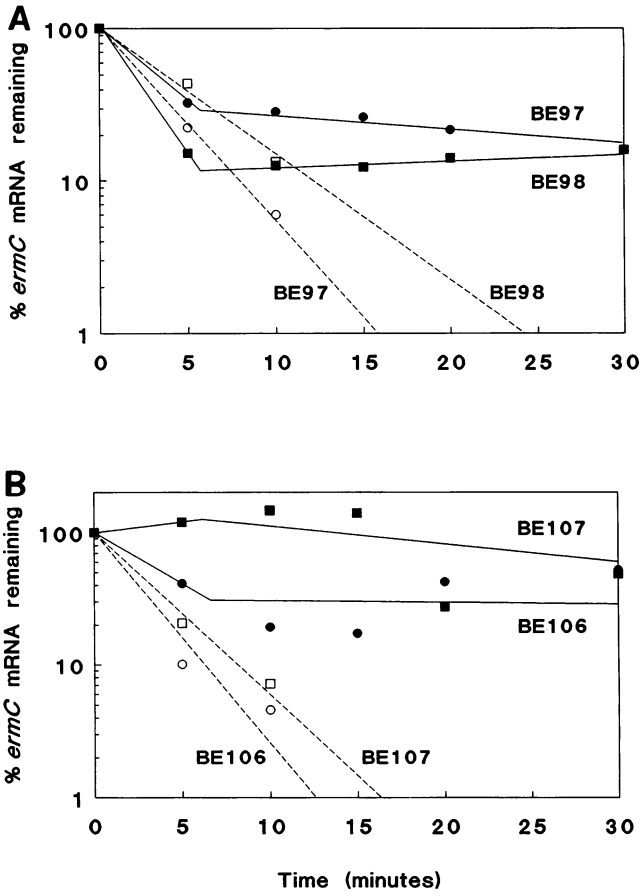

FIG. 3. Induced stability of *ermC* mRNA encoded by *Bal* 31 deletion mutants BE97 and BE98 (A) and BE106 and BE107 (B). Relative amounts of *ermC* mRNA remaining at various times after rifampin addition were measured by densitometric analysis of autoradiograms of Northern blots. The values obtained were plotted on a semilogarithmic scale as a percentage of *ermC* mRNA remaining versus time after rifampin addition. Symbols: ● and ■, RNA samples isolated from the indicated strains in the presence of erythromycin; ○ and □, RNA samples isolated from the indicated strains in the absence of erythromycin. Straight lines are linear regressions. Curved lines indicate the best fit through the data points.

last two codons and resulting in an in-frame fusion between the *ermC* leader peptide coding sequence and the middle of the methylase coding sequence. The RNA secondary structure prediction program of Zuker (PCFOLD version 3.0) was used to examine possible secondary structure of the 5′ region (140 nt) of the RNA encoded by this deletion mutant, as well as the deletion mutants carried in strains BE98 and BE107 (below). Although the computer program accurately predicted the secondary structure for the wild-type sequence (Fig. 2), the secondary structure predicted for the 5′ region of the deletion mutant RNAs was not more stable than the structures predicted for random 140-nt segments of downstream *ermC* mRNA. The finding that *ermC* mRNA encoded in strain BE106 was nevertheless inducibly stable (Fig. 3) showed that erythromycin-induced ribosome stalling in the leader peptide coding sequence is independent of RNA secondary structure. Finally, BE107 carried a plasmid containing a 423-nt deletion that extended well into the leader peptide coding sequence, leaving only 11 of the 19 leader peptide codons and resulting in an out-of-frame fusion to the middle of the methylase coding sequence. Induced *ermC* mRNA stability was observed in this strain as well, demonstrating that the entire leader peptide coding sequence is not required for ribosome stalling, in agreement with the results of Mayford and Weisblum (20; see below).

Construction of point mutations in the leader peptide coding sequence. Results from the *Bal* 31 deletions indicated that the first 11 codons of the leader peptide coding sequence were sufficient for ribosome stalling. The DNA sequence encoding these 11 codons was a reasonable target size for oligonucleotide-directed mutagenesis to determine the importance of specific codons for ribosome stalling. We constructed point mutations in this region by using oligonucleotides representing the upstream portion of the leader peptide coding sequence that contained randomly generated changes (see Materials and Methods). Although the synthesis of the

TABLE 1. Growth on tylosin and erythromycin of strains containing *ermC* leader region point mutations

Strain	Leader peptide amino acids 1 through 10[a]	Mutated codon(s)	Growth on tylosin-erythromycin[b]	Mutant in reference 20[c]
BE177	M G I F S I F V I S	Wild type	++	
BE188	– – V – – – – – – R	3 (ATT→GTT)	+	
		10 (AGC→AGA)		m31
BE248	– – – – – – – – – R	10 (AGC→AGG)	++	m31
BE220	– – V – – – – – – –	3 (ATT→GTT)	+	
BE194	– – – L – L – – – –	4 (TTT→TTA)	+	
		6 (ATT→CTT)		m13
BE246	– – # – – L – – – –	3 (ATT→ATC)	++	
		6 (ATT→CTT)		m13
BE189	– – – Y G – – – – –	4 (TTT→TAT)	–	
		5 (AGT→GGT)		m7
BE222	– # – Y – – – – – –	2 (GGC→GGT)	+	
		4 (TTT→TAT)		
BE192	– – – – – – V – – –	7 (TTT→GTT)	±	
BE245	– – – L – – – – F –	4 (TTT→TTA)	±	
		9 (ATC→TTC)		m56
BE247	– – – – – T – – – –	6 (ATT→ATC)	++	

[a] The wild-type amino acids (single-letter code) are given for strain BE177. –, no change; #, silent mutation.
[b] Growth on medium containing tylosin (1.0 μg/ml) and erythromycin (0.02 μg/ml) was judged by the size of colonies observed after incubation at 32°C for 48 or 72 h. –, no growth; ±, 1 to 2 mm after 72 h; +, 1 to 2 mm after 48 h; ++, 2 to 3 mm after 48 h.
[c] Mutations that are identical to those reported by Mayford and Weisblum (20) are indicated.

doped oligonucleotides was designed to produce mostly single-nucleotide changes, many of the mutations recovered by mutagenizing with these oligonucleotides had missense mutations in more than one codon. While this work was in progress, a report by Mayford and Weisblum was published (20), describing a large number of point mutations in the leader peptide coding sequence and the effects of these mutations on induction of an *ermC*::*lacZ* fusion product. They found that particular mutations in codons 5 through 9 affected inducibility, indicating that these codons constitute the ribosome stall site. We chose to characterize several mutations that might complement the results obtained by Mayford and Weisblum.

Strains carrying missense mutations in the leader peptide coding sequence were tested for inducibility of *ermC* expression by plating on selective media containing tylosin and erythromycin (Table 1). Tylosin is a noninducing MLS antibiotic, and *B. subtilis* cells carrying *ermC* can grow on plates containing tylosin only if induction of *ermC* by erythromycin (i.e., ribosome stalling in the leader peptide coding sequence) takes place. A mutation that decreases ribosome stalling will affect growth on tylosin since induction of methylase translation is decreased. BE188 carried missense mutations in codons 3 (Ile to Val) and 10 (Ser to Arg), which caused a decrease in the inducibility of *ermC* expression. The change in codon 10 alone (Ser to Arg; strain BE248) had no effect. We constructed a mutation that had the change in codon 3 alone (BE220) and found that inducibility of *ermC* expression was also decreased, similar to the decrease with BE188. The induced stability of *ermC* mRNA in strain BE188 was analyzed by Northern blotting; a decrease of inducibility on plate tests could be correlated with a decrease of induced *ermC* mRNA stability (Fig. 4). This is to be expected, since both induction of stability and growth on tylosin are consequences of ribosome stalling. These results demonstrated that, in addition to the codons identified by Mayford and Weisblum (i.e., codons 5 through 9), codon 3 is involved in erythromycin-induced ribosome stalling.

BE194 contained an *ermC* gene that had a Phe-to-Leu change in leader peptide codon 4 and an Ile-to-Leu change in codon 6. The inducibility of *ermC* expression (Table 1) and the induced *ermC* mRNA half-life (data not shown) were decreased in this strain, similar to the effect seen in BE188, which had changes in codons 3 and 10. Another strain (BE246) that had the same Ile-to-Leu change in codon 6 (and a silent mutation in codon 3) showed a wild-type phenotype. These results suggested that the decreased inducibility of *ermC* expression in BE194 was caused by the change in codon 4, thus implicating this codon also in erythromycin-induced ribosome stalling. BE189 contained a Phe-to-Tyr change in codon 4 and a Ser-to-Gly change in codon 5, and this strain could not grow at all on tylosin and erythromycin, indicating a total loss of inducibility. Northern blot analysis of RNA isolated from BE189 was consistent with the lack of inducibility (Fig. 4); there was little difference between the stabilities of *ermC* mRNAs in the presence and absence of erythromycin. Since the Ser-to-Gly change at codon 5 alone does not affect inducibility (20), this result also showed that codon 4 is involved in erythromycin-induced ribosome stalling. To determine whether the change in codon 4 by itself could confer the noninducible phenotype, we constructed a mutation that had a Phe-to-Tyr change at codon 4; the resultant strain (BE222) was partially inducible, with a growth phenotype similar to that of BE188. The complete loss of inducibility in BE189, therefore, indicates that changes in certain codons can act synergistically to affect ribosome stalling, as has been noted previously (20).

Two additional mutant strains were isolated that grew poorly on tylosin and erythromycin. BE192 had a Phe-to-Val change in codon 7. Mayford and Weisblum showed that a Cys or Leu in codon 7 also reduced inducibility. BE245 had a Phe-to-Leu change in codon 4 and an Ile-to-Phe change in codon 9. Northern blot analysis of RNA isolated from both of these strains showed a lack of *ermC* mRNA stabilization in the presence of erythromycin (data not shown). Since the Phe-to-Leu change in codon 4 (BE194) and the Ile-to-Phe change in codon 9 by itself (20) had only a small effect on inducibility, but these changes had a large effect on inducibility when present together in BE245, it appears that these two mutations act in concert to give the poorly inducible

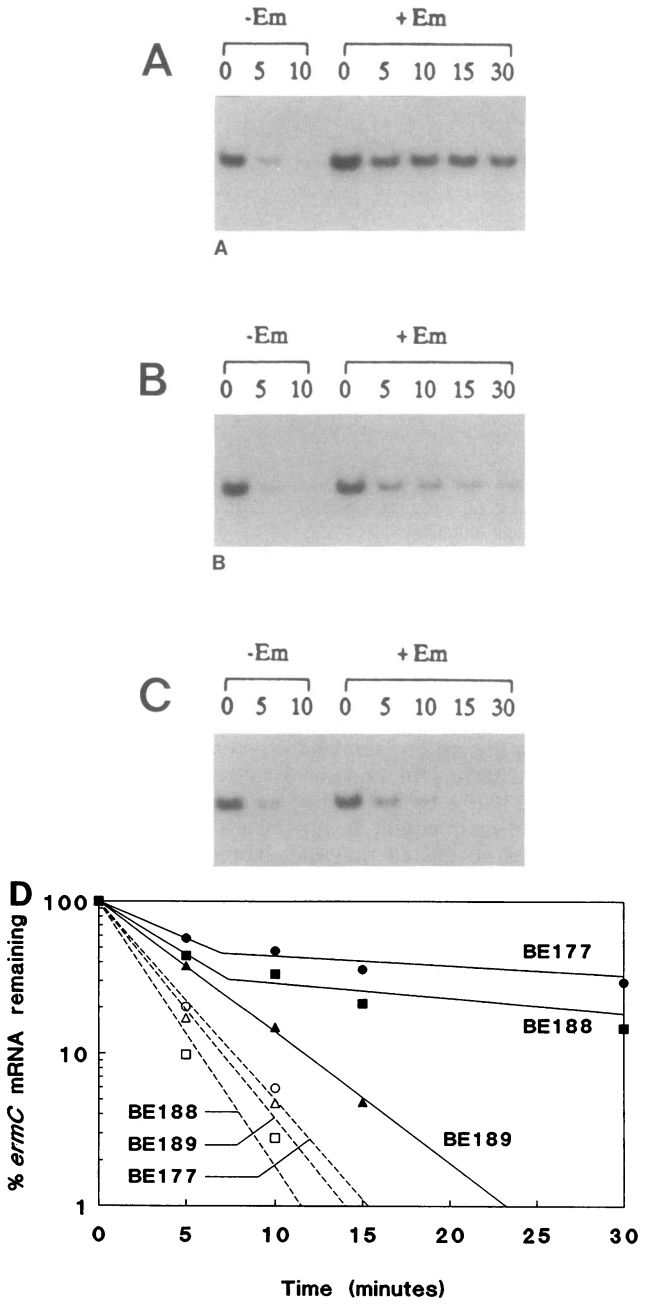

FIG. 4. Induced stability of *ermC* mRNA encoded by point mutations. RNAs isolated from BE177 (wild-type leader region) (A), BE188 (point mutations in codons 3 and 10) (B), and BE189 (point mutations in codons 4 and 5) (C) in the absence and presence of erythromycin were subjected to Northern blot analysis. The time (in minutes) after rifampin addition is indicated above each lane. (D) Plot of the data obtained from densitometric analysis of the Northern blots shown in panels A, B, and C.

phenotype of BE245. We also found a mutation in codon 6 (BE247; Ile to Thr) that did not affect inducibility. Mayford and Weisblum found previously that Asn in codon 6 caused a much-reduced inducibility, that Phe in codon 6 caused a small reduction in inducibility, and that Leu in codon 6 had no effect.

Induction of *ermC* mRNA stability without induction of translation. From the deletion mutations described above, we knew that neither the *ermC* leader stem-loop structure nor the entire leader peptide coding sequence was required for induced ribosome stalling. We therefore designed an *ermC* leader sequence that encoded an RNA in which ribosome stalling could confer induced mRNA stability but could not induce methylase translation. That is, we wished to obtain an RNA molecule in which a ribosome would stall in a position that was close enough to the 5′ end to provide protection against mRNA decay but that was upstream of the stem-loop structure, preventing induction of translation. Construction of such a molecule could enable us to assess separately the contribution of induced mRNA stabilization to *ermC* expression.

Construction of a leader peptide coding sequence in which ribosome stalling would occur upstream of the stem-loop structure was accomplished in stepwise fashion. Using oligonucleotide-directed mutagenesis we first constructed an *ermC* leader sequence that had a 9-nt insertion after codon 3 (BE207, Fig. 5B). The inserted sequence (Fig. 5B) represented alternate codons for the wild-type amino acids in positions 4, 5, and 6 (Phe, Ser, and Ile, respectively). We used alternate codons to avoid the possibility that repeated wild-type codons could lead to the formation of different leader stem-loop structures. In this construct the ribosome stall site is located at the same position in the stem structure as that in the wild type (BE177, Fig. 5A). Strain BE207, which contained this construct, was phenotypically wild type with respect to both plating on medium containing tylosin and erythromycin and induced *ermC* mRNA stability, as determined by Northern blot analysis (Fig. 6). This construct was then modified by an additional three-codon insert (BE218, Fig. 5C). In this construct, the first nine codons of the leader peptide sequence specified the same amino acids as those in the wild type, but they were predicted to be located in the RNA leader sequence upstream of the stem-loop. Strain BE218, which contained this construct, was poorly inducible as evidenced by the length of time required to form colonies on medium containing tylosin and erythromycin. Presumably this was because erythromycin-induced ribosome stalling occurred in the mutant leader peptide coding sequence upstream of the normal ribosome stall site, leading to inadequate opening of the stem-loop structure. Northern blot analysis was performed on RNA isolated from BE218, and *ermC* mRNA was found to be inducibly stable (Fig. 6). Finally, a mutation was constructed (BE258; Fig. 5D) that had an insertion of two codons and a stop codon after codon 8 of BE218. In this construct, leader peptide codons 1 through 10 encoded the wild-type amino acids and were located upstream of the stem-loop structure. These were followed by a stop codon, which would prevent ribosomes from translating codons that constitute part of the stem-loop structure. Strain BE258, which carried the plasmid with this last insert, did not grow at all on medium containing tylosin and erythromycin. However, Northern blot analysis showed that the *ermC* mRNA encoded by this plasmid was inducibly stable (Fig. 6). The results with strain BE258 are similar to those observed by Mayford and Weisblum with mutant m79 (21), in which the ribosome stall site was displaced upstream by the insertion of three serines after codon 9. Although ribosome stalling did occur at the displaced ribosome stall site in m79 leader mRNA, induction of expression was greatly reduced.

The finding that strain BE258 could not grow on medium containing tylosin and erythromycin was interpreted to mean

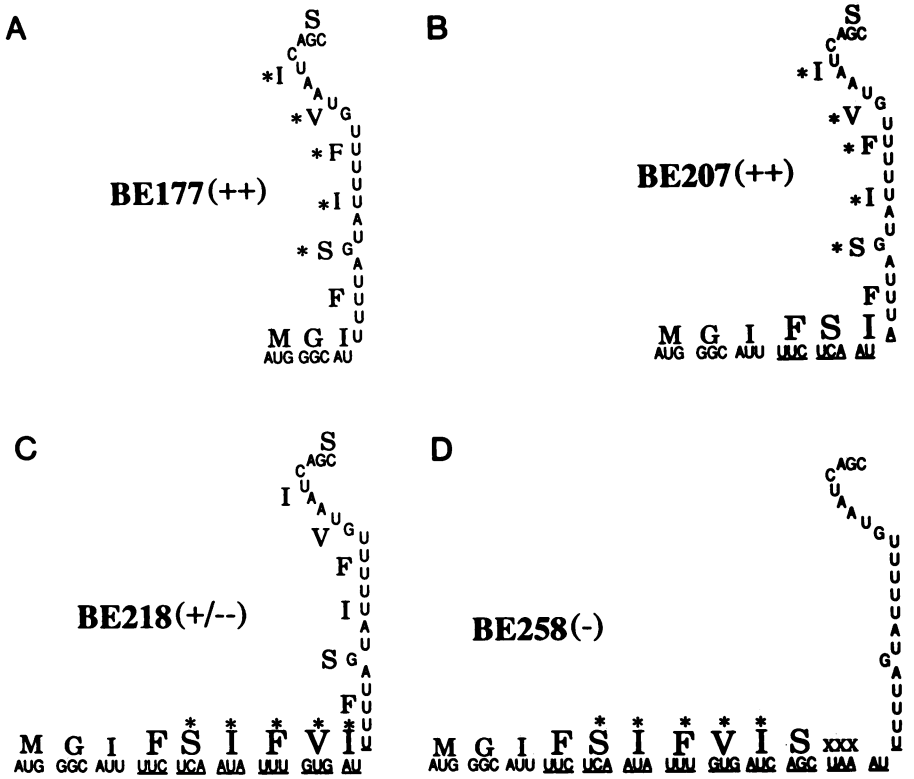

FIG. 5. Insertion mutations in the leader region. Partial structures of the wild-type *ermC* leader region RNA from BE177 (A) and of insertion mutations carried in BE207 (B), BE218 (C), and BE258 (D) are shown. Inserted sequences are underlined. The amino acids (single-letter code) encoded by the inserted codons are in large type. Starred amino acids indicate the ribosome stall site. The growth of strains carrying these constructs on medium containing tylosin and erythromycin is shown in parentheses (Table 1); +/−−, colonies of 1-to-2 mm were observed after 120 h at 32°C.

that translational induction cannot occur on this *ermC* mRNA because the site of ribosome stalling is too far removed from the stem-loop structure. Since induction of *ermC* mRNA stabilization was observed in strain BE258, it was possible that quantitative analysis of *ermC* expression in BE258 could provide an opportunity to measure the effect of *ermC* mRNA stabilization alone on *ermC* expression. Induction of methylase synthesis in the BE177 and BE258 strains was assayed by Western blot analysis. No increase in methylase expression in the BE258 strain could be detected up to 4 h postinduction (data not shown). As a more sensitive assay of *ermC* expression, an *ermC*::*lacZ* translational fusion was constructed. In previous work with *ermC*::*lacZ* fusions, we found that erythromycin-induced RNA stability for *ermC*::*lacZ* fusion RNAs was similar to that of *ermC* mRNA itself (4, 30). Therefore, expression of β-galactosidase activity from an *ermC*::*lacZ* fusion is an accurate measure of expression from mRNA that is stabilized by erythromycin-induced ribosome stalling. Strain BE259 carried an *ermC*::*lacZ* fusion in which the *ermC* coding sequence of BE258 was fused in frame with the *lacZ* coding sequence, similar to a previously described *ermC*::*lacZ* fusion (15). In this fusion, β-galactosidase expression was controlled by the *ermC* leader region. Induction of β-galactosidase in BE259 was measured in the presence and absence of erythromycin. The addition of erythromycin resulted in a 60% increase in the level of β-galactosidase (Table 2). This 60% increase could be a measure of the effect on expression of mRNA stabilization, or it could reflect a residual translational induction that was measurable by enzyme assay but that was not detectable by Western blotting and was not enough to allow the BE258 strain to grow on tylosin and erythromycin.

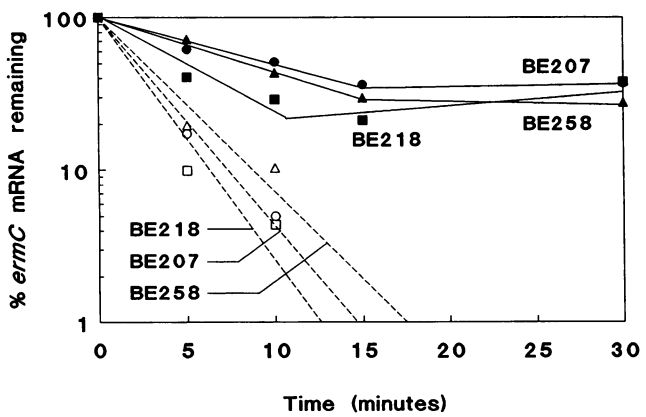

FIG. 6. Induced stability of *ermC* mRNA from strains carrying the insertion mutations depicted in Fig. 5.

To test the effect of mRNA stabilization on *ermC* gene expression in the absence of any possible translational induction, an *ermC*::*lacZ* transcriptional fusion (carried in strain BE232) was constructed in which the *lacZ* gene contained its own translational signals and was fused to the

TABLE 2. β-Galactosidase assays of translational and transcriptional *ermC*::*lacZ* fusions

Strain	Leader region[a]	Increase[b]	
		Without erythromycin	With erythromycin
BE261	Wild-type *ermC* leader sequence	1.03 ± 0.27	5.07 ± 1.04
BE259	*ermC* leader sequence with upstream erythromycin stall site (as in BE258)	0.94 ± 0.14	1.59 ± 0.21[c]
BE232	Wild-type *ermC* leader sequence up to the *Eco*RI site in pSD101	1.43 ± 0.11	1.45 ± 0.11

[a] The nature of the leader sequence of the *ermC*::*lacZ* fusion is indicated. BE261 and BE259 contain translational fusions at the *ermC Bcl*I site. BE232 contains a transcriptional fusion at the *Eco*RI site in the pSD101 leader region (Fig. 2).

[b] The values represent the fold increase in β-galactosidase activity after growth for 60 min in the presence of 0.2 μg of erythromycin per ml. Mean values ± standard deviations are given; $n = 4$.

[c] $P < 0.0001$.

wild-type *ermC* leader region sequence such that translation of the *lacZ* coding sequence was not regulated by *ermC* RNA secondary structure (see Materials and Methods). Northern blot analysis of RNA isolated from BE232 showed that the RNAs encoded by this *ermC*::*lacZ* fusion were inducibly stable (data not shown). Expression of β-galactosidase in BE232 was measured in the presence and absence of erythromycin. Since β-galactosidase expression in BE232 was constitutive, the level of β-galactosidase activity in this strain in the absence of erythromycin was 30-fold higher than the basal level in strain BE261. If induction of *ermC* mRNA stability alone could increase *ermC* expression, we would expect to be able to detect an increase in β-galactosidase activity in BE232 in response to the addition of erythromycin. In fact, the data in Table 2 show that there was no difference in β-galactosidase expression in the presence or absence of erythromycin. We conclude that stabilization of *ermC* mRNA does not cause a significant increase in *ermC* expression and that induced *ermC* gene expression in the presence of erythromycin is due primarily, if not entirely, to induction of translation.

DISCUSSION

According to the translational attenuation model for regulation of *ermC* gene expression, the RNA stem-loop structure of the *ermC* leader region plays a major role in controlling methylase translation. In the absence of erythromycin, the stem-loop structure sequesters the translational signals required for high-level methylase translation. Stalling of an erythromycin-bound ribosome in the *ermC* leader peptide coding sequence disrupts base pairing in the stem structure, allowing rapid induction of methylase synthesis. We have shown previously that ribosome stalling also results in stabilization of the *ermC* message. The goal of the present work was to study the requirements for induction of *ermC* mRNA stabilization. A priori, several components of the *ermC* regulatory system could have been necessary for induced stability. First, methylase protein itself could play a role in mRNA stability, since Dubnau and co-workers have shown that *ermC* gene expression is regulated by translational autorepression (9), probably by binding of methylase protein to the *ermC* leader region (6). Second, according to the view that message stability is a function of coverage by translating ribosomes (7), induced translation of the methylase coding sequence could have been required for induced mRNA stability. Although we had shown previously that a mutation of SD2 that severely decreased methylase translation did not affect induced mRNA stability (3), a small amount of methylase protein could still be detected in minicells carrying this SD2 mutation, so that we could not rule out completely a role for methylase translation in *ermC* mRNA stability. Third, the secondary structure of the *ermC* leader region could have been required to act together with erythromycin binding to slow down and stall the ribosome at the correct location in the leader peptide coding sequence. Finally, when these studies were initiated, it was not known how much of the leader peptide coding sequence was actually required for ribosome stalling.

The decay pattern of *ermC* mRNA from four strains carrying *ermC* deletion mutations is presented in Fig. 3. We chose to examine these particular mutations, since the extent of the deletions in these mutations was such that we could test the relevance of the *ermC* components mentioned above to induced mRNA stability. *ermC* mRNA encoded by all four of these deletion mutations was inducibly stable in the presence of erythromycin, demonstrating that erythromycin-induced ribosome stalling was occurring in all cases. The fact that ribosome stalling occurs in the absence of methylase protein shows that induction is independent of the autorepression mechanism, in agreement with the results of Breidt and Dubnau (6). Induction of *ermC* mRNA stability in the strain deleted for SD2 rules out the hypothesis that stability is a result of induced methylase translation, i.e., that the flow of ribosomes along the *ermC* message provides protection against decay. The results of Sandler and Weisblum with *ermA*, a related MLS resistance gene, also showed that induced mRNA stability could not be explained by increased ribosome density on *ermA* mRNA (25). Finally, the dispensability of *ermC* leader region secondary structure and the carboxy-terminal portion of the leader peptide coding sequence for induced stability indicate that the codons constituting the ribosome stall site are sufficient for stalling of an erythromycin-bound ribosome and protection of the 5′ end from decay. We note that the difference between the induced and uninduced half-lives of *ermC* mRNA encoded by the deletion mutations was variable (Fig. 3). The *ermC* mRNA from strain BE98, for example, is somewhat stable even in the absence of erythromycin. It may be that alterations at the 5′ end that change the structure of the mRNA lead to differences in recognition by the host decay machinery and in the rate of initiation of decay.

Mayford and Weisblum constructed missense mutations in the leader peptide coding sequence and measured the effect of these mutations on induction of translation by assaying for β-galactosidase activity from an *ermC*::*lacZ* fusion (20). They concluded that codons 5 through 9 constitute the critical codons required for ribosome stalling. A more recent report by Weisblum's group showed that codon 4 was involved in induction by celesticetin, another inducing antibiotic, and that deletion of codons 2 through 4 resulted in reduced inducibility by erythromycin (22). New missense mutations described here (Table 1) show that codons 3 and 4 are also involved in erythromycin induction. The erythromycin stall site therefore begins at codon 3 and extends through codon 9. The inclusion of codons 3 and 4 in the erythromycin stall site correlates with previous reports on the inhibition of protein synthesis by erythromycin that suggested an inhibition of chain elongation when the nascent

peptide reached between two and five residues (1, 28). We speculate that the particular codon sequence of the leader peptide has been selected to ensure that ribosome stalling occurs at a position on *ermC* leader mRNA that allows maximal induction of methylase translation. It would be of interest to determine whether ribosome stalling could occur if the sequence of codons 3 through 9 would be located elsewhere, in a different coding sequence.

We observed that combinations of single changes, which by themselves caused little reduction of inducibility, resulted in poor or no growth on tylosin and erythromycin. This could suggest that ribosome stalling in the *ermC* leader peptide coding sequence may be due to a gradual retardation of an erythromycin-bound ribosome that is translating codons 3 through 9. Missense mutations in the leader peptide coding sequence can prevent this slowdown, but the effect of certain single mutations is not enough to result in a mutant phenotype unless additional mutations are also present.

Since erythromycin-induced ribosome stalling causes induction of both translation and mRNA stability, we were unable previously to assess whether *ermC* mRNA stabilization was a contributing factor to the induced level of methylase expression. Earlier experiments with *ermC* in vitro excluded the possibility that induction of *ermC* expression was due entirely to mRNA stabilization (23). However, it was possible that the final induced level of methylase translation was due to the combination of increased translation and increased mRNA stability. The insertion mutations in the leader peptide coding sequence that are described in this report (Fig. 5) were designed to position the ribosome stall site such that ribosome stalling could not cause induction of translation but could still cause mRNA stabilization. Increasing the distance of the ribosome stall site from the stem-loop structure resulted in decreased induction, as evidenced by growth on tylosin and erythromycin, but had little effect on induced stability (Fig. 6).

The construct in which the erythromycin stall site was located entirely upstream of the stem-loop sequence and was followed by a stop codon (carried in strain BE258) resulted in a total loss of inducibility, since this strain could not grow on tylosin and erythromycin. However, *ermC* mRNA encoded by this construct was still inducibly stable. The addition of erythromycin to strain BE259, which contained an *ermC*::*lacZ* fusion that had the *ermC* leader region with this upstream stall site, resulted in a reproducible 60% increase in β-galactosidase expression (Table 2). Since the addition of erythromycin did not cause induction of β-galactosidase expression in a strain containing an *ermC*::*lacZ* transcriptional fusion (BE232; Table 2), despite the fact that the addition of erythromycin did result in mRNA stabilization in this strain, we conclude that mRNA stabilization is not an important factor in induced *ermC* gene expression. The 60% increase in expression observed in BE259 is probably due to a small amount of translational induction.

The earlier report of Shivakumar et al. (27), which was the first demonstration of induced *ermC* mRNA stability, showed clearly, at least in minicells, that stabilized *ermC* mRNA in the presence of rifampin was capable of being translated. In studies on the *ermA* gene, Sandler and Weisblum reported that induced stabilization of a mutant *ermA*::*lacZ* mRNA that was constitutively expressed (*ermA C26*) did not lead to an increase in β-galactosidase expression (25). This is consistent with our results with the *ermC*::*lacZ* fusion in strain BE232 (Table 2). However, in a subsequent report on *ermA* stabilization, Sandler and Weisblum found that expression of the *cat-86* gene product from an *ermA*::*cat-86* construct increased upon induction of *ermA*::*cat-86* mRNA stability (26). We suggest that, although the stabilization of an mRNA should increase total message concentration and should therefore have an effect on translational yield (17), the presence of a stalled ribosome in the 5′-leader region could result in long-range mRNA-ribosome interactions that negatively affect translation of the coding sequence. The level of induced methylase translation observed with wild-type *ermC* would then be the combined result of the positive effect of translational induction and the negative effect of the stalled ribosome. The negative effect of the stalled ribosome on downstream translation would explain why increased β-galactosidase expression is not observed for constitutively expressed *lacZ* sequences fused to the ribosome stalling moieties of *ermC* or *ermA*. On the other hand, this negative effect may be specific to certain structural elements in the coding sequence and so may not occur in the *ermA*::*cat-86* fusion mRNA.

That the stalled ribosome-*ermC* mRNA complex may have unique properties is also suggested by the fact that stalling of a ribosome in the leader region alone can protect diverse downstream sequences from decay (3). In our current understanding of induced stability, the stalled ribosome protects against initiation of decay from the 5′ end, perhaps by a 5′-to-3′ exoribonuclease (4, 26). It remains to be explained how stalling of a ribosome in the leader region protects *ermC* mRNA from exonucleolytic attack at the 3′ end or endonucleolytic attack at internal sites, which, by analogy to *Escherichia coli* (5), should be major modes of mRNA decay also in *B. subtilis*. A novel conformation or localization of *ermC* mRNA that has a stalled ribosome at its 5′ end could explain both induced stability and the lack of increased expression by mRNA stabilization.

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