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The *ermC* Leader Peptide: Amino Acid Alterations Leading to Differential Efficiency of Induction by Macrolide-Lincosamide-Streptogramin B Antibiotics

MARK MAYFORD[1] AND BERNARD WEISBLUM[2]*

Department of Molecular Biology, University of Wisconsin,[1] and Department of Pharmacology, University of Wisconsin Medical School,[2] Madison, Wisconsin 53706

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The inducibility of *ermC* by erythromycin, megalomicin, and celesticetin was tested with both wild-type *ermC* and several regulatory mutants altered in the 19-amino-acid-residue leader peptide, MGIFSIFVISTVHYQP NKK. In the model test system that was used, the ErmC methylase was translationally fused to β-galactosidase. Mutational alterations that mapped in the interval encoding Phe-4 through Ile-9 of the leader peptide not only affected induction by individual antibiotics, but did so differentially. The subset of mutations that affected inducibility by the two macrolides erythromycin and megalomicin overlapped and were distinct from the subset of mutations that affected induction by celesticetin. These studies provide a model system for experimentally varying the relative efficiencies with which different antibiotics induce the expression of *ermC*. The possibility that antibiotics with inducing activity interact directly with the nascent leader peptide was tested by using a chemically synthesized decapeptide, MGIFSIFVIS—, attached at its C-terminus to a solid-phase support. This peptide, however, failed to bind erythromycin in vitro.

The *ermC* gene encodes an inducible rRNA methylase that confers resistance to the macrolide-lincosamide-streptogramin B (MLS) group of procaryotic translation inhibitors (24). Exposure of cells containing *ermC* to nanomolar concentrations of erythromycin (Em), a macrolide antibiotic, results in about a 10-fold increase in the intracellular level of methylase protein during a 60-min period (22), and a model for regulation of *ermC* has been proposed (9, 12). According to the model, *ermC* mRNA is transcribed constitutively, but only small amounts of methylase protein are produced due to sequestration of the methylase ribosome-binding site and initiator Met codon by mRNA secondary structure, as shown in Fig. 1.

In the presence of Em, an antibiotic-bound ribosome is thought to stall while translating the 19-amino-acid leader peptide located upstream from the methylase-coding region. Genetic (18) and biochemical (19) studies have suggested that the Em-bound ribosome stalls at leader peptide codons Val-8 and Ile-9, causing the *ermC* transcript to assume an alternative (active) conformation in which segments 2 and 3 become associated and the methylase translation initiation sequence becomes unpaired. This realignment of complementary segments allows ribosomes to initiate methylase translation at a higher rate than they can with mRNA in the repressed conformation shown.

Inducible MLS resistance is found in a wide range of bacterial genera. The induction specificity, defined as the subset of MLS antibiotics that induce expression of a particular MLS resistance determinant, can differ depending on the organism (8, 11). Inducible MLS resistance determinants from several different organisms have been cloned and studied in detail, and they appear to be regulated by a translational attenuation mechanism (6, 11, 13). The molecular basis for the variability of induction specificity is not clear; however, it has been shown that two different determinants, *ermC* and *ermAM*, with different induction specificities, behave in *Bacillus subtilis* as they did in their original hosts, *Staphylococcus aureus* and *Streptococcus sanguis*, respectively. These observations suggest that induction specificity is determined by a *cis*-acting element associated with a particular MLS resistance determinant.

In transcriptional attenuators that regulate amino acid-biosynthetic operons, there is a clear correlation between the amino acid sequence of the leader peptide and the amino acid whose abundance regulates expression of the operon (15). The *Salmonella typhimurium leu* operon, in particular, is regulated by transcriptional attenuation; it contains four adjacent Leu codons in the leader peptide, and expression is enhanced under conditions of leucine deficiency (3). However, when the four sequential Leu codons are converted to four sequential Thr codons by a frameshift in the leader peptide, expression is enhanced by threonine deficiency instead (3). The amino acid sequence of the leader peptide thus determines the efficiency with which a stimulus will regulate the transcriptional attenuator.

For translational attenuators, diversity in both the structure of the ribosome (10) and the amino acid sequence of the leader peptide (13, 14) has been shown to contribute to differences in the induction specificity of translational attenuators, i.e., differences in the relative efficiency with which antibiotics function as inducers. In the present study, we examined a set of leader peptide mutants with respect to their inducibility by MLS antibiotics and indeed found that the levels of induction by Em, megalomicin (Meg), and celesticetin (Cel), respectively, did not vary in the same proportion. These findings provide useful model system with which to study systematic alterations in leader peptide amino acid sequences and the consequent altered induction efficiency of an antibiotic.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *ermC* leader peptide base substitution mutations were carried on plasmid pMM222 and have been described previously (18). Several

* Corresponding author.

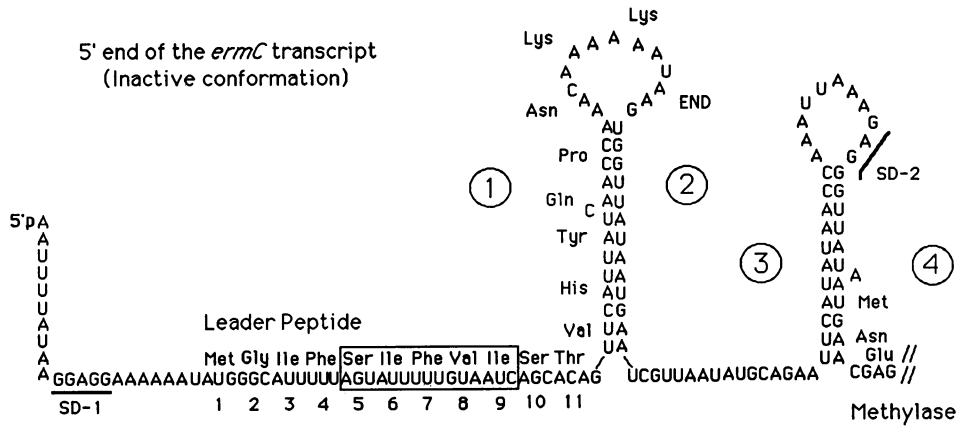

FIG. 1. Structure of the 5' end of the *ermC* transcript. Nucleotide sequence and predicted secondary structure of the 5' end of the translationally inactive form of the *ermC* transcript. Translation of the boxed amino acids is critical for Em-induced expression of *ermC*. SD-1 and SD-2 refer to the ribosome-binding sites for translation initiation of the leader peptide and methylase, respectively. The numbers 1, 2, 3, and 4 in circles denote segments of the transcript capable of forming stem-loop structures by intramolecular base-pairing. In the induced form of the *ermC* message, segments 2 and 3 are paired, while segments 1 and 4 are unpaired. Leader peptide residues 1 through 11 are numbered.

additional mutants were constructed for this work by replacement of the 127-nucleotide *Sac*I-*Pst*I fragment of pMM222 with double-stranded synthetic oligonucleotides containing the desired sequence alterations, as described (18), leading to leader peptide insertion mutants m79, m80, and m81, deletion mutants m82, m83, m84, and m85, and codon 9 stop mutant m86.

Antibiotics. The following antibiotics were used and were gifts from the pharmaceutical firms indicated: celesticetin (Upjohn), erythromycin (Abbott Laboratories and Upjohn), fluorothiamphenicol (Schering-Plough Corporation), lincomycin (Upjohn), maridomycin (Takeda Chemical Co.), megalomicin (Schering-Plough), oleandomycin (Chas. Pfizer Inc.), and tylosin (Eli Lilly & Co.).

Construction of leader peptide deletion mutants d17 and d33. Plasmid pMM210, which was identical to pMM222 except that it contained an intact *ermC* gene rather than an *ermC-lacZ* fusion, was digested with *Apa*I, followed by phenol extraction and ethanol precipitation. The DNA was suspended in 800 μl of Bal-31 buffer (20 mM Tris hydrochloride [pH 8.0], 600 mM NaCl, 12.5 mM $MgCl_2$, 12.5 mM $CaCl_2$) and digested with 8 U of Bal-31 slow enzyme (International Biotechnologies, Inc., New Haven, Conn.) at 30°C. Samples (80 μl) were removed every 3 min for up to 30 min and placed in 50 mM EDTA-saturated phenol. The DNA was phenol extracted, ethanol precipitated, and treated with DNA polymerase Klenow fragment to fill in the ends. *Apa*I linkers were added, followed by digestion with *Pst*I and electrophoresis on a 10% polyacrylamide gel. Fragments that migrated with mobilities corresponding to 50 to 80 base pairs were excised and eluted from the gel. The resultant DNA fragments were ligated with plasmid pMM222 that had been digested with *Apa*I and *Pst*I. The ligated DNA was used to transform *Escherichia coli* CSH26. Colonies were picked, and plasmid DNA within the leader peptide region was sequenced.

Induction assays. Induction of *ermC* was measured in terms of β-galactosidase activity produced by a translational fusion between *ermC* and *E. coli lacZ*. Determination of enzymatic activity (in terms of Miller units [19a]) with a microtiter plate technique was described previously (18).

RESULTS

Inducibility of base substitution mutants. The construction of a series of *ermC* leader peptide base substitution mutants was described previously (18). The mutations were carried on plasmid pMM222 as part of an in-phase *ermC-lacZ* translational fusion. The macrolide Meg and the lincosamide Cel have both been shown previously to induce staphylococcal *erm* genes (2, 20). In the present study, the ability of these antibiotics to induce β-galactosidase expression in *B. subtilis* cells carrying the wild-type *ermC-lacZ* fusion was measured, and optimal concentrations were determined to be 30 μg/ml for Meg and 10 μg/ml for Cel. The inducibility of *ermC* leader peptide base substitution mutants by Meg at 30 μg/ml and Cel at 10 μg/ml was assayed as described previously (18), and the results are summarized in Table 1 along with the inducibility data for Em obtained previously, which are included for comparison. A summary of the effect of leader peptide changes on the inducibility of *ermC* by Em, Meg, and Cel is shown in Fig. 2. The comparison only went as far as Ile-9 because, as we shall show below, a nonsense codon at the Ser-10 codon position permitted inducibility by Em, Meg, and Cel.

Several patterns of altered specificity were distinguished. Mutants m5, m8, and m11 showed reduced inducibility by Cel and unaltered inducibility by both Em and Meg. Mutants m15 and m18, in contrast, showed reduced inducibility by both Em and Meg and unaltered inducibility by Cel. Mutants m20 and m24 were predominantly affected in inducibility by Em, whereas mutant m28 was predominantly affected in inducibility by Meg. Mutant m22 showed reduced inducibility by Em, Meg, and Cel, whereas mutants m19, m25, m26, m27, and m29 showed no altered induction. In the collection of mutants studied, there may be an association between reduced inducibility by Cel and the location of critical amino acids close to the amino terminus, e.g., m5, m8, m11, and m46. Thus, single-base substitutions in codons for the leader peptide altered the induction specificity of *ermC*.

Inducibility of nonsense mutants. The above results suggest that the ribosome stall induced by Cel and possibly Meg occurs upstream from the codon for leader peptide residue Ser-10, as demonstrated previously for Em-induced stall

TABLE 1. Induction of *ermC* base substitution mutants by Em, Meg, and Cel[a]

Mutant	Leader peptide sequence	Codon change	Inducibility (relative β-galactosidase activity)		
			Em	Meg	Cel
WT	M G I F S I F V I S T V	None	7.7 ± 1.1	6.5 ± 1.3	6.0 ± 1.1
m1	– C – – – – – – – – –	GGC→TGC	8.7 ± 1.2	9.1 ± 0.8	10.2 ± 3.8
m2	– – L – – – – – – – –	ATT→CTT	9.3 ± 2.2	6.9 ± 1.2	6.5 ± 1.0
m3	– – P – – – – – – – –	ATT→CCT	6.6 ± 1.9	6.5 ± 0.7	6.5 ± 1.5
m4	– – – V – – – – – – –	TTT→GTT	9.3 ± 2.2	6.1 ± 0.5	6.7 ± 2.4
m5	– – – I – – – – – – –	TTT→ATT	8.5 ± 1.9	5.7 ± 0.8	3.8 ± 1.4
m6	– – – C – – – – – – –	TTT→TGT	8.3 ± 1.7	9.3 ± 1.3	9.9 ± 0.7
m7	– – – – G – – – – – –	AGT→GGT	9.7 ± 1.7	6.4 ± 1.5	6.6 ± 2.5
m8	– – – – R – – – – – –	AGT→CGT	8.6 ± 1.9	7.1 ± 0.9	2.7 ± 1.2
m9	– – – – C – – – – – –	AGT→TGT	8.3 ± 1.2	6.8 ± 0.8	7.3 ± 1.5
m10	– – – – T – – – – – –	AGT→ACT	8.3 ± 2.7	6.3 ± 1.2	7.9 ± 2.8
m11	– – – – N – – – – – –	AGT→AAT	9.1 ± 2.3	7.4 ± 1.6	4.6 ± 1.7
m12	– – – – I – – – – – –	AGT→ATT	4.6 ± 1.1	4.8 ± 1.7	7.7 ± 3.3
m13	– – – – – L – – – – –	ATT→CTT	8.7 ± 1.6	6.7 ± 1.7	8.2 ± 1.5
m14	– – – – – F – – – – –	ATT→TTT	5.4 ± 1.1	4.8 ± 1.1	6.2 ± 1.1
m15	– – – – – N – – – – –	ATT→AAT	2.9 ± 0.2	3.2 ± 0.8	7.9 ± 3.0
m16	– – – – – – Y – – – –	TTT→TAT	11.9 ± 0.6	8.6 ± 1.6	10.1 ± 3.7
m17	– – – – – – L – – – –	TTT→CTT	2.6 ± 0.3	4.6 ± 0.9	4.6 ± 0.8
m18	– – – – – – C – – – –	TTT→TGT	3.0 ± 0.3	3.1 ± 0.6	9.7 ± 3.2
m19	– – – – – – – I – – –	GTA→ATA	7.7 ± 1.5	5.8 ± 1.8	6.4 ± 1.7
m20	– – – – – – – A – – –	GTA→GCA	3.7 ± 0.9	5.6 ± 0.7	7.7 ± 3.0
m21	– – – – – – – E – – –	GTA→GAA	2.9 ± 0.8	4.3 ± 0.8	4.3 ± 0.5
m22	– – – – – – – K – – –	GTA→AAA	1.8 ± 0.4	3.7 ± 0.5	3.9 ± 0.7
m23	– – – – – – – L – – –	GTA→CTA	1.4 ± 0.3	7.4 ± 1.9	6.5 ± 2.5
m24	– – – – – – – L – – –	GTA→TTA	1.7 ± 0.3	7.0 ± 1.3	6.4 ± 2.5
m25	– – – – – – – – V – –	ATC→GTC	8.0 ± 2.6	8.7 ± 1.2	8.6 ± 2.3
m26	– – – – – – – – L – –	ATC→CTC	7.8 ± 1.9	5.8 ± 1.1	6.7 ± 1.1
m27	– – – – – – – – N – –	ATC→AAC	7.3 ± 2.4	5.6 ± 1.2	6.9 ± 1.4
m28	– – – – – – – – S – –	ATC→TCC	6.7 ± 0.9	4.6 ± 0.1	7.3 ± 1.4
m29	– – – – – – – – I – –	ATC→ATT	6.2 ± 1.8	8.2 ± 2.2	6.4 ± 1.2
m45	– C N – – – – – – – – –		9.4 ± 2.5	9.4 ± 1.3	8.8 ± 1.9
m46	– S – – R – – – – – – –		8.7 ± 2.3	8.0 ± 2.0	2.9 ± 1.1
m47	– – – – G – Y – – – – –		7.3 ± 3.1	4.7 ± 0.3	5.6 ± 2.2
m56	– – – – – – – – F – S –		4.4 ± 0.1	3.8 ± 0.4	7.6 ± 2.6
m57	– – – V – – L – – – – –		2.1 ± 0.8	2.6 ± 0.4	3.3 ± 0.9
m60	– – – – I – – Q – – – –		1.4 ± 0.6	2.5 ± 0.4	4.5 ± 0.8
m67	– – – C T – – I – – – –		3.7 ± 0.2	4.1 ± 0.6	6.7 ± 1.6
m68	– – – – – – L – S – – A		1.6 ± 0.2	3.3 ± 0.5	3.9 ± 0.8
m71	– – – – – – C E L – – G		1.6 ± 0.3	2.6 ± 0.4	4.4 ± 1.2
m72	– – – – – – L *		1.5 ± 0.1	2.6 ± 0.2	3.4 ± 0.6
m73	– – – – – F L *		1.5 ± 0.4	2.6 ± 0.1	3.6 ± 0.9
m74	– – – – – – – – – *	AGT→TAA	4.7 ± 1.6	5.2 ± 0.5	4.4 ± 0.7
m75	– – – – – – – – – – – *	GTT→TAA	3.2 ± 0.2	3.2 ± 0.4	3.6 ± 0.6

[a] The sequence of the first 12 amino acids of the leader peptide is shown for the wild type (WT). Amino acid substitutions are shown, and the associated codon change is listed for mutants in which a single codon was changed. An asterisk indicates a stop codon. Concentrations of antibiotics used for induction were: Em, 0.1 μg/ml; Meg, 30 μg/ml; and Cel, 10 μg/ml. Inducibility is defined as β-galactosidase activity measured 90 min after the addition of antibiotic divided by the activity of the time-zero sample. The values listed and their standard deviations from the mean were based on averaging three measurements. Induced β-galactosidase activities of less than 5 are underlined. The uninduced 90-min control sample activity was approximately 1. Mutants m74 and m75 were considered inducible after taking into consideration the twofold-higher basal level of expression seen (about 500 U) compared with the basal level (about 250 U) found in the other mutants listed above.

(18). The ability of Em, Meg, and Cel to induce expression was therefore tested in two *ermC* leader peptide mutants in which UAA stop codons had been inserted at the codons for Ile-9 (mutant m86) and Ser-10 (mutant m74). The results are shown in Fig. 3. In both mutants the basal level of expression was elevated compared with the wild type for reasons discussed previously (18). All three antibiotics were able to induce normal maximal levels of β-galactosidase activity in mutant m74 (UAA at the codon for Ser-10); however, for mutant m86 (UAA at the codon for Ile-9), Meg and Cel were more effective inducers than Em. These observations are consistent with a model in which SIFVI constitutes the critical sequence for induction by Em but FSIFV constitutes the critical sequence for induction by Cel and possibly Meg.

Inducibility of leader peptide deletion mutants. The effect of single-base substitutions on the inducibility of *ermC* suggested that the codons for leader peptide residues Gly-2, Ile-3, and, depending on the antibiotic, Phe-4 were dispensable for antibiotic-induced expression. A set of leader peptide deletion mutants were therefore constructed in which the codons for Gly-2 and Ile-3, Gly-2 through Phe-4, and Gly-2 through Ser-5 were removed. The inducibility of these deletion mutants by Em, Meg, and Cel was assayed, and the results are shown in Table 2.

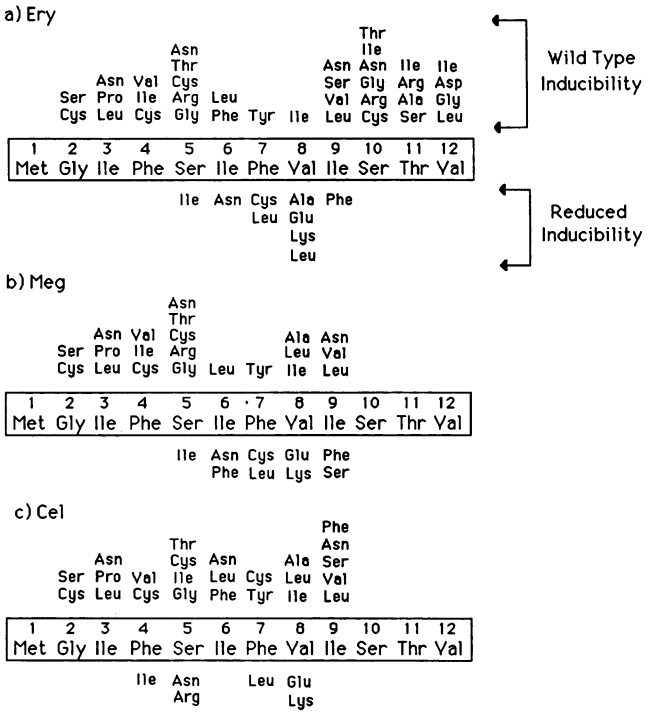

FIG. 2. Effect of leader peptide base substitution mutations on *ermC* inducibility. The wild-type sequence of the first 12 amino acids of the *ermC* leader peptide is shown within the box. Substitutions that reduced the inducibility of *ermC* to less than 5 (see Table 1, footnote *a*) are shown below the wild-type amino acid sequence, and substitutions that maintained an inducibility of greater than 5 are shown above the sequence. Ery, Erythromycin (Em).

Mutant m83, in which the codons for leader peptide residues Gly-2 and Ile-3 were deleted, remained fully inducible by Em, Cel, and Meg. Mutant m84 was especially noteworthy because it was not inducible by Em despite the intact SIFVI sequence that we have shown plays a critical role in induction. This observation points to additional physical properties of the leader peptide that are critical for induction and will be discussed below. In any event, mutant m84 was still inducible by Cel. Deletion of the codons for leader peptide residues Gly-2 through Ser-5 (mutant m85) reduced the inducibility by all three antibiotics to less than threefold.

Inducibility of leader peptide insertion mutants. The *ermC* regulatory model postulates that antibiotic-induced ribosome stall within the leader peptide directly disrupts association of stem segment 1 with either segment 2 or segment 4. The genetic localization of the Em-induced ribosome stall site at or upstream from the codon for Ser-10 (18) coupled with in vivo footprinting experiments involving protection of *ermC* mRNA during induction (19) suggest that a stalled ribosome would cover only the lower portion of stem segment 1. If the respective stall sites for Em and Cel are indeed located as suggested by the experiments described above, upstream displacement of these stall sites by an inserted stuffer sequence should result in differential changes of inducibility when stem segment 1 is out of range of the disruptive influence of the stalled ribosome. Additionally, downstream displacement of the Em and Cel stall sites to bring them closer to the Val-12 codon at the base of stem segment 1 should allow inducibility to remain at wild-type levels.

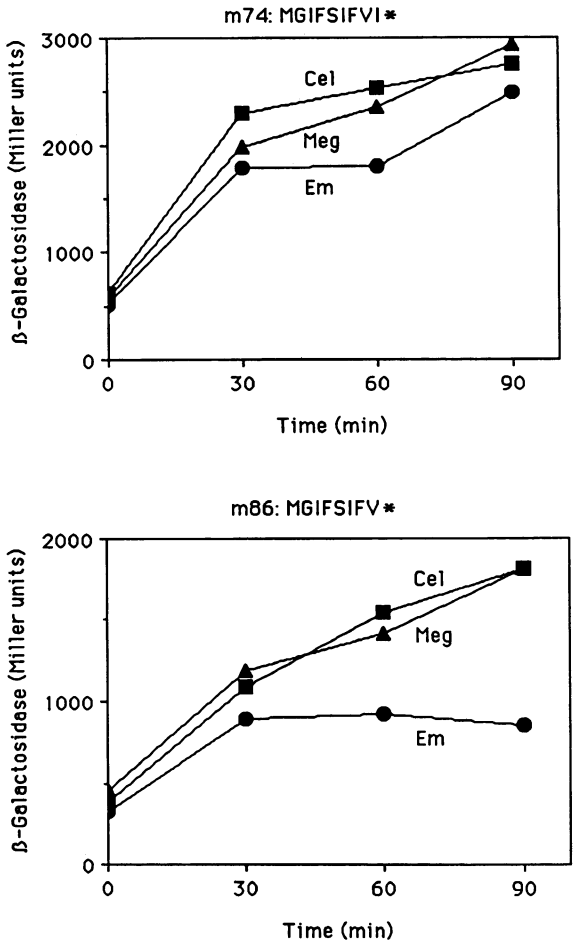

FIG. 3. Induction of *ermC* leader peptide nonsense mutants. β-galactosidase activity was determined by a microtiter plate assay as described in the text. Concentrations of antibiotics used for induction were: Em, 0.1 μg/ml; Meg, 30 μg/ml; and Cel, 10 μg/ml. Inducibility is defined as the β-galactosidase activity 90 min after the addition of antibiotic divided by the activity before the addition of antibiotic.

To test the differential effects of displacing the stall site on *ermC* inducibility, the distance between the codon for leader peptide residue Ile-9 and stem segment 1 was altered systematically by deletion or insertion of DNA between the codons for Ile-9 and Val-12. The inducibility of the mutants that were generated was analyzed, and the results are summarized in Table 2. Deletion of the codons for Ser-10 and Thr-11 (mutant m82) or the insertion of a single AGC (Ser) between the codons for leader peptide residues Ile-9 and Ser-10 (mutant m79) did not affect *ermC* inducibility by Em, Meg, or Cel. However, the insertion of two or three Ser codons, $(AGC)_2$ or $(AGC)_3$, between the codons for leader peptide residues Ile-9 and Ser-10 (mutants m80 and m81, respectively) reduced the inducibility by Em, Meg, and Cel to about threefold. These observations point to a maximal allowable distance of about 12 nucleotides between the ribosome stall codon (Ile = 9) and the critical stem-loop structure whose integrity is disrupted during induction of *ermC*.

Inhibition of protein synthesis near stem segment 1 does not suffice for induction of *ermC*. Our findings leave open the question of whether amino acids at the beginning of the

TABLE 2. Induction of *ermC* leader peptide amino-terminal insertion and deletion mutants

Mutant	Leader peptide sequence[a]	Inducibility (relative β-galactosidase activity)[b]		
		Em	Meg	Cel
WT	MGIFSIFVISTV—stem 1	7.7 ± 1.1	6.5 ± 1.3	6.0 ± 1.1
m79	MGIFSIFVISSTV—stem 1	7.2 ± 0.8	5.2 ± 0.4	5.8 ± 0.5
m80	MGIFSIFVISSSTV—stem 1	3.0 ± 0.6	2.6 ± 1.1	3.5 ± 0.8
m81	MGIFSIFVISSSSTV—stem 1	3.3 ± 1.4	2.5 ± 0.9	3.8 ± 0.3
m82	MGIFSIFVI--V—stem 1	6.8 ± 1.0	6.1 ± 1.2	6.7 ± 0.9
m83	M--FSIFVISTV—stem 1	7.3 ± 0.9	7.3 ± 1.7	6.7 ± 0.5
m84	M---SIFVISTV—stem 1	3.7 ± 1.2	3.6 ± 1.5	5.8 ± 0.6
m85	M----IFVISTV—stem 1	1.9 ± 0.6	2.6 ± 0.6	2.5 ± 0.2

[a] The wild-type (WT) sequence is shown with the critical SIFVI sequence underlined (see text for discussion). Deletions (-) in the various mutants are indicated.
[b] See Table 1, footnote *a*.

leader peptide could provide a stall site for induction by MLS antibiotics but that induction is not seen for many MLS antibiotics because the relevant amino acids are too far upstream from stem segment 1 to be able to destabilize it. Two deletion mutants, d17 and d33, were therefore constructed that brought the initiator Met closer to segment 1. Stem segments 1 and 2 in the two deletion mutants were identical to their wild-type counterparts, and the difference in amino acids encoded by segment 1 is ascribed to the frameshift resulting in the construction.

The structures of d17 and d33 are shown in Fig. 4. No induction was seen with the set of 15 MLS antibiotics listed. From these observations, we infer that the failure of an MLS antibiotic to induce expression cannot be ascribed to inhibition of protein synthesis per se, but to the stabilized association between ribosome and messenger that occurs during translation of the critical codons. Part of the critical function of these codons is dictated by the amino acids that they encode; part is specified by the distance of these amino acids from both the amino terminus and from stem segment 1, whose association with segment 2 is either prevented or reversed.

Leader peptide affinity chromatography. A model to explain the sensitivity of antibiotic-induced expression of *ermC* to changes in the leader peptide sequence involves the direct interaction of the nascent leader peptide with the inducing antibiotic to produce ribosome stall. We were unable to demonstrate such binding to a chemically synthesized peptide consisting of the first 10 amino acids of the *ermC* leader peptide (data not shown).

DISCUSSION

Induction in a series of *ermC* leader peptide base substitution mutants by Em, Meg, and Cel was measured. The ability of each of these three antibiotics to induce *ermC* expression was differentially affected in the mutants analyzed. The subsets of mutants in which reduced *ermC* inducibility was seen for the two macrolide antibiotics (Em and Meg) involved base substitutions in the codons specifying the residues Ser-5 through Ile-9; they overlapped strongly. The subset of mutants displaying reduced inducibility by Cel had alterations in approximately the same region but was distinct from the subset of mutants displaying reduced inducibility by the two macrolides.

Leader peptide amino acid alterations in other translational attenuators. The role of individual amino acids in leader peptides associated with *cat* translational attenuators has also been examined. Dick and Matzura (5) have studied the 9-amino-acid leader peptide MKKSEDYSS of the pUB112-

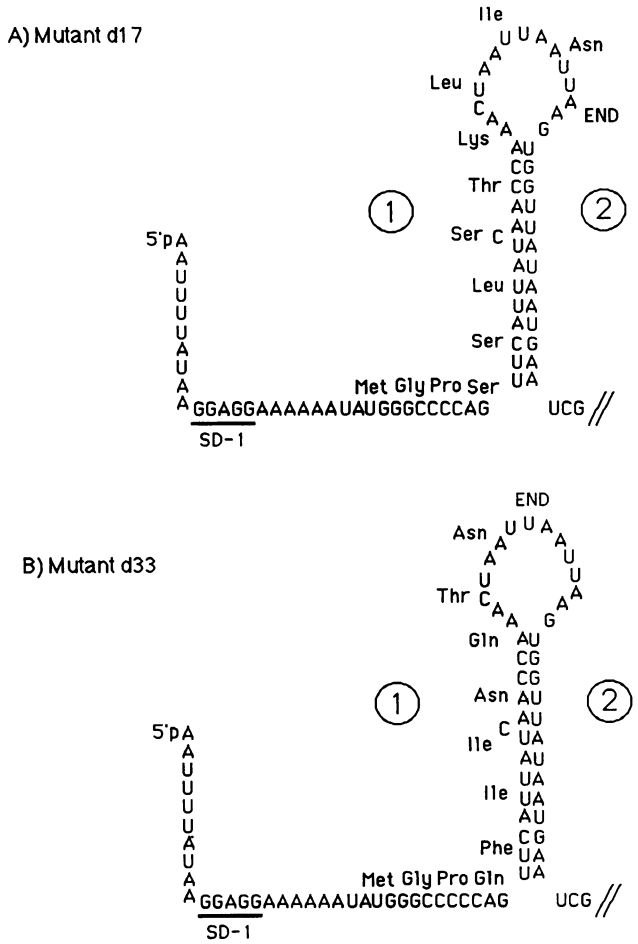

FIG. 4. Sequence of the 5′ end of two mutants in which the leader peptide initiator Met codon is in proximity to stem segment 1. The following 15 MLS antibiotics were tested for the ability to induce expression in mutants d17 and d33 over the concentration ranges indicated: Em, 0.003, 0.01, 0.03, 0.1, 1.0, and 3.0 μg/ml; Cel, matromycin, Meg, oleandomycin, and lankamycin 0.1, 0.3, 1.0, 3.0, 10, 30, and 100 μg/ml; and tylosin, lincomycin, kitasamycin, cirramycin, methymycin, chalcomycin, ostreogrycin B, maridomycin, and carbomycin, 0.01, 0.03, 0.1, and 0.3, 1.0, 3.0, and 10 μg/ml. None of the antibiotics tested produced detectible induction of *ermC*. See Fig. 1 legend for details of the diagram.

cat attenuator. In systematically replacing the individual codons for Lys-2, Ser-4, Glu-5, and Asp-6 with the termination codon UAA, they observed that the substitutions at Lys, Ser, and Glu but not at Asp led to loss of inducibility by chloramphenicol. In similar stop codon studies of the *cat-86* leader peptide MVKTDKISS, Alexieva et al. (1) successively replaced the individual codons for Lys-3, Thr-4, Asp-5, and Lys-6 with the termination codon UAA and found that the substitutions at Lys-3, Thr-4, and Asp-5 but not at Lys-6 led to loss of inducibility by chloramphenicol. Collectively, these findings suggest that the codons for residues 2, 3, 4, and 5 in both pUB112-*cat* and *cat-86* specify the amino acid sequence that is critical for induction. The extent to which amino acid residue 2 of either peptide actively determines induction specificity or simply provides the continuity needed for the ribosome to reach critical amino acids requires a test of inducing activities in leader peptide missense mutants.

Experimental alteration of induction specificity associated with a missense mutation has been reported by Kim et al. (14) for the *cat-86* attenuator, which is inducible by both chloramphenicol and amicetin. pC221-*cat*, pUB112-*cat*, and pC194-*cat*, in contrast, are inducible by chloramphenicol but not by amicetin. The leader peptide associated with the *cat-86* attenuator has the deduced sequence MVKTDKISS, and one mutational alteration that was analyzed, Val-2 to Lys, resulted in a loss of inducibility of *cat-86* by amicetin but not by chloramphenicol. The full extent of the determinants of inducibility by chloramphenicol and by amicetin, including possible leader peptide amino acid context effects, will require analysis of inducibility in a larger set of missense mutants.

How *ermC* leader peptide mutations can influence induction specificity. Two models can be proposed to account for the altered induction specificity seen in several mutants. While the mechanisms of translation inhibition by each of the three inducing antibiotics tested may be similar, they are clearly not identical. Each of the antibiotics may alter the ribosome structure in a slightly different way so that the altered ribosomes respond differently to the nascent leader peptide sequence translated. The correlation between antibiotic structure and the leader peptide sequence capable of supporting induction of *ermC* would reflect a greater similarity in the ribosomal alterations caused by binding of the macrolides Em and Meg than that caused by binding of the lincosamide Cel. Alternatively, the antibiotics may interact directly with the nascent leader peptide chain. In this case the growing peptide chain would interact with the ribosome-bound antibiotic, and when the appropriate sequence of the nascent peptide was reached, ribosome movement would be blocked. The sequence of the nascent leader peptide that would allow ribosome stall would be directly related to the structure of the antibiotic with which it interacts.

We tested the ability of Em to bind to a chemically synthesized *ermC* leader decapeptide. No detectable binding was observed in an affinity chromatography experiment under the solution conditions used previously by Mao and Robishaw (17), in which the ability of Em to inhibit the transfer of peptides to puromycin varied depending on the amino acid sequence of the peptide transferred. There are several alternative explanations for the lack of Em-peptide binding. The peptide may not be presented properly for Em binding under the solution conditions used. Since the peptide is highly hydrophobic, it may not be available to Em because of strong interaction with the polystyrene resin. Alternatively, the structure of the nascent leader peptide or of the antibiotic, while constrained on the ribosome, may differ from the respective structures in solution.

Effects of amino acid changes near the amino terminus of the leader peptide. The region of the leader peptide required for antibiotic-induced expression of *ermC* was further localized by progressive deletion of the leader peptide from the amino-terminal end. Mutant m83, in which Gly-2 and Ile-3 were deleted, was fully inducible by Em, Meg, and Cel, suggesting that these codons are in fact dispensable for antibiotic-induced expression. Deletion of the codons for Gly-2 through Phe-4 (mutant m84) resulted in reduced *ermC* inducibility by Em and Meg but not by Cel. No base substitution mutations were obtained in the codon for Phe-4 which reduced inducibility by Em or Meg, yet deletion of the codons for Gly-2 through Phe-4 (mutant m84) did reduce inducibility by Em and Meg. However, *ermC* inducibility by Cel, which was reduced by a Phe-4 to Ile substitution (mutant m5), was not reduced in mutant m84. It is not clear whether the above results reflect the deletion of the codon for Phe-4 or the simple substitution of fMet at Phe-4. Deletion of the codons for Gly-2 through Ser-5 (mutant m85) resulted in reduced inducibility by Em, Meg, and Cel. Together, these results extend the amino-terminal region of the leader peptide necessary for antibiotic-induced expression to include the codon for Phe-4 but not those for Gly-2 or Ile-3.

Effects of amino acid changes on the carboxy side of the leader peptide. The extent to which the carboxy half of the leader peptide must be translated for inducible *ermC* expression has been defined by the substitution of UAA stop codons to replace those for Ile-9 and Ser-10 (18). Mutant m74, in which leader peptide translation terminates at the codon for Ser-10, remained inducible by Em, Meg, and Cel. Mutant m86, in which leader peptide translation terminates at the codon for Ile-9, remained inducible by Meg and Cel but showed reduced inducibility by Em. This suggests that for induction by Em, ribosomes must translate through codon 9 but not as far as the codon for Ser-10. For induction by Cel and Meg, translation of the codon for Ile-9 may not be necessary. These results place the putative antibiotic-induced ribosome stall sites upstream from the codon for Ser-10 for Em and upstream from that for Ile-9 for Meg and Cel.

The translational attenuation model predicts that a ribosome stalled within the leader peptide-coding region directly disrupts the base-pairing association of segment 1 with segment 2 or segment 4. The effect of altering the distance between the putative ribosome stall site (at or upstream from the codon for Ile-9) was investigated. The deletion of the codons for Ser-10 and Thr-11 or the insertion of a single AGC Ser codon between the codons for Ser-10 and Thr-11 had no effect on the inducibility of *ermC* by Em, Meg, or Cel, whereas the insertion of two or three Ser codons [$(AGC)_2$ or $(AGC)_3$; mutants m80 and m79, respectively] reduced the inducibility by all three antibiotics. These results are consistent with the translational attenuation model.

Based on the genetic localization of the ribosome stall site and the amount of mRNA protected from nuclease attack by a bound ribosome, during induction of the wild-type *ermC* gene, the antibiotic-stalled ribosome is expected to cover only the 5′ side of stem segment 1 to the extent of 4 to 6 nucleotides (Fig. 1). Increasing the distance between the stall site and stem segment 1 by 3 nucleotides (mutant m81) might still allow partial disruption of stem segment 1 base-pairing. Larger increases in this spacing would place the

stalled ribosome too far upstream from stem segment 1 to directly disrupt base-pairing and thus reduce the inducibility.

Ribosomal contribution to induction specificity. *ermAM* from *Streptococcus sanguis* appears to be inducible by all MLS antibiotics both in its original background and after transfer to *B. subtilis* by transformation. This specificity differs from that of *ermC*, which likewise is characteristically and indistinguishably expressed in *S. aureus* and *B. subtilis*. Thus, two different *erm* determinants in the same host can show different specificities of induction. In contrast, Horinouchi and Beppu (10) have reported that *ermC* translationally fused to a pigment gene as a reporter, when introduced into *Streptomyces lividans*, is inducible by lincomycin. Thus, the same *erm* in two different backgrounds can show two different specificities. These observations suggest that both the leader peptide sequence and the structure of the translating ribosome determine the induction specificity of a given MLS resistance gene.

Some MLS antibiotics are unable to induce. Our previous findings correlating *ermC* messenger stability (21) and the location of the ribosome stall site in vivo (18, 19) suggested that the ability of the antibiotic-ribosome complex to remain stably associated with the message plays a significant role. The action of the 16-membered-ring macrolides appears to differ from that of Em when they are assayed for the ability to inhibit translation in vitro (16, 17). While the inhibitory action of Em is dependent, in part, on the amino acid composition of the peptide chain and allows some polypeptide chain growth to proceed before inhibition occurs, the 16-membered-ring macrolides tend to inhibit chain growth early in translation, usually at the formation of the first or second peptide bond. The inability of the majority of the MLS antibiotics to induce *ermC* expression may be explained in terms of a model in which the noninducing antibiotics cause inhibition early in leader peptide translation, before the ribosome has reached a position that would enable it to disrupt stem segment 1 base-pairing. Fifteen MLS antibiotics were tested for the ability to induce *ermC* mutants d17 and d33, in which the leader peptide initiator Met codon was in proximity to stem segment 1 (Fig. 4). None of the MLS antibiotics produced any detectable enhancement of *ermC* expression in these mutants. Under the assumption that all the antibiotics tested can inhibit leader peptide synthesis somewhere within the first seven or eight amino acids, these observations suggest that inhibition of peptide bond formation per se, even at a critically located amino acid in the leader peptide, does not lead to induction.

Several explanations can be proposed for the inability of the antibiotics tested to induce the *ermC* mutants or the wild-type *ermC* gene. One possibility is that the noninducing antibiotics inhibit leader peptide translation in a manner which does not cause the ribosome to remain bound to the transcript. Consistent with this idea are the observations that Em stabilizes *Bacillus megaterium* polysomes in vivo, whereas other MLS antibiotics, such as spiramycin (a 16-membered-ring macrolide) and lincomycin, cause their rapid breakdown (4, 7). Likewise, Em has been found to stabilize *E. coli* polysomes (22).

Ribosomal contribution to induction specificity. The *ermAM* determinant, which appears to be regulated by a translational attenuation mechanism, however, was inducible in *B. subtilis* by all MLS antibiotics tested by Horinouchi et al. (11). This observation suggests that there is nothing inherent in *B. subtilis* ribosomes that precludes induction of *ermC* by all MLS antibiotics. It will be of interest to determine where ribosome stall occurs on the *ermAM* message in vivo in response to MLS antibiotics that do not induce *ermC* and whether these sites are distinct.

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