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Studies on Regulation of the Colicin El Gene Expression Using Promoter-operator Mutants

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Abstract Promoter-operator mutations of the colicin El gene on ColEl plasmid of *Escherichia coli* were isolated using site-directed chemical mutagenesis. The operator region of the gene is constituted by two overlapped SOS boxes which has high affinity to LexA repressor. DNA sequence analysis of the mutants revealed that base changes were in both the SOS boxes as well as the downstream region. Studies on gene expression *in vivo* and *in vitro* suggest the followings : 1) multiple point mutations within the terminal trinucleotide consensus sequences of the SOS boxes are necessary for derepression of the colicin El gene, 2) the intermediary portion between terminal consensus sequences has no significant effect, 3) the Pribnow boxes which are created by base substitutions shift the transcription initiation sites towards downstream, and 4) the downstream sequence together with the central overlapping position of the trinucleotide consensus sequence may be important in the LexA repressor-DNA interaction.

Key Words : Bacterial toxin, SOS response, Localized mutagenesis, Gene expression, DNA sequencing

Introduction

Bacteriocins are a group of bacterial toxins which attack bacterial cells. They are distinct from other bacterial toxins such as diphtheria toxin, pertussis toxin, and cholera toxin with respect to their target cells. The latter toxins attack mammalian cells and alter cellular activities in various ways. Although the target cells are different, both types of toxins are considered to have similar features in the mechanism of production in the cells. In the present study, therefore, I chose the colicin El-producing system to analyze the molecular mechanisms involved in the synthesis of the bacteriocin. Knowledge obtained from this study would be useful in understanding physiological and pathological process elicited by the bacterial infection.

Colicin El is a channel forming bacteriocin encoded by plasmid ColEl (1). The synthesis of colicin El can be induced by treating *Escherichia coli* cells harboring ColEl plasmid with agents that either damage DNA or interfere with DNA replication (2). Colicin El induction is one of the SOS responses which include phenomena such as prophage induction, induced DNA repair, mutagenesis, cessation of respiration and filamentous growth of cells (3). The genes involved in the SOS responses are repressed by a common repressor, lexA protein (LexA) (4). Upon DNA

damage, an inducing signal is generated and activates the recA protein (RecA). The activated form of RecA stimulates the specific cleavage of LexA at an alanine-glycine bond near the center of the protein, leading to

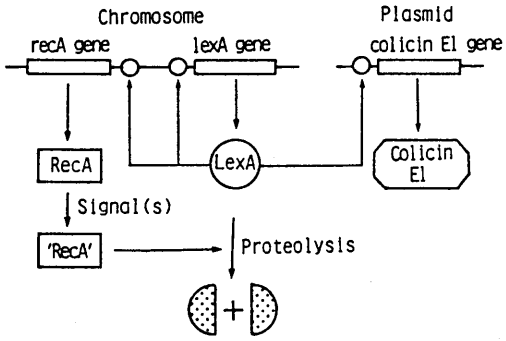

Fig. 1 Model of the colicin E1 gene expression (see text for discussion). RecA and LexA denotes respective proteins. "*RecA*" represents the activated form of RecA protein.

an inactivation of LexA and the derepression of all SOS genes including the colicin E1 gene (Fig. 1 ; for review see refs. 5, 6).

In a previous work, it has been established that the *in vivo* transcription of the colicin E1 gene starts at the point 75 base pairs (bp) upstream from the initiation codon of colicin E1 (7). It has been demonstrated that LexA binds with a high affinity to the approximately 40-bp long sequence around the transcription initiation site. The sequence of the binding site is composed of two overlapped "SOS boxes" which are found in other SOS genes (4).

To clarify further the molecular mechanism of transcription regulation of the colicin E1 gene, I report here isolation of some mutants of the promoter-operator region of the colicin E1 gene using site-directed chemical mutagenesis. Effects of those mutations on transcription both *in vivo* and *in vitro* were studied.

Materials and Methods

Bacterial strains and plasmids : All bacterial strains used were derivatives of *E. coli* K12. MC1000 (*lac*$^-$) and JM103 were our laboratory stock, K58 (*ung*$^-$) was a gift from Dr. M. Takanami. Plasmids used were ColE1 and pMC1396 (8).

Enzymes and chemicals : All restriction endonucleases were purchased from Takara Shuzo except *Sst* II which was from Bethesda Research Laboratories. T4 DNA polymerase, T4 DNA ligase and T4 polynucleotide kinase were supplied by Takara Shuzo. Calf intestinal alkaline phosphatase was supplied by Sigma. S1 nuclease was purchased from Boehringer-Mannheim. All restriction endonuclolytic and enzymatic incubations were performed according to instructions of the suppliers. RNA polymerase was purified by a modification of the method of Chamberlin and Berg (9). The purification procedures of the LexA repressor were described by Brent and Ptashne (10) and modified by Ebina *et al.* (4). Sodium bisulfite, sodium sulfite and hydroquinone were

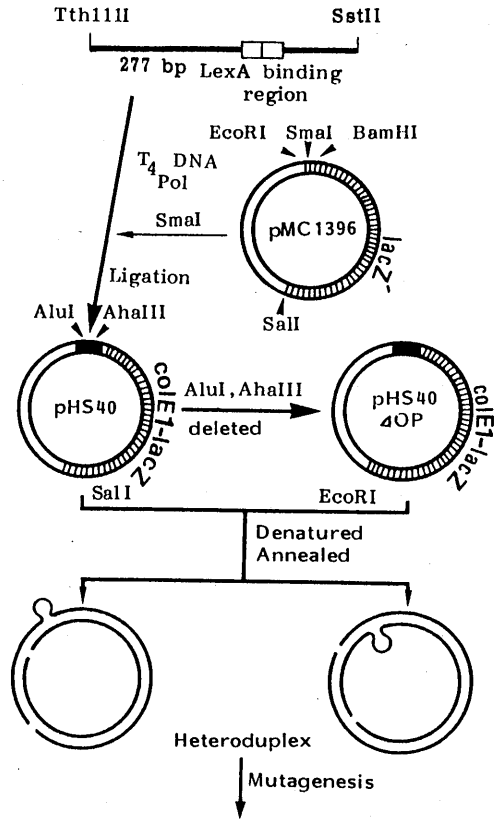

Fig. 2 Construction of the colE1-*lacZ* fusion and *in vitro* mutagenesis. Only restriction enzyme cleavage sites which were used in this study are shown.

purchased from Sigma ; mitomycin C from Kyowa Hakko (Tokyo) ; X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside from Wako Pure Chemical Industries. [γ-^{32}P] ATP (5000 Ci/mmol) was purchased from Radiochemical Center (Amersham).

Preparation of DNA : Covalently closed circular plasmid DNA was isolated by CsCl-ethidium bromide equilibrium centrifugation as described (11). Crude plasmid DNA was prepared by the modified alkaline method (12). Digested DNA was analyzed electrophoretically as described previously (13).

Construction of colE1-lacZ translational fusion : The vector plasmid pMC1396 which has the *lacZ* structural gene was used to construct the colE1-*lacZ* fusion (Fig. 2). If any appropriate regulatory sequence with the NH_2-terminal portion is plased in front of the *lacZ* gene in the same coding frame, the gene codes for functional β-galactosidase. The 277-bp *Tth*111 I-*Sst* II fragment was isolated from polyacrylamide gel and treated with T4 DNA polymerase at 37℃ for 2 h in the reaction mixture (20 μl) containing 67 mM Tris-HCl, pH 8.3, 10 mM 2-mercaptoethanol, 6.7 mM $MgCl_2$, 0.3 mM 4 dNTPs (dATP, dGTP, dCTP and TTP). The blunt ended fragment was then inserted into the *Sma* I site of the plasmid pMC1396. In the resulting plasmid pHS40, the NH_2-terminal segment of the colicin E1 gene was fused to *lacZ* structural gene in the same coding frame. Plasmid pHS40ΔOP was constructed from the plasmid pHS40 by deleting the 64-bp *Alu* I-*Aha* III fragment within the regulatory sequence.

Mutagenesis with sodium bisulfite : Mutagenesis with sodium bisulfite was performed as described (14). Plasmid DNA from each of the plasmids pHS40 and pHS40ΔOP was cut at a unique but different sites with *Sal* I and *Eco*R I, respectively (Fig. 2). Hybridization was carried out by mixing two kinds of linear DNA molecules (one pmol each) in 20 μl mixture to which 4 μl 1 M NaOH was added. The mixture was incubated for 10 min at room temperature followed by addition of 40 μl 0.1 M HCl and 8 μl 1 M Tris-HCl, pH 8.0 and incubation at 65℃ for 2 h. Hybrids were precipitated with ethanol and dissolved in 50 μl TE butter (10 mM Tris-HCl, pH 8.0, 0.1 M EDTA). Mutagenesis was carried out by addition of 280 μl 4 M bisulfite solution, pH 6.0 (freshly prepared by dissloving 156 mg of sodium bisulfite plus 64 mg of sodium sulfite in 0.43 ml distilled water) and 12 μl 0.1 M hydroquinone (freshly prepared). The mixture was incubated in the dark under paraffin at 37℃ for 1 h. The mutagenized DNA was separated from bisulfite by passage through a Sephadex G-100 column equilibrated with 0.2 M Tris-HCl, pH 9.2, 50 mM NaCl, 2 mM EDTA. The DNA fraction was incubated overnight at 37℃, precipitated with ethanol and redissolved in 40 μl TE buffer. The DNA was transferred into *E. coli* (*ung*$^-$) cells. Crude plasmid DNA prepared from these transformants was then retransferred into *E. coli* MC1000. Mutants showing deep blue color were selected on L-agar plates containing ampicillin (100 μg/ml) and X-gal (50 μg/ml).

Sl nuclease mapping assay : Sl-nuclease mapping assay was performed as described previously (7). *In vitro* transcription was performed using the covalently closed circular form of each mutant plasmid in the presence or absence of LexA repressor (100 nM). The standard transcription mixture (20 μl) contained 40 mM Tris-HCl, pH 8.0, 50 mM mercaptoethanol, 4 mM $MgCl_2$, 120 mM KCl, 1 mM each ATP, CTP, UTP, GTP, 1.5 μg RNA polymerase and 0.25 pmol of DNA template. Heparin was added at 1 mg/ml to avoid reinitiation of transcription by RNA polymerase. Incubations were carried out in three stages : (i) The reaction mixture containing LexA repressor and DNA template was incubated for 10 min at 37℃ to allow repressor to bind to the operator ; (ii) RNA polymerase was added and incubated for 10 min to allow it to bind to the promoter ; (iii) nucleotides mixture and heparin were added to initiate transcription. After 10 min at 37℃ DNase I (1μg/μl) and RNAsin (1U/μl) were added and incubation continued for another 10 min. The reaction was terminated by adding 5 μg of tRNA and 25 μl phenol (phenol, chloroform and isoamylalcohol at the ratio of 25 : 24 : 1), and the transcripts were ethanol precipitated. Transcripts were dissolved in hybridization buffer (80% formamide, 20 mM piperazine-N, N'-bis(2-ethanesulfonic acid), to which the ^{32}P-labeled probe was added (the sense strand of the 291-bp *Eco*RI-*Bam*HI fragment). The mixture was heated at 75℃ for 10 min and cooled down gradually to 37℃. The incubation was further continued for 2 h. After adding 240 μl of water and 30 μl of the 10 X Sl buffer (0.3 M sodium acetate, pH 4.6, 0.5 M NaCl, 10 mM $ZnSO_4$ and 50% glycerol), the reaction mixture was treated with 500 units of Sl nuclease for 45 min. The reaction was terminated by adding 5 μg tRNA and 300 μl phenol. After phenol extraction, the reaction products were precipitated with ethanol. The precipitate was

dissolved in 10 μl of 80% formamide, 10 mM NaOH, 1 mM EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol. The mixture was heated at 90℃ for 2 min and electrophoresed on a 10% polyacrylamide gel in the presence of 8 M urea. The S1 nuclease-protected DNA bands were visualized autoradiographically.

DNA sequence analysis : Sequencing of the mutated DNA region was performed by the dideoxy chain termination method using a M13 vector system (15). The *Eco*R I-*Bam*H I fragment (291-bp) from each mutant was isolated and cloned into the replicative form of M13mp8. The commercially available M13 primer was used for primed synthesis of the cloned region.

Enzyme assay and chemical analysis : β-Galactosidase activity was determined according to Miller (16). Protein concentrations were measured by the Lowry method with bovine serum albumin as standard (17).

Results and Discussion

In vitro mutagenesis

Starting with the plasmid pHS40, a colE1-*lacZ* fusion, the heteroduplex molecules were constructed in which either of the strands in the 64-bp *Alu* I-*Aha*III segment looped out (Fig. 2). The heteroduplexes thus formed were treated with sodium bisulfite that preferentially modified cytosine residues within the exposed single stranded region to uracil. The *in vitro*-modified molecules were transferred into the *E. coli* (*ung*$^-$) host. Due to the defective uracil N-glycosylase gene, this host allow a replication of DNA molecules containing uracil residues. Because linear homoduplexes transform poorly, transformants would arise predominantly from heteroduplex molecules. Upon subsequent replication, sodium bisulfite-modified molecules would produce mutant progeny molecules with GC-AT transitions. Plasmid DNA prepared from these primary transformants was retransferred into strain MC1000. Mutants were detected on agar plates containing ampicillin and the chromogenic compound X-gal as deep blue colonies compared with lighter ones, the wild type. The deep blue colonies were expected to contain mutant plasmids in which binding of LexA repressor to the operator was weakened. From each of such mutants, the 291-bp *Eco*R I-*Bam*H I fragment which contained the target region was isolated and cloned into the M13mp8 vector. Sequence of the mutated region of each plasmid was determined, and the results are summarized in Fig. 3. Only in case of mutant pHS40-6 the base substitutions were from C to T. All other mutants contained G to A substitutions. Although mutations were expected to occur in both strands, the base substitutions obtained here were primarily those on antisense strand. The secondary structure of the exposed single-stranded DNA might hinder the reactivity of the mutagen. Mutants pHS40-6 and pHS40-8 contained multiple point mutations, while others double or single.

β-Galactosidase activity directed by mutant plasmids

In the colE1-*lacZ* fusion, expression of the β-galactosidase is absolutely dependent on

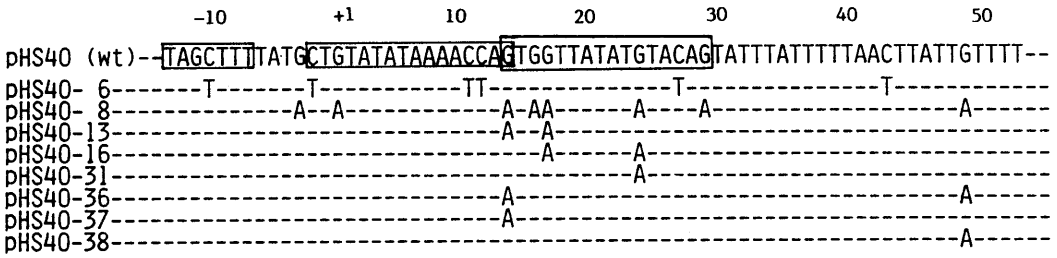

Fig. 3 Nucleotide sequences of colicin E1 promoter-operator mutants. Base changes found in mutants are shown below the wild type sequence, while unchanged bases are shown by dotted lines. Numbers above the sequence are counted from the position of the initiation site for colicin E1 mRNA. The LexA binding site and −10 region (Pribnow box) are boxed. Only mutagenized portion is shown here.

the promoter-operator derived from the colicin E1 gene. Thus, β-galactosidase synthesis is induced in the presence of mitomycin C, an inducer of the colicin E1 gene. To assess the mutations obtained above β-galactosidase activity was measured with extracts from the plasmid-carrying cells that had been preincubated with or without mitomycin C (Table 1). Mutant pHS40-6 showed higher expression of β-galactosidase at both the basal and induced levels than any other mutants and gave a low induction ratio. This mutant had multiple point mutations within the LexA binding region. Changes from C to T in the conserved terminal trinucleotide CTG of SOS box would weaken the binding affinity of the LexA repressor (18).

Table 1 β-Galactosidase activity directed by mutant plasmids. MC1000 harboring each mutant plasmid was grown at 37℃ in L-broth. At the early exponential phase, mitomycin C (1 μg/ml) was added to induce the expression of colE1-*lacZ* fusion gene. β-galactosidase activities were measured after 2 h incubation with or without mitomycin C.

plasmid	β-Galactosidase activity (%)[a]		Induction ratio[b]
	Basal	Induced	
pHS40 (wt)	100	800	8.0
pHS40-6	670	1200	1.8
pHS40-8	270	900	3.3
pHS40-13	190	800	4.2
pHS40-16	130	700	5.3
pHS40-31	120	700	5.8
pHS40-36	210	400	1.9
pHS40-37	90	1000	11.1
pHS40-38	110	1200	10.9

a β-Galactosidase activity is shown as % of wild type

b Induction ratio is the level of β-galactosidase activity at the induced state divided by the level at the non-induced state

The production of LexA repressor itself is autoregulated and it has been shown to contain two SOS boxes (10, 19). Since mutaions are obtained in both boxes of the lexA gene, it seems both sites are important for repression for the lexA gene (20, 21). The colicin E1 gene has two LexA binding sites with G in common at the joining position (4). Mutation at this position along with other positions as in mutant pHS40-8 raised the basal level of β-galactosidase. Among base substitutions occurred in mutant pHS40-8, the changes which were common in mutants pHS40-16 and pHS40-31 can be considered neutral. These changes that appear in the intermediary region between the consensus terminal trinucleotide did not affect the basal and induced levels of β-galactosidase. Mutant pHS40-13 which had G to A mutation at the central overlapping position of the SOS boxes together with another change in the intermediary portion showed considerable elevation of β-galactosidase expression at the basal level. Interestingly, mutant pHS40-36 which had a single point mutation at the overlapping position and another in the downstream region showed higher basal level with lower induction ratio. In contrast mutants pHS40-37 which had only single point mutation at the joining position of the SOS boxed and pHS40-38 which contained single point mutation at the downstream region as did mutant pHS40-36 did not show any effect on the β-galactosidase expression. Simultaneous change at these two different positions was required for the non-inducible phenotype. Thus, there seems to be an interaction between these two mutated nucleotides in derepression of the colicin E1 gene by LexA.

In vitro transcription analysis

S1-nuclease is a single-strand specific endonuclease. Only double-stranded region in hybrids are protected from digestion by S1-nuclease. *In vitro* transcription was carried out with the covalently closed circular form of each plasmid using purified RNA polymerase. If this form of plasmid is used, the transcription of the colicin E1 gene *in vitro* starts at the same position as *in vivo* (7). The tran-

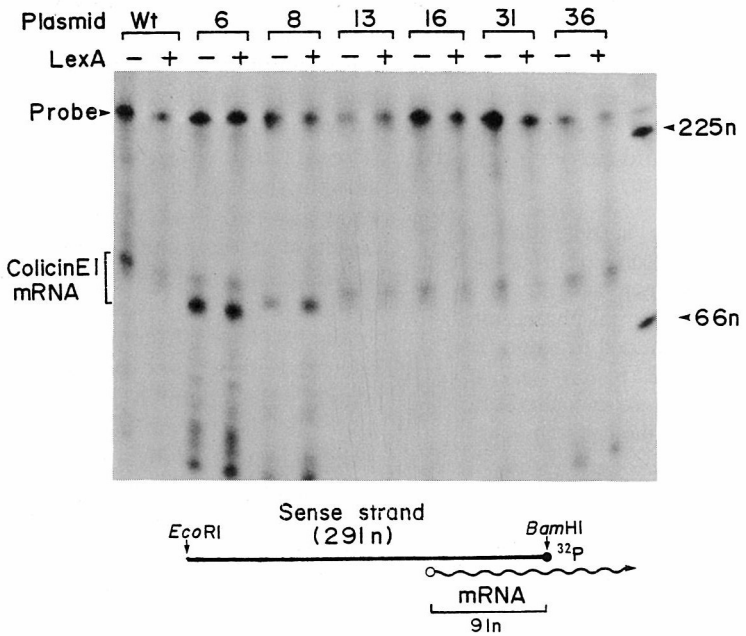

Fig. 4 S1-mapping analysis of *in vitro* transripts of the mutants. The number above each pair of lanes indicates the pHS40 number of the mutant. Minus and plus signs above the lanes indicate the absence and presence of LexA repressor respectively. On the bottom is illustrated the principle of the S1-mapping assay using the 291-nucleotide probe.

scription products were analyzed by the S1-nuclease protection assay and the results are shown in Fig. 4. the probe used was the ^{32}P-labeled sense strand of the *Eco*RI-*Bam*HI fragment. As expected, the colicin E1 transcript protected about 90 nucleotides portion of the probe. In mutant pHS40-6 there were two transcripts. The upper minor band corresponded to the authentic transcript. The lower major band was thought to be derived from a new transcript which has a length about 10 nucleotides shorter than the authentic one. The initiation site would have shifted from G(+1) to C(+11) in the latter transcript. The intense lower band might contribute to elevating the basal level of this mutant. Since both the transcripts were not repressed, it is likely that LexA repressor would bind with low affinity to the operator of this mutant. In case of mutant pHS40-8 the entire transcript was 7 nucleotides shorter than the authentic one. This might be interpreted by transcriptional activation from a new Pribnow box TTTATACT (from −8 to −1) that was created upon mutation. The initiation site was shifted from G(+1) to A(+7). In case of this mutant, the basal level of β-galactosidase was higher in the *in vivo* analysis (Table 1). This may due either to low affinity of LexA repressor to the operator or to the shift of transcription initiation site. The latter interpretation seemed unlikely, since the newly created transcription initiation site was still within the LexA binding region. In mutants pHS40-13, pHS40-16 and pHS40-31, expression of the colE1-*lacZ* fusion gene was clearly repressed in the presence of LexA. Mutant pHS40-36 showed almost the same amount of transcript in the absence and presence of LexA repressor. These results were essentially consistent with those of *in vivo* expression as judged by

β-galactosidase activity.

Conclusion

The LexA binding region of the colicin E1 gene is composed of two overlapping SOS boxes. There are four terminal trinucleotides that are conserved in many SOS boxes. However, two of them share a common nucleotide in the central position. The conclusions from the present study are the followings : (1) multiple point mutations within the terminal trinucleotide consensus sequences are necessary for derepression of the colicin E1 gene, (2) the intermediary portion between terminal consensus sequences of the SOS boxes has little effect as evidenced by mutations in the right box, (3) the Pribnow boxes which are created by base substitutions shift the transcription initiation sites towards downstream, and (4) mutaion in the downstream region indicates that the downstream sequences together with the overlapping terminal trinucleotide portion plays some role in repressor-DNA interaction.

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