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NOTES

Regulation of a Macrolide Resistance–β-Galactosidase (*ermC-lacZ*) Gene Fusion in *Escherichia coli*

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A fusion constructed between the putative attenuator plus the first 219 nucleotides of the *ermC* (erythromycin resistance) structural gene and a 5′ terminally deleted *lacZ* gene produced a moderate, basal level of β-galactosidase which was increased by erythromycin addition. Another construction containing an intact *ermC* gene in addition to the fusion produced lower levels of β-galactosidase, suggesting that the *ermC* gene product exerts negative feedback control on expression.

Plasmid pE194 was originally isolated from *Staphylococcus aureus* (9) and found to carry the *ermC* gene, which codes for inducible resistance to macrolide-lincomycin-streptogramin (MLS)-type antibiotics (16). Resistance is mediated by a 29,000-dalton methylase which acts by catalyzing the N^6,N^6-dimethylation of adenine in 23S RNA (16). Induction is produced by as little as 15 nM erythromycin and is highly antibiotic specific, as other MLS antibiotics (except oleandomycin) do not act as inducers. The synthesis of other proteins coded by pE194 is not affected by *ermC* induction, and induction occurs in the presence of rifampin and streptolydigin, indicating that induction is specific and occurs in the absence of transcription (14). Induction requires nonmethylated ribosomes; consistent with this is the observation that methylase structural mutants are hyperinducible. However, methylase synthesis is not dependent upon the presence of nonmethylated ribosomes, since *ermC* constitutive mutants carry methylated ribosomes, and the synthesis of methylase continues in induced cultures (14). On the basis of these data and DNA sequence analysis, two laboratories have proposed a translational attenuator model for the control of methylase synthesis (4, 7).

The *ermC* gene has a 141-base-pair leader sequence which is located between the putative promoter and the ATG start codon for the methylase. This leader sequence contains a Shine-Dalgarno (ribosome-loading) sequence (12), a short-reading frame which could code for a 19-amino-acid peptide, and a second Shine-Dalgarno sequence just 5′ to the methylase start codon. This leader sequence is theoretically capable of forming secondary structures which could make the second Shine-Dalgarno sequence unavailable to the ribosome. The model for *ermC* regulation postulates that erythromycin interacts with the ribosome-mRNA complex in a fashion which stalls the ribosome and allows changes in mRNA secondary structure which unmask the second Shine-Dalgarno sequence, leading to the translation of the methylase sequence. Methylation of the ribosome would be expected to suppress the effect of erythromycin and thus produce a feedback control which would lead to a steady state of methylase production at a given erythromycin concentration. Shivakumar et al. (14) have reported that erythromycin-sensitive, *ermC* deletion mutants are hyperinducible, suggesting that ribosome methylation has a negative feedback effect on induction. In addition, this model is strongly supported by DNA sequence analysis of constitutive mutations which lie within the 141-base-pair leader sequence and which would theoretically destabilize the secondary structure of the mRNA in a fashion which would predictably lead to constitutive expression (4, 7, 8). Hahn et al. (5) have analyzed a series of in vitro deletions within the leader sequence which would be expected to alter secondary structure. These deletions show altered induction behavior which correlates with the anticipated effect of the deletion on the stability of the secondary structure of the leader sequence. These experiments provide tests of the attenuator model by studying the effects on induction produced by alterations of the putative attenuator sequence.

We wished to test the ability of the putative attenuator to confer erythromycin inducibility on an adjacent DNA sequence by ligating the attenuator to a readily assayable structural gene. Such gene fusions should also provide independent evidence for the observed feedback control on synthesis produced by the methylase. We therefore constructed and studied the induction behavior of gene fusions containing the 5′ portion of the *ermC* gene and a 5′ deleted version of the *Escherichia coli* β-galactosidase gene originally described by Casadaban et al. (3).

To construct these fusions, we first subcloned a *Taq*I fragment of pE194 which is known to carry the entire *ermC* gene (4, 7) into the *Cla*I site of pBR322 to produce plasmid pML1, which was then used to transform *E. coli* RR1 (2) to ampicillin resistance (Fig. 1). The expression of constitutive (1, 10) and inducible (6) MLS antibiotic resistance from gram-positive bacteria has previously been reported for *E. coli*. Strain RR1 was found to be inhibited by about 50 μg of erythromycin per ml in tube dilution assays. After transfection, cells carrying pML1 were found to be resistant to greater than 400 μg of erythromycin per ml, demonstrating the expression of the *ermC* gene in this construction after its introduction into *E. coli*.

To determine whether the *ermC* gene was inducible in *E. coli*, we employed the macrolide antibiotic rosamicin, which shows unusually high activity against gram-negative bacteria (15). In tube dilution assays, RR1 cells and pML1-transformed RR1 cells were both inhibited by between 2 and 4 μg of rosamicin per ml. Plasmid pML1-transformed RR1 cells

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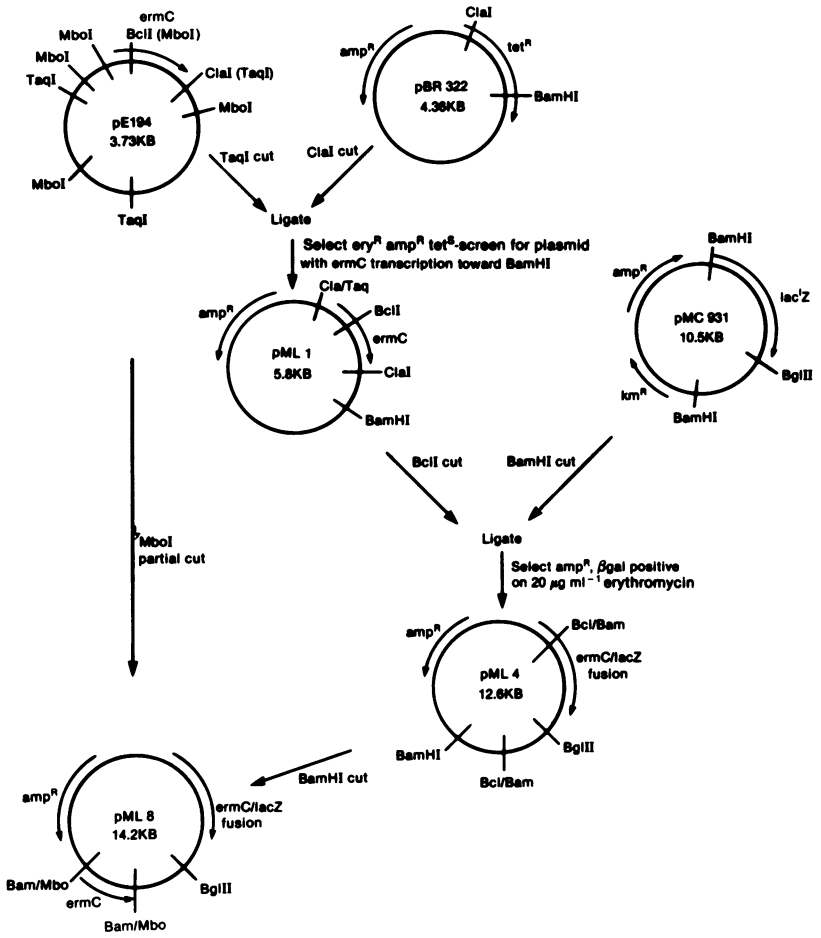

FIG. 1. Construction of *ermC-lacZ* gene fusion plasmids. The indicated restriction endonuclease sites were predicted from published values and checked by restriction endonuclease site mapping. Restriction fragment orientations were determined by mapping asymmetric restriction sites. To clone the erythromycin resistance (*ermC*) gene from pE194 into *E. coli*, plasmid pML1 was constructed by ligating a 1.5-kb *Taq*I fragment from pE194 carrying the *ermC* gene into the *Cla*I site of pBR322. To fuse the postulated attenuator region of *ermC* to *lacZ*, plasmid pML1 was purified from a *dam*$^-$ *E. coli* strain in which the *Bcl*I site remains unmethylated, cut with *Bcl*I, and ligated to a 6.8-kb *Bam*HI fragment from pMC931 which carries a 5′ terminally deleted *lacZ* gene to generate pML4. To study the effect of a functional *ermC* gene on the expression of the fusion, pML4 was cut with *Bam*HI and ligated with *Mbo*I partially digested fragments of pE194. Plasmid pML8 carries a 1.6-kb fragment from PE194 which contains an intact, functional *ermC* gene.

were inoculated into LB agar plates containing inhibitory concentrations of rosamicin, and disks containing various MLS antibiotics were placed onto the surface of the agar. Two of the tested MLS antibiotics (erythromycin and lincomycin) were able to induce resistance as shown by a zone of bacterial growth surrounding the drug disk, whereas four other MLS antibiotics (clindamycin, oleandomycin, streptogramin B, and tylosin) did not induce resistance and showed no zone of growth (Fig. 2). Under the same conditions, RR1 cells which do not carry plasmid pML1 are inhibited by erythromycin, lincomycin, clindamycin, and tylosin, whereas oleandomycin and streptogramin B produce no effect (data not shown). The zones of decreased growth seen clearly around the clindamycin disk and more faintly around the tylosin disk are probably due to the fact that these antibiotics are not only unable to induce resistance to rosamicin, but also show inhibitory activity toward the strain. In some cases, the antibiotics which were found to induce *ermC* in this experiment are different from the antibiotics which have been observed to induce *ermC* in *S. aureus*. Although this could be due to any of a number of factors, such as differences between ribosomes of gram-negative and gram-positive bacteria or differences in the rate of entry of drugs into the cell, this experiment does clearly indicate that the *ermC* gene retains the ability to be induced by a subset of MLS antibiotics after transfer to *E. coli*.

To study the mechanism for the induction of *ermC*, we performed the following experiment. The attenuator model postulates that control is provided by sequences which lie 5′ to the AUG codon for the *ermC* methylase. One would predict that ligating these sequences to another structural gene should lead to the induction of that structural gene by erythromycin. There is a unique *Bcl*I site within the *ermC* gene which lies 219 bases downstream from the *ermC* methylase AUG codon. An examination of the published nucleotide sequences for *ermC* (4, 7) and *lacZ* (3) indicated that the respective *Bam*HI (GGATCC) and *Bcl*I (TGATCA) sites contain the codon for aspartic acid (GAT) in phase for both genes. Therefore, the ligation of the *lacZ*-containing *Bam*HI fragment from pMC931 to the *Bcl*I site of pML1 in the proper orientation should fuse the two (*ermC* and *lacZ*) structural genes in phase, leading to the production of a

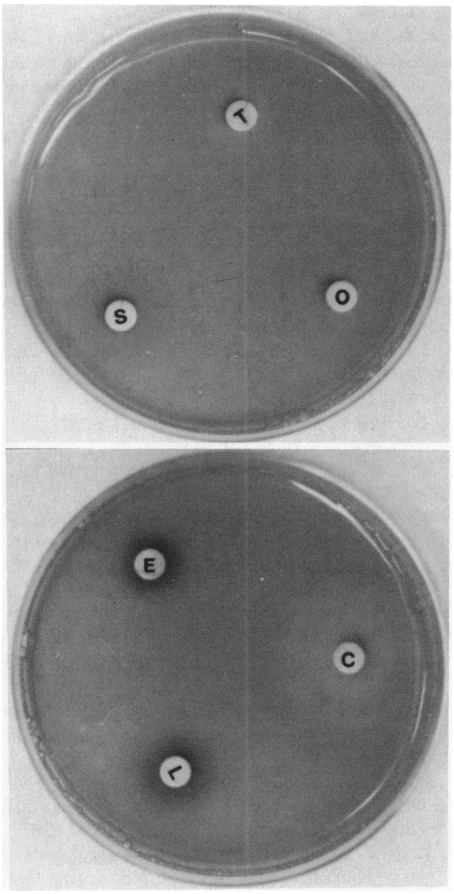

FIG. 2. Inducible expression of the *ermC* gene in *E. coli*. *E. coli* RR1 cells carrying plasmid pML1 were inoculated into media containing inhibitory concentrations of the macrolidic drug rosamicin. Disks, each containing 200 μg of an MLS antibiotic, were placed on the agar to induce resistance to rosamicin. Two of the drugs (erythromycin and lincomycin) induced resistance, as demonstrated by a zone of bacterial growth surrounding the disk. Two other MLS antibiotics (streptogramin B and oleandomycin) did not produce zones of growth. Clindamycin and, more faintly, tylosin produced zones of decreased growth rather than inducing resistance. Triphenyltetrazolium chloride was added to the plates for photographic purposes to enhance the appearance of growth zones. E, Erythromycin; L, lincomycin; S, streptogramin B; C, clindamycin; T, tylosin; O, oleandomycin.

hybrid protein containing the first 74 amino-terminal amino acids from the *ermC* methylase and all but the first seven amino-terminal amino acids of β-galactosidase.

Plasmid pML1 was purified from a *dam*$^-$ strain of *E. coli* in which the *Bcl*I site remains unmethylated and cleaved with the restriction endonuclease *Bcl*I. This DNA was ligated to *Bam*HI-cleaved pMC931 and used to transform *E. coli* MC1061 (a strain deleted for the *lac* operon) to ampicillin resistance. Ampicillin-resistant isolates were then screened, and several erythromycin- and kanamycin-sensitive strains were selected, from which small-scale plasmid DNA preparations were made. Restriction enzyme analysis indicated the presence of two types of plasmids, pML4, in which the *lacZ* and *ermC* genes are fused (Fig. 1), and plasmids in which the pMC931 fragment was inserted in the opposite orientation.

We next wished to add a functional *ermC* gene to this construction. Plasmid pML4 DNA was restricted with *Bam*HI and ligated with pE194 DNA which had been partially digested with *Mbo*I. This ligation mixture was then used to transform strain MC1061 to ampicillin resistance, and ampicillin-resistant colonies were screened for erythromycin resistance. Restriction enzyme analysis of rapid DNA preparations from several such strains demonstrated the insertion of DNA at the *Bam*HI site. Plasmid pML8 carried an insert of ca. 1.6 kilobases (kb) which is composed of three *Mbo*I fragments from pE194: two *Mbo*I fragments of 0.93 and 0.41 kb which are required to code for erythromycin resistance plus an adjacent 0.27-kb fragment. Restriction mapping indicated that these sequences were inserted in the opposite orientation relative to pE194 sequences already present on pML4.

Cells carrying pML4 and cells carrying pML8 were then tested for β-galactosidase activity in the presence and absence of erythromycin, using the assay described by Miller (11). Both pML4 and pML8 strains produced moderate levels of β-galactosidase in the absence of erythromycin (Fig. 3). The *ermC* gene in *Bacillus subtilis* also shows a basal level of uninduced synthesis of methylase, but probably not as much as that seen for β-galactosidase in this fusion (13, 14). This difference could be due to a variety of factors, including differences in the interaction of the attenuator sequence between gram-positive and gram-negative ribosomes. With the addition of erythromycin, both strains

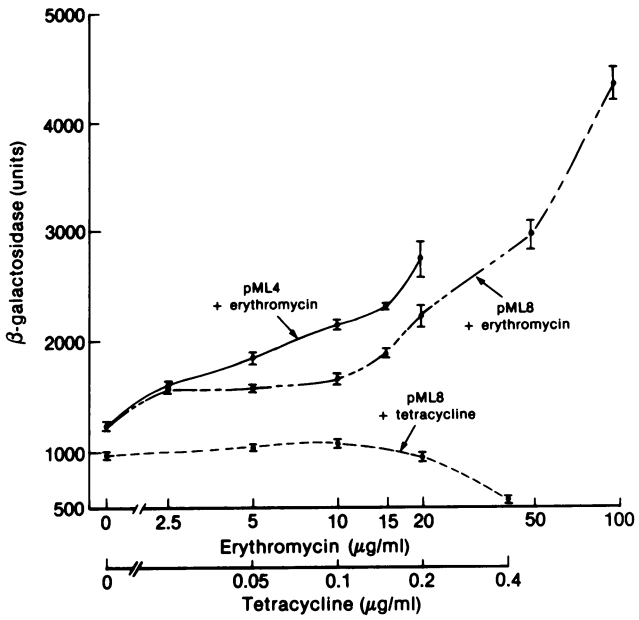

FIG. 3. Induction of β-galactosidase by antibiotics in *ermC-lacZ* fusion constructions. Cells were grown in Luria broth in the presence of various levels of drug to the same stage in late log phase (7×10^8 to 8×10^8 cells per ml). Growth rates at different drug concentrations varied, but in all cases cells were assayed at the same stage of growth. β-Galactosidase assays were performed, and enzyme activity was calculated and expressed in units as described by Miller (11). Levels of β-galactosidase activity for each data point were determined by three independent assays. The bars indicate the standard deviation calculated for each point. Symbols: ——, pML4-transformed cells grown on different levels of erythromycin; ----, pML8-transformed cells grown on different levels of erythromycin; -----, pML8-transformed cells grown on different levels of tetracycline.

showed an increase in β-galactosidase activity which was dose related to the increase in erythromycin concentration.

It has been postulated that methylation of ribosomes will negatively modulate expression in this system. Strains carrying plasmid pML4 presumably have no methylase activity since they do not carry an intact *ermC* gene and are erythromycin sensitive. Thus, these cells should not show negative feedback effects due to methylation. Consistent with this is our observation that at comparable levels of erythromycin, pML4 produced significantly more β-galactosidase activity than pML8 (Fig. 3). The pML4-carrying strain is sensitive to erythromycin concentrations of about 40 to 50 μg/ml and could thus only be tested at lower levels of the drug. The pML8 construction is erythromycin resistant and was tested at higher levels of erythromycin. At a concentration of 200 μg of erythromycin per ml, the pML8-carrying strain produced 6,145 U of β-galactosidase activity, which is an increase of about fivefold relative to cells grown in the absence of erythromycin.

To control for the possibility that the observed increase in β-galactosidase activity by erythromycin might be an artifact due to protein synthesis inhibition, we treated the pML8 strain with subinhibitory levels of the protein synthesis inhibitor tetracycline and assayed for β-galactosidase activity (Fig. 3). The pML8 strain (which was inhibited by tetracycline concentrations of about 0.5 to 1 μg/ml) showed no stimulation of β-galactosidase activities at tetracycline concentrations up to 0.4 μg/ml. In addition, a *lac*$^+$ strain of *E. coli* was grown in low levels (0.25%) of lactose to induce about 1,000 U of β-galactosidase and was treated with various levels of erythromycin. The erythromycin produced no increase and at some concentrations produced a decrease in the level of β-galactosidase in this experiment (data not shown).

These experiments indicate that nucleotide sequences at the 5′ end of the *ermC* gene are capable of controlling the expression of downstream sequences. Expression is induced by erythromycin and not by all protein synthesis inhibitors. Induction does not require the presence of a functional *ermC* gene in the cell, and the presence of a functional *ermC* gene produces a negative effect on induction at certain erythromycin concentrations.

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