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ermK Leader Peptide : Amino Acid Sequence Critical for Induction by Erythromycin

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The *ermK* gene from *Bacillus lichenformis* encodes an inducible rRNA methylase that confers resistance to the macrolide-lincosamide-streptogramin B antibiotics. The *ermK* mRNA leader sequence has a total length of 357 nucleotides and encodes a 14-amino acid leader peptide together with its ribosome binding site. The secondary structure of *ermK* leader mRNA and a leader peptide sequence have been reported as the elements that control expression. In this study, the contribution of specific leader peptide amino acid residues to induction of *ermK* was studied using the PCR-based megaprimer mutation method. *ermK* methylases with altered leader peptide codons were translationally fused to *E. coli* β-galactosidase reporter gene. The deletion of the codons for Thr-2 through Ser-4 reduced inducibility by erythromycin, whereas that for Thr-2 and His-3 was not. The replacement of the individual codons for Ser-4, Met-5 and Arg-6 with termination codon led to loss of inducibility, but stop mutation of codon Phe-9 restored inducibility by erythromycin. Collectively, these findings suggest that the codons for residue 4, 5 and 6 comprise the critical region for induction. The stop mutation at Leu-7 expressed constitutively *ermK* gene. Thus, ribosome stalling at codon 7 appears to be important for *ermK* induction.

Key words: *ermK* leader peptide, Induction, Ribosome stalling, Transcriptional and translational attenuation

INTRODUCTION

The macrolides are largely prescribed for empiric therapy of community-acquired respiratory tract infections and may be useful in case of intolerance or resistance to â-lactams. They act by binding to the 50S subunit of bacterial ribosomes and inhibit protein synthesis by blocking elongation of the nascent peptide chain (Leclerq and Couvalin, 1991). The macrolides, erythromycin (EM) is thought to block the entrance of tunnel that channels the nascent peptides away from the peptidyl transferase center (Yonath *et al.*, 1987; Frank *et al.*, 2001).

But after the introduction of erythromycin into clinical practice in 1953, many clinical isolates of macrolide-resistant Gram-positive organisms were reported and disseminated. The molecular mechanisms by which they become resistant involve drug efflux or alterations in the antibiotic target site. Ribosomal target site modification due to methylases encoded by *erm* class genes is the most common and investigated mechanism of erythromycin resistance (Weisblum, 1995). From studies with an *ermC* model system, it has been shown that methylase expression occurs by a translational attenuation mechanism (Dubnau, 1984). In the absence of erythromycin, the ribosome binding site for the *ermC* methylase coding sequence is sequestered by leader region RNA secondary structure. In contrast, the addition of nanomolar concentrations of erythromycin cause ribosome stalling in a 19-amino-acid coding sequence contained within the leader region, which results in destabilization of the leader region RNA stem-loop structure and frees the methylase ribosome binding site and initiation codon for translation (Mayford and Weisblum, 1989a, 1989b).

The *ermK* gene from *Bacillus licheniformis* encodes an inducible rRNA methylase that confers resistance to the macrolide-lincosamide-streptogramin B (MLS) antibiotics. The *ermK* mRNA leader sequence has a total length of 357 nucleotides and encodes a 14-amino-acid leader peptide together with its ribosome binding site. The *ermK*

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regulation had been studied and it appeared that *ermK* methylase expression is regulated by transcriptional attenuation (Kwak *et al.,* 1991) in contrast other inducible *erm* genes, which are regulated translationally. The *ermK* leader peptide contains two rho-factor independent transcriptional terminators. In the absence of inducer, truncated transcription products only are synthesized and the full length transcription product is not detected. But the stalling of erythromycin-bound ribosome in the *ermK* leader peptide disrupts terminator structures, allowing rapid induction of *ermK* methylase transcription.

The present study was designed to determine (1) where ribosome stall occurs on the *ermK* mRNA (2) the amino acid sequence that is critical for erythromycin-mediated induction. To determine the role of the leader peptide sequence in *ermK* regulation, we altered leader peptide amino acid codons using the PCR-based megaprimer method. And then expression of *ermK* gene was measured in terms of β-galactosidase activity produced by a translational fusion between mutated *ermK* leader peptide and *Escherichia coli lacZ*.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacillus subtilus UOTO277 and plasmid pEC101 were used in all of our experiments (Kwak *et al,* 1991). *E. coli* CSH26, *B. subtilus* BR151, and plasmid pMM156 were used for the β-galactosidase assay (Choi *et al.,* 1997).

Construction of the mutated ermK leader peptide:lacZ reporter plasmids

Plasmid template pEC101, which carries the entire *ermK* gene, was purified by using the Qiagen Plasmid Mini Prep kit. In order to introduce stop, deletion or point mutations throughout the leader peptides, we isolated the mutated *ermK* leader peptide by megaprimer PCR with mutagenic primers (Sarkar and Sommer, 1990). The mutated 750bp amplification products were digested with *BamH*I and *EcoR*I. The fragments were ligated in-frame into the *BamH*I – *EcoR*I site of promoterless *lac*-containing pMM156. The resultant ligation products were transformed into *E. coli* CSH26, followed by selection for chloramphenicol resistance. Finally plasmid DNA preparations from transformants introduced *B. subtilis* BR151 with chloramphenicol selection. Plasmid constructs were characterized further by restriction analysis and DNA sequencing to determine each mutation.

Inducibility assay of the mutated ermK leader peptide:lacZ reporter plasmids

The inducibility assays of the mutated *ermK* leader peptide-*lacZ* fusion plasmid were performed as described previously (Choi *et al.*, 1997; Miller, 1972).

Growth curve of the leader peptide mutated *ermK* plasmids

B. subtilis BR151 harboring the pEC101 with stop mutation at the leader peptide were designated as like pEC101_t4 for the stop codon mutant at 4th codon. Overnight cultures were grown in LB medium containing 10 μg/mL of chloramphenicol. Cultures were diluted with LB medium containing erythromycin induction concentration (0.1 μg/mL) to the final density of A_{590}=0.2. Two mL of each culture was incubated at 37°C with constant shaking. After 3 h incubation, cultures were diluted with fresh medium to A_{590}=0.05 and challenged with 150 μg/mL of EM. The optical densities of cultures were measured at 2, 4, 8 and 10 h of incubation. Growth was compared with that of wild type pEC101.

RESULT AND DISCUSSION

Inducibility of leader peptide stop mutants

We constructed pJA4, pJA5, pJA6, pJA7 and pJA9, in which the codons for Ser-4, Met-5, Arg-6, Leu-7 and Phe-9 were mutated to stop codon, respectively. The construction and inducibility of series of leader peptide stop mutants was described in Table I and Fig. 1. The mutations were carried on plasmid pMM156 as part of an in-phase *ermK-lacZ* translational fusion. Whereas mutant pJA9 showed no altered induction, mutants pJA4, pJA5 and pJA6 showed reduced inducibility by EM. Especially, replacement of Ser-4 with the ochre codon (TAA) strongly affected inducibility by EM. Mutants pJA5 and pJA6 showed partially reduced inducibility by EM, even though they had higher basal level of expression than either the wild-type or the mutant that terminate at Ser-4 (pJA4). This suggests that for induction by EM it is only necessary for the EM-bound ribosome to translate up through Arg-6. One question is involves the location of the stalled EM-bound ribosome on the *ermK* message. Replacement of Leu-7 with stop codon (TAG) resulted in high level constitutive expression of *ermK*, the level of β-galactosidase activity in this mutant in the absence of EM was about twenty–fold higher than the basal level in wild type. In fact, there was no difference in *ermK* gene expression in the presence or absence of EM. This result suggests the stalled ribosome with Arg-6 at the P-site and Leu-7 at the A-site can activate *ermK* expression, even in the absence of inducing condition.

Growth curves of EM-induced stop mutants against EM

To confirm the result of β-galactosidase assays, we constructed the plasmids pEC101_t4, pEC101_t5, pEC101_t6, pEC101_t7, in which the stop mutation was taken at the

Table I. Construction and induction of *ermK* leader peptide mutants[a]

	Amino acid change														Codon change	β-galactosidase	
																No inducer	EM
wt	M	T	H	S	M	R	L	R	F	P	T	L	N	Q	None	107± 23	2244±221
pJA4	-	-	-	*	-	-	-	-	-	-	-	-	-	-	TCATAA	263± 12	337±150
pJA5	-	-	-	-	*	-	-	-	-	-	-	-	-	-	ATGTAA	504± 24	645±128
pJA6	-	-	-	-	-	*	-	-	-	-	-	-	-	-	AGATGA	447± 45	562± 37
pJA7	-	-	-	-	-	-	*	-	-	-	-	-	-	-	CTGTAG	2535± 68	2742±850
pJA9	-	-	-	-	-	-	-	-	*	-	-	-	-	-	TTCTAA	333± 92	2160±480
pJD23	-			-	-	-	-	-	-	-	-	-	-	-		91± 15	1866±253
pJD234	-				-	-	-	-	-	-	-	-	-	-		80± 26	150±25
pJA_S4Q	-	-	-	Q	-	-	-	-	-	-	-	-	-	-	TCACAG	134± 32	983±56
pJA_S4I	-	-	-	I	-	-	-	-	-	-	-	-	-	-	TCAATC	102± 16	394±26
pJA_M5T	-	-	-	-	T	-	-	-	-	-	-	-	-	-	ATGACA	203± 22	2265±210
pJA_R6T	-	-	-	-	-	T	-	-	-	-	-	-	-	-	AGAACC	531± 34	2563±278
pJA_R6P	-	-	-	-	-	P	-	-	-	-	-	-	-	-	AGACCC	143± 12	487± 32
pJA_R6Y	-	-	-	-	-	Y	-	-	-	-	-	-	-	-	AGATAC	475± 24	2679±442
pJA_R6F	-	-	-	-	-	F	-	-	-	-	-	-	-	-	AGATTC	302± 11	2230±326
pJA_R6A	-	-	-	-	-	A	-	-	-	-	-	-	-	-	AGAGCC	389± 27	2366±245
pJA_L7A	-	-	-	-	-	-	A	-	-	-	-	-	-	-	CTGGCA	234± 15	2934±363
pJA_L7T	-	-	-	-	-	-	T	-	-	-	-	-	-	-	CTGACT	1010±203	2969±269
pJA_L7E	-	-	-	-	-	-	E	-	-	-	-	-	-	-	CTGGAA	784± 54	2535±198

[a]The sequences of the first 14 amino acid residues of the leader peptide are shown for the wild-type (wt). Amino acid changes are listed in column 2 and associated codon change is listed in column 3 for the mutants in which a single codon is changed. β-galactosidase activity is expressed in Miller units (Miller, 1972).

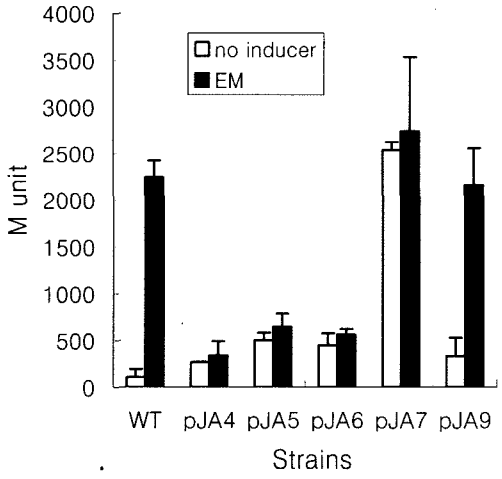

Fig. 1. Induction of *β*-galactosidase activity in *ermK* leader peptide stop mutants. Induction levels of *ermK* after 2 h of incubation with (■) or without (□) 0.1 μg of EM per mL.

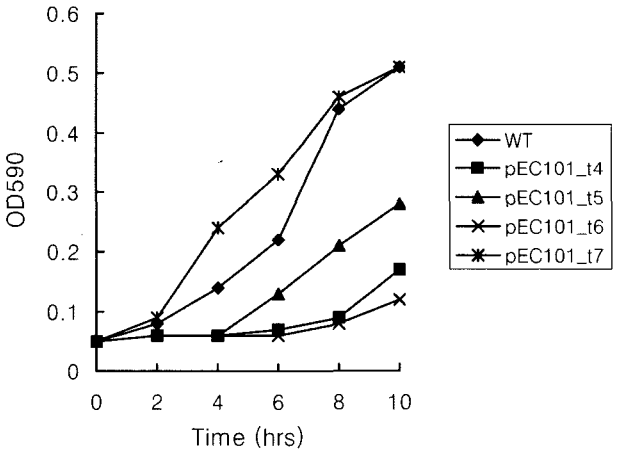

Fig. 2. Growth curves of EM (0.1 μg/mL)-induced stop mutant against EM (150 μg/mL)

4th, 5th, 6th and 7th codon of leader peptide. Each mutant was induced with a subinhibitory concentration of 0.1 μg/mL EM for 2 h. After induction, they were challenged with concentrations higher than the MICs of EM (150 μg/mL). EM-induced mutants of pEC101_t4, pEC101_t5 and pEC101_t6 showed a delay in growth for up to 10h incubation, consistent with induction experiments on *ermK* leader peptide-*lacZ* fusion (Fig. 2). pEC101_t7 showed constitutive resistance phenotype, which is consistent with *β*-galactosidase assay.

Inducibility of leader peptide deletion mutants

Mutant pJD23, in which the codons for leader peptide residues Thr-2 and His-3 were deleted, remained fully inducible by EM. But deletion of the codons for leader peptide residues Thr-2 through Ser-4 (mutant pJD234) resulted in reduced inducibility by EM. Therefore, the EM-bound ribosome stalling might begin at codon Ser-4 and extend through codon Arg-6 and Ser-4 through Arg-6 constitute the critical codons required for ribosome stalling

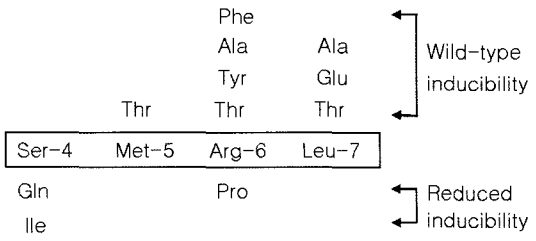

Fig. 3. Effect of leader peptide point mutation on *ermK* inducibility. The wild-type sequence of codons for 4 through 7 of the *ermK* leader peptide is shown within the box. Substitutions that reduced the inducibility of *ermK* are shown below the wild-type amino acid sequence, and substitutions that maintained an inducibility of *ermK* are shown above the sequence.

(Fang *et al.*, 2000).

Inducibility of leader peptide point mutants

We constructed point mutations in the region of Ser-4 through Leu-7 by using primers representing leader peptide coding sequence that contained randomly generated change (Table I) and summarized the inducibility of leader peptide mutations in Fig. 3. In pJA_S4Q, the inducibility of *ermK* expression by EM was decreased and moreover, Ser-to-Ile change in codon 4 (pJA_S4I) strongly affected inducibility by EM. These results suggest that the decreased inducibility of *ermK* expression is caused by change in codon 4, thus implicating this codon in EM-induced ribosome stalling. The change in codon 5 alone (pJA_M5T) had no effect. But other point mutations in codon 5 might reduce inducibility. The Arg-to-Pro change in codon 6 (pJA_R6P) showed reduced inducibility whereas replacement of Arg-6 with several other codons had no effect. These data indicate that the codons for residues Ser-4 to Arg-6 of the leader peptide comprise the critical region in which ribosome stalls in the presence of EM. Although base substitutions of three types in codons 7 showed a wild-type phenotype, this codon is important as a site at which the EM-bound ribosome stalls. To determine whose peptide sequence is important for ribsome to stall, more extensive mutations in which more than one codon is altered should be examined.

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