JOURNAL OF BACTERIOLOGY, Aug. 1989, p. 4518–4520
0021-9193/89/084518-03$02.00/0

Vol. 171, No. 8

# Induction of *ermC* Methylase in the Absence of Macrolide Antibiotics and by Pseudomonic Acid A

SUNIL K. KADAM

*Anti Infective Research Division, D47M, Abbott Laboratories, Abbott Park, Illinois 60064*

Received 17 January 1989/Accepted 18 May 1989

**The methylase encoded by *erm* genes and induced by erythromycin modifies the 23S rRNA and confers resistance to macrolide-lincosamide-streptogramin B antibiotics. Induction is due to a posttranscriptional mechanism in which the inducer activates translation of methylase mRNA by binding to unmethylated (erythromycin-sensitive) ribosomes and stalling them in the leader region. It is shown in this study that pseudomonic acid A, an inhibitor of isoleucyl-tRNA synthetase, can also induce methylase synthesis. Isoleucine starvation has a similar effect on ribosomes translating the *ermC* leader region to cause induction of methylase synthesis. These observations support the requirement for ribosome stalling and destabilization of a stem-loop structure and demonstrate that stalling can occur without macrolide-bound ribosomes.**

*erm* genes code for an rRNA methylase and are inducibly expressed by certain 14-membered macrolides, such as erythromycin (3). The methylase modifies adenine 2058 in the 23S rRNA, conferring resistance simultaneously to macrolides, lincosamides, and streptogramin type B antibiotics as well as certain 16-membered macrolides, such as niddamycin and tylosin, which are not themselves inducers (13, 20). This effect is explained by a posttranscriptional mechanism called translational attenuation (18). Accordingly, low concentrations of an inducing macrolide can bind to the 50S subunit of procaryotic ribosomes and stall the ribosome during the translation of the *erm* leader region to destabilize the mRNA structure, rendering the ribosome-binding site for the *erm* methylase (SD2) free to initiate methylase synthesis. Several studies support this model (8, 10). The induction of *cat* genes is also due to a similar mechanism which modulates primarily translation of *cat* mRNA. In this case, chloramphenicol modifies ribosomal function, permitting drug-bound ribosomes to destabilize a stem-loop structure in *cat* mRNA which normally sequesters the *cat* ribosome-binding site (17). The mechanism of induction is compatible with ribosome stalling in the leader region (6). However, drug-free ribosomes can also induce chloramphenicol acetyltransferase synthesis by ribosomal stalling due to amino acid starvation (5). This stall site was recently determined to be lysine codon 6 in the *cat* leader region by amino acid starvation and site-specific mutagenesis (1). In contrast, the mechanism of methylase induction by erythromycin is less well understood. There is evidence that erythromycin stimulates dissociation of peptidyl-tRNA from the ribosome during translation (15) and that erythromycin-bound ribosomes have trouble translating hydrophilic and bulky amino acids (14). Recent reports suggest that the leading edge of the stalled ribosome on the *ermC* transcript includes the last two lysine residues of the 19-amino-acid leader peptide (16). However, the data mentioned above were obtained with synthetic RNA templates (14, 15) and in in vitro systems (16), which may not reflect the situation in whole cells. Furthermore, there is considerable diversity among the methylase leader regions in *erm* genes from different sources, and there is no apparent consensus for a ribosomal stall sequence in the various leaders. The aim of this study was to test the possibility that ribosomes translating the leader region stall at a discrete location when complexed with inducing antibiotics, leading to mRNA destabilization and synthesis of drug resistance determinant. A unique feature of the *ermC* leader region is the presence of 3 isoleucine residues among the first 10 amino acids, while the *ermD* leader has no isoleucines (7, 11). In this study, pseudomonic acid A (PA), a potent inhibitor of isoleucyl-tRNA synthetase (12), was found to induce *ermC* methylase but not *ermD* methylase. These findings are supported by *ermC* methylase induction during isoleucine starvation. This is the first demonstration of induction of an *erm* methylase in the absence of a macrolide antibiotic. The results confirm that destabilization of mRNA structure by ribosome stalling is required for methylase induction and that it can be achieved without macrolide-bound ribosomes. The results presented in this study, together with the data on transcript protection by erythromycin-bound ribosomes (16), suggest that methylase induction can occur by ribosomal stalling at more than one site in the *ermC* leader.

**PA induction of *ermC* methylase.** Plasmids pE194, pBD246, and pBD247, carrying *ermC*, *ermC-lacZ*, and *ermD-lacZ*, respectively, were supplied by D. Dubnau (Public Health Research Institute, New York, N.Y.) (9). The plasmids were transformed into *Bacillus subtilis* BD170 *trpC2 thr-5* (2, 4). PA was prepared from *Pseudomonas fluorescens* and purified by the method of Klein et al. (L. L. Klein, C. M. Yeung, P. Kurath, J. C. Mao, P. B. Fernandes, P. A. Lartey, and A. G. Pernet, J. Med. Chem., in press). Since the *lacZ* gene is translationally fused in frame with the leader regions of *ermC* and *ermD* in pBD246 and pBD247, respectively, induction of β-galactosidase was achieved with low concentrations of erythromycin and was found to be linear over a period of 2 h in Spizizen minimal medium (19) with amino acid supplementation (data not shown). To demonstrate methylase induction by PA in solid medium, BD170 cells carrying pE194 grown overnight in Luria broth were spread in a lawn on L-broth agar, and filter paper disks soaked with various concentrations of PA and erythromycin were placed in an inner circle (Fig. 1). The outer disks all had niddamycin (10 μg per disk). The plates were incubated at 30°C for 4 to 5 h. Disk sets (inner and outer) 1 and 2 (Fig. 1) show the characteristic "D" zone due to erythromycin induction of *ermC* methylase, which confers resistance to erythromycin as well as niddamycin, hence the area of growth between the two disks which gives D-shaped zones. However, as seen in

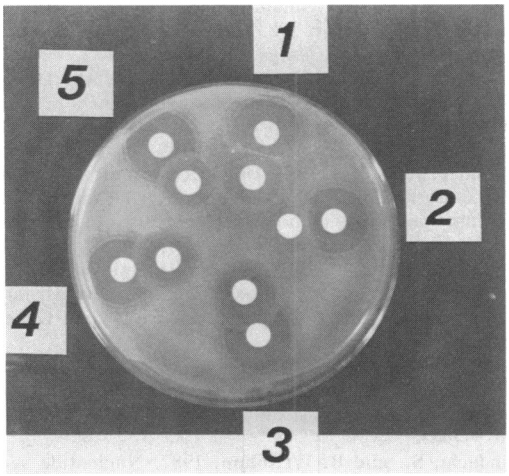


FIG. 1. Plate assay for methylase induction. *B. subtilis* BD170(pE194) cells grown in L broth were spread on an L-agar plate, and 5-mm filter disks with 10 and 2 μg of erythromycin (1 and 2, respectively) and 10, 5, and 2 μg of PA (3, 4, and 5, respectively) were placed in the inner circle. The outer disks all had niddamycin (10 μg).

disk sets 3, 4, and 5 (Fig. 1) with inner disks containing different concentrations of PA, a different kind of resistance zone appears which looks C shaped. This type of zone has a C-shaped area of growth within the niddamycin zone due to induction of methylase by low concentrations of PA, which confers resistance to niddamycin but not to PA. To confirm these results, BD170 cells with pBD246 and pBD247 were grown separately in Spizizen minimal medium containing chloramphenicol (5 μg/ml) at 30°C overnight. The cells were harvested by centrifugation and washed with fresh medium without antibiotic, and the optical density at 550 nm was adjusted separately so that the cell density of pBD247 (*ermD-lacZ*)-carrying cells was fivefold greater than that of the cells carrying plasmid pBD246 (*ermC-lacZ*). Previous experiments had shown that under these conditions the same level of β-galactosidase was produced in 60 min when the cells were treated with equal amounts of an inducing concentration of erythromycin. This is probably due to a higher pBD246 copy number per cell (9). Samples (5 ml) of medium containing BD170 with pBD246 and pBD247 were then separately treated with different concentrations of PA and incubated with shaking for 60 min at 30°C. Cells were harvested by centrifugation and treated with toluene, and the level of β-galactosidase produced at each concentration of PA was determined by the method of Gryczan et al. (9). In cells with pBD246, β-galactosidase was induced by PA with peak induction at 0.01 μg/ml (Fig. 2). In contrast, the β-galactosidase level in cells with pBD247 was close to background. The appearance of C-shaped resistance zones due to PA (Fig. 1) and the β-galactosidase induction from *ermC-lacZ* and not from *ermD-lacZ* can be explained by the presence of the isoleucine residues in the *ermC* leader and none in the *ermD* leader. PA inhibition of isoleucyl-tRNA synthetase prevents the formation of charged isoleucyl-tRNA, thus causing ribosomes to stall during the translation of the leader peptide. This would release the ribosome-binding site (SD2) on methylase mRNA, allowing the synthesis of methylase and, in turn, the methylation of ribosomes, conferring resistance to niddamycin in cells carrying pE194, and the induction of β-galactosidase in cells carrying pBD246.

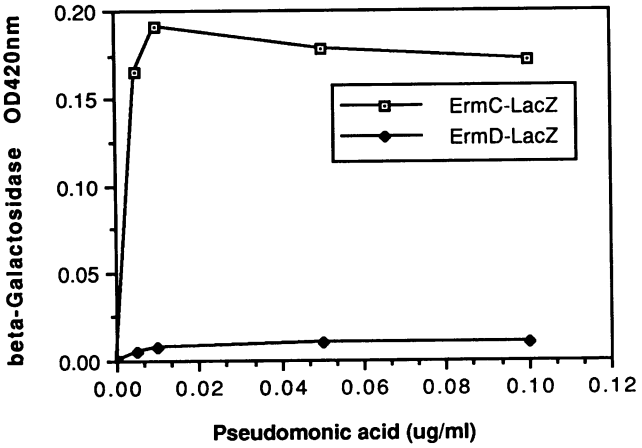


FIG. 2. Effect of PA on β-galactosidase induction by *B. subtilis* BD170(pBD246 [*ermC-lacZ*]) and BD170(pBD247 [*ermD-lacZ*]) grown in Spizizen minimal medium with appropriate nutritional amino acid supplementation.

**Antibiotic-free induction of *ermC* methylase.** To test the hypothesis that ribosomal stalling can occur due to a shortage of charged isoleucyl-tRNA, *ermC* induction was tested in isoleucine auxotrophs during starvation. Cells were grown at 30°C in Spizizen minimal medium with 50 μg of appropriate amino acid per ml. *B. subtilis* BD54 *leu met ile* transformed with pBD246 was used for isoleucine starvation and β-galactosidase induction after the addition of PA (0.01 μg/ml) and erythromycin (0.1 μg/ml). Cells harvested from log phase were washed with warm amino acid-free growth medium and suspended in the same medium containing all amino acids (50 μg/ml) except the one for which cells were starved. Media for fully supplemented controls and cells used for erythromycin and PA induction experiments contained all 20 amino acids. Cell samples were taken at 30-min intervals for 2 h, treated with toluene, and used for β-galactosidase assay. Fully supplemented cells produced a low background level of β-galactosidase, whereas erythromycin addition resulted in much higher levels (Fig. 3). The enzyme was induced by isoleucine starvation to the same extent as with PA addition, although the activity appeared to level off after 90 min without isoleucine (Fig. 3). The effect of isoleucine starvation on β-galactosidase induction from *ermC-lacZ* was similar to that reported for lysine starvation (1) on the induction of chloramphenicol acetyltransferase by the *cat* gene. Amino acid starvation is thought to lead to events in which the required amino acids, in short supply, are acquired during turnover of preexisting proteins to continue protein synthesis (1). By starving for specific amino acids or by supplying them in limiting quantities, ribosome stalling has been shown to occur and cause localized melting of RNA structure (5). The data presented here support ribosomal stalling during *ermC* methylase induction and demonstrate that destabilization of the RNA stem-loop structure can occur without macrolide-bound ribosomes, leading to synthesis of methylase and macrolide-lincosamide-streptogramin B resistance.

Thanks are due to John C. Capobianco for providing purified preparations of PA, David Dubnau for sending several plasmids and strains, and A. Shivakumar for critically reviewing the manuscript.

## LITERATURE CITED

1. **Alexieva, Z., E. Duvall, N. P. Ambulos, Jr., U. J. Kim, and P. S. Lovett.** 1988. Chloramphenicol induction of *cat-86* requires

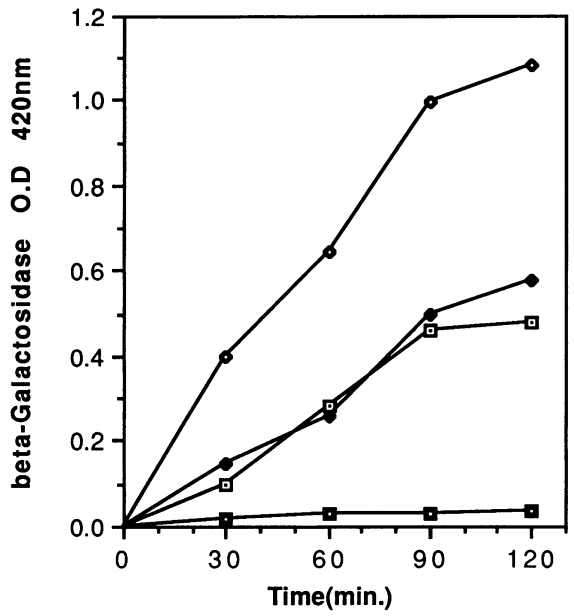


FIG. 3. β-Galactosidase induction resulting from isoleucine starvation (⊡), PA addition (◆), and erythromycin addition (◇) to growing cultures of *B. subtilis* BD54(pBD246). Fully supplemented control (□) represents BD54(pBD246) grown in the presence of all amino acids.

ribosome stalling at a specific site in the leader. Proc. Natl. Acad. Sci. USA **85**:3057–3061.

2. **Contente, S., and D. Dubnau.** 1979. Characterization of plasmid transformation in *Bacillus subtilis*: kinetic properties and the effect of DNA conformation. Mol. Gen. Genet. **167**:251–258.
3. **Dubnau, D.** 1984. Translational attenuation: the regulation of bacterial resistance to the macrolide-lincosamide-streptogramin B antibiotics. Crit. Rev. Biochem. **16**:103–132.
4. **Dubnau, D., and R. Davidoff-Abelson.** 1971. Fate of transforming DNA following uptake by *Bacillus subtilis*. I. Formation and properties of the donor-recipient complex. J. Mol. Biol. **56**: 209–221.
5. **Duvall, E. J., N. P. Ambulos, Jr., and P. S. Lovett.** 1987. Drug-free induction of a chloramphenicol acetyltransferase gene in *Bacillus subtilis* by stalling ribosomes in a regulatory leader. J. Bacteriol. **169**:4235–4241.
6. **Duvall, E. J., D. M. Williams, P. S. Lovett, C. Rudolph, N. Vasantha, and M. Guyer.** 1983. Chloramphenicol inducible gene expression in *Bacillus subtilis*. Gene **24**:171–177.
7. **Gryczan, T., M. Israeli-Reches, M. Del Bue, and D. Dubnau.** 1984. DNA sequence and regulation of *ermD*, a macrolide-lincosamide-streptogramin B resistance element from *Bacillus licheniformis*. Mol. Gen. Genet. **194**:349–356.
8. **Gryczan, T. J., G. Grandi, J. Hahn, R. Grandi, and D. Dubnau.** 1980. Conformational alteration of mRNA structure and the posttranscriptional regulation of erythromycin-induced drug resistance. Nucleic Acids Res. **8**:6081–6097.
9. **Gryczan, T. J., M. Israeli-Reches, and D. Dubnau.** 1984. Induction of macrolide-lincosamide-streptogramin B resistance requires ribosomes able to bind inducer. Mol. Gen. Genet. **194**: 357–361.
10. **Horinouchi, S., and B. Weisblum.** 1980. Posttranscriptional modification of mRNA conformation: mechanism that regulates erythromycin-induced resistance. Proc. Natl. Acad. Sci. USA **77**:7079–7083.
11. **Horinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. J. Bacteriol. **150**:804–814.
12. **Hughes, J., and G. Mellows.** 1980. Interaction of pseudomonic acid A with *Escherichia coli* B isoleucyl-tRNA synthetase. Biochem. J. **191**:209–219.
13. **Lai, C. J., and B. Weisblum.** 1971. Altered methylation of ribosomal RNA in an erythromycin resistant strain of *Staphylococcus aureus*. Proc. Natl. Acad. Sci. USA **68**:856–860.
14. **Mao, J. C.-H., and E. E. Robishaw.** 1972. Erythromycin, a peptidyltransferase effector. Biochemistry **11**:4864–4872.
15. **Menninger, J. R., and D. P. Otto.** 1982. Erythromycin, carbomycin, and spiramycin inhibit protein synthesis by stimulating the dissociation of peptidyl-tRNA from ribosomes. Antimicrob. Agents Chemother. **21**:811–818.
16. **Narayanan, C. S., and D. Dubnau.** 1987. Demonstration of erythromycin-dependent stalling of ribosomes on the *ermC* leader transcript. J. Biol. Chem. **262**:1766–1771.
17. **Shaw, W. V.** 1983. Chloramphenicol acetyltransferase: enzymology and molecular biology. Crit. Rev. Biochem. **14**:1–43.
18. **Shivakumar, A. G., J. Hahn, G. Grandi, Y. Kozlov, and D. Dubnau.** 1980. Posttranscriptional regulation of an erythromycin resistance protein specified by plasmid pE194. Proc. Natl. Acad. Sci. USA **77**:3903–3907.
19. **Spizizen, J.** 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. USA **44**:1072–1078.
20. **Weisblum, B.** 1984. Inducible erythromycin resistance in bacteria. Br. Med. Bull. **40**:47–53.