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Plasmid-Encoded Regulation of Colicin E1 Gene Expression

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A plasmid-encoded factor that regulates the expression of the colicin E1 gene was found in molecular cloning experiments. The 2,294-base-pair *Ava*II fragment of the colicin E1 plasmid (ColE1) carrying the colicin E1 structural gene and the promoter-operator region had the same information with respect to the repressibility and inducibility of colicin E1 synthesis as the original ColE1 plasmid. An operon fusion was constructed between the 204-bp fragment containing the colicin E1 promoter-operator and *xylE*, the structural gene for catechol 2,3-dioxygenase encoded on the TOL plasmid of *Pseudomonas putida*. The synthesis of the dioxygenase from the resulting plasmid occurred in *recA*$^+$, but not in *recA*$^-$ cells and was derepressed in the *recA lexA*(Def) double mutant. These results indicate that the ColE1 plasmid has no repressor gene for colicin E1 synthesis and that the *lexA* protein functions as a repressor. Colicin E1 gene expression was adenosine 3',5'-phosphate (cAMP) dependent. Upon the removal of two *Pvu*II fragments (2,000 bp in length) from the ColE1 plasmid, the induced synthesis of colicin E1 occurred in the adenylate-cyclase mutant even without cAMP. The 3,100-bp *Tth*111I fragment of the ColE1 plasmid cloned on pACYC177 restored the cAMP dependency of the deleted ColE1 plasmid. Since the deleted fragments correspond to the mobility region of ColE1, the cAMP dependency of the gene expression should be somehow related to the plasmid mobilization function.

The synthesis of colicin E1 is coded for by the colicin E1 plasmid (ColE1) in *Escherichia coli* cells. The synthesis is repressed under ordinary conditions, but can be induced by treatments with UV light or mitomycin C. The induction of colicin E1 requires the SOS response (20), which also includes such phenomena as enhanced DNA repair capacity, induced mutagenesis, inhibition of cell division, cessation of respiration, and prophage induction. The SOS response is controlled coordinately by two regulatory elements, the *recA* and *lexA* proteins (8). The *lexA* protein operates exclusively as a repressor of SOS genes. The *recA* protein, which is activated as a protease by signals occurring after SOS-inducing treatments, inactivates the *lexA* protein. Thus, the SOS genes are derepressed to elicit the SOS response.

We previously demonstrated that the colicin E1 gene was repressed by the *lexA* protein in vivo (5) and in vitro (Y. Ebina, Y. Takahara, F. Kishi, A. Nakazawa, and R. Brent, J. Biol. Chem., in press). In addition to the *lexA* protein of chromosomal origin, a plasmid-encoded repressor has been suggested (13). In the present study, such a factor was extensively sought by evaluating colicin E1 synthesis in the cells bearing deletion variants of plasmid ColE1. Furthermore, we constructed an operon fusion between the promoter-operator region of the colicin E1 gene and *xylE*, the structural gene for catechol 2,3-dioxygenase (10). The results of experiments using these recombinant plasmids strongly suggested that ColE1 has no repressor gene for the regulation of the colicin E1 gene expression.

The expression of the colicin E1 gene was dependent on cAMP in vivo (6, 12). The transcription in vitro of the colicin E1 gene, however, occurred in the absence of cAMP and its receptor protein (CRP) (4). Using the deletion plasmids of ColE1, we present evidence that the mobility region of the plasmid is required for cAMP dependency of colicin E1 synthesis.

MATERIALS AND METHODS

Bacterial strains and plasmids. All strains used were derivatives of *E. coli* K-12. W3110, KY7231 (*recA1 trpB9578 tna-2 rpsL*), KH720 (Δ*cya phoS trp lac-2 rpsL rpoB*), DM1187 [F$^-$ *lexA51*(Def) *lexA3*(Ind$^-$) *recA441 sulA11 sup-37 thr leu his ilv*(Ts) *pro gal rpsL*], and NS872 (same as DM1187 except *recA1* and *his*$^+$) were described previously (5, 6). W3110-1 was a colicin E1-resistant derivative of W3110. The plasmids used were pACYC184 (2), pACYC177 (2), and pTS115 (10).

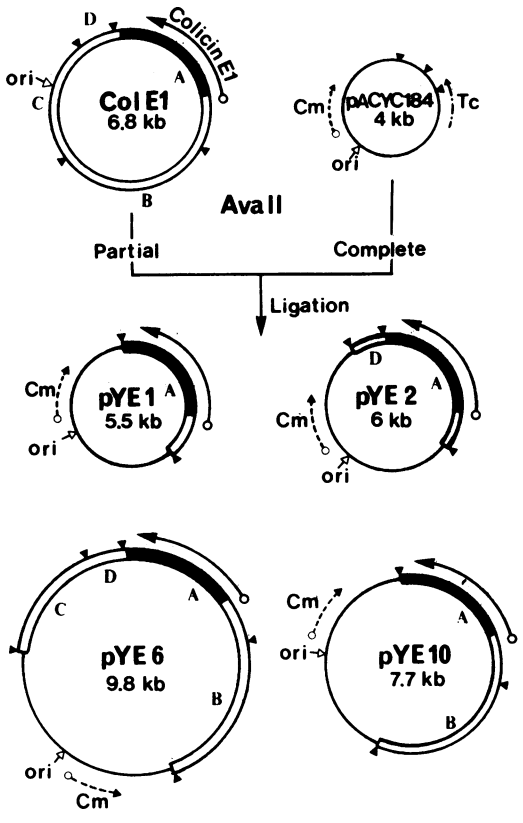

FIG. 1. Construction of ColE1-pACYC184 hybrid plasmids. Detailed methods for the construction of the hybrid plasmids were described in the text. The sites for *Ava*II are shown by solid triangles. Cm and Tc represent the genes that confer chloramphenicol and tetracycline resistance, respectively. The replication origins of ColE1 and pACYC184 are shown by *ori*. The thick and thin lines represent DNA from ColE1 and pACYC184, respectively. The filled portion indicates the structural gene for colicin E1. The directions of transcription are shown by arrows.

Media and induction conditions. Colicin E1 induction was performed in modified M9-Casamino Acids medium as described previously (11, 12). Catechol 2,3-dioxygenase from the operon fusion was induced by mitomycin C (1 μg/ml) in L-broth medium for strains W3110 and KY7231 and in modified M9-Casamino Acids medium for the adenylate cyclase-deletion mutant KH720. Colicin E1 (12) and catechol 2,3-dioxygenase (7) activities were assayed with sonic extracts as previously reported. Colicin E1 activity was expressed as units synthesized per cell culture turbidity (absorbance at 660 nm). Catechol 2,3-dioxygenase activity was expressed as milliunits per milligram of protein.

Restriction enzyme cleavage, ligation, and transformation. The isolation of covalently closed circular plasmid DNA was previously described (4). Crude plasmid DNA was prepared by the alkaline extraction method (1). Restriction endonucleases *Ava*II and *Sst*II were products of Bethesda Research Laboratories, Bethesda, Md. *Hae*III, *Sma*I, *Tth*111I, *Eco*RI, and T4 DNA polymerase were purchased from Takara Shuzo Co. Ltd., Kyoto, Japan. Digestions with restriction endonucleases were carried out according to the instructions of the manufacturers of the enzymes. The analysis of restriction fragments was done by electrophoresis as described previously (7, 10).

The ligation of DNA fragments and the transformation of *E. coli* cells were carried out as described previously (7). Transformants were selected on L agar containing antibiotics. The antibiotic concentrations used were 10 μg/ml (chloramphenicol), 25 μg/ml (kanamycin), and 100 μg/ml (ampicillin).

RESULTS AND DISCUSSION

Construction of ColE1-pACYC184 hybrid plasmids. To examine the minimal region of ColE1 essential for the controlled synthesis of colicin E1, we cloned the *Ava*II fragments of ColE1 into the vector pACYC184. ColE1 was cleaved with *Ava*II into four fragments, the sizes of which were 2.3 (A), 2.2 (B), 1.7 (C), and 0.5 (D) kilobase pairs. *Ava*II-A includes the whole sequence of the colicin E1 structural gene (1,566 base pairs) (21) and its promoter-operator region (4), and *Ava*II-D contains the gene for colicin E1 immunity (14). ColE1 DNA was partially digested by *Ava*II, and the products were ligated with the fragments of pACYC184 that were obtained through complete cleavage by *Ava*II. After the transformation of strains W3110 and W3110-1 by the ligated DNA, Cm^r Tc^s and colicin E1-producing clones were isolated. Four recombinant plasmids that carried one to four of the *Ava*II

TABLE 1. Colicin E1 synthesis by ColE1-pACYC184 hybrid plasmid[a]

Strain	Plasmid	Colicin E1 (U/A_{660})[b]			
		Without mitomycin C		With mitomycin C	
		Expt 1	Expt 2	Expt 1	Expt 2
W3110-1	pYE1	3	8	280	2,000
W3110-1	pYE2	2	5	300	8,500
W3110	pYE2	3	5	450	10,000
W3110-1	pYE6	3	ND[c]	480	2,400
W3110-1	pYE10	4	8	200	3,200
W3110	ColE1	1	2	960	2,600

[a] Colcin E1 synthesis was induced by mitomycin C (2 μg/ml) for 2 h at 37°C.

[b] The activity was expressed as units synthesized per cell culture turbidity (absorbance at 660 nm [A_{660}]). Colicin E1 units were defined as the highest dilution of the sonicated culture giving a clear zone of growth inhibition of the colicin E1-sensitive cells that were spread on a nutrient broth petri dish. Since the colicin assay used here was a biological method, the activities obtained sometimes varied. Accordingly, the results of two independent experiments are shown.

[c] ND, Not determined.

fragments of ColE1 were obtained (Fig. 1). Since pYE1 and pYE10 did not have *Ava*II-D, which contained the immunity region, the transformants carrying the plasmids were obtained only in W3110-1, a colicin E1-resistant strain. The relative orientations of the cloned *Ava*II fragments were the same as those in ColE1, judging from restriction enzyme analysis.

Colicin E1 synthesis from the hybrid plasmids. Spontaneous and induced levels of colicin E1 were determined with the cells carrying the recombinant plasmids described above. Spontaneous colicin E1 synthesis levels in the wild type and in the colicin E1-resistant cells carrying these hybrid plasmids were as low as that in the cells carrying ColE1 (Table 1). Under inducing conditions with mitomycin C, colicin E1 synthesis from these hybrid plasmids increased as greatly as did that from ColE1. In these cells, the copy numbers of the plasmids were essentially the same, judging from agarose gel electrophoresis of the plasmids obtained by the rapid alkaline extraction procedure (data not shown). When *Ava*II-A of ColE1 was cloned onto a derivative of plasmid R1, a multicopy vector with runaway replication, the level of the spontaneous colicin E1 synthesis from the recombinant plasmid was almost the same as that from ColE1 (unpublished data). Thus, whichever vector was used, *Ava*II-A gave the same repressibility of colicin E1 synthesis as did the entire ColE1.

We have found a new gene in the 5'-flanking region of the colicin E1 gene, the transcription of which started from the site 179 base pairs away from the start site of colicin E1 transcription and proceeded in the opposite direction (unpublished data). The transcription in vivo of the gene was induced by mitomycin C and stimulated by cAMP. The determination of the nucleotide sequence of this gene revealed that about 90% of the sequence of the structural gene was included in *Ava*II-A. We constructed a deletion plasmid of pYE2 in which the C-terminal-half region down from the unique *Bcl*I site in the structural gene was deleted. Spontaneous and induced colicin E1 syntheses from this plasmid did not differ from those from pYE2 or from ColE1 (data not shown). Therefore, this unknown gene appeared to play no significant role in colicin E1 induction. These results suggested that colicin E1 synthesis was not repressed by any factor encoded by the ColE1 region other than *Ava*II-A.

Construction of ColE1-*xylE* fusion plasmid. The only significant coding sequence on *Ava*II-A is that for colicin E1. Therefore, colicin E1 itself might control autogeneously its own gene function. To exclude this possibility, we constructed a recombinant plasmid, pYT3, in which the promoter-operator region of the colicin E1 gene was fused to *xylE*, the structural gene for catechol 2,3-dioxygenase (Fig. 2). The dioxygenase is one of the enzymes, responsible for the degradation of toluene, which are encoded by the TOL plasmid of *Pseudomonas putida* mt-2 (7). We previously determined the DNA sequence of *xylE* (10). The gene was expressed in *E. coli* cells when it was joined to the appropriate promoter of *E. coli*. A fused protein with catechol 2,3-dioxygenase should not be synthesized from the hybrid plasmid, since within 30 bases upstream from the coding sequence, stop codons are in the same coding frame as *xylE*. A 204-base-pair fragment carrying the promoter-operator region of the colicin E1 gene (4) was obtained by digesting ColE1 with *Sst*II and *Hae*III. The fragment was ligated to the *Sma*I and *Sst*II sites of pACYC177, and pYT6 was obtained. On the other hand, the *Sst*II fragment (1.2 kilobase pairs) of pTS115, a *xylE*-pACYC177 hybrid plasmid previously constructed, included the structural gene for catechol 2,3-dioxygenase, but not its promoter (10). Therefore, the *Sst*II fragment was inserted into the *Sst*II site of pYT6, and pYT3 was obtained.

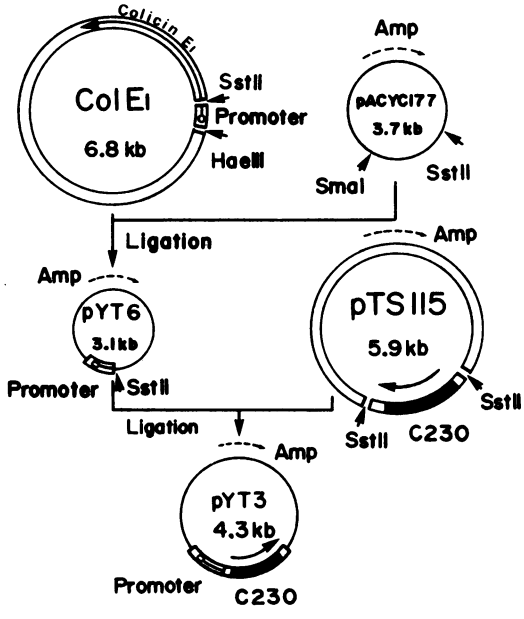

FIG. 2. Construction of ColE1-*xylE* fusion plasmid. pYT6 was obtained from an Am^r colony which was sensitive to kanamycin. pTS115 is a *xylE*-pACYC177 hybrid plasmid. The filled portion indicates the structural gene for catechol 2,3-dioxygenase (C230). Cells carrying pYT3 were selected on the plate containing ampicillin by the development of yellow color due to 2-hydroxymuconic semialdehye after 0.1 M catechol was sprayed over colonies as described previously (7). For other detailed procedures to construct these plasmids, see the text.

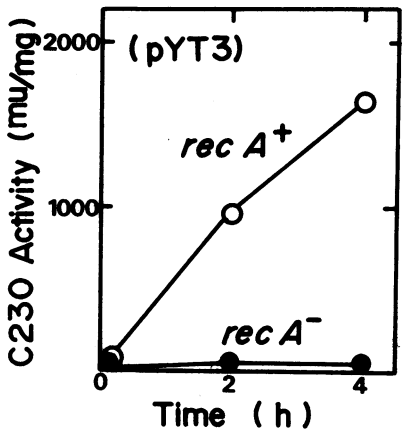

FIG. 3. Induction of catechol 2,3-dioxygenase by mitomycin C in *recA*$^+$ (W3110) and *recA*$^-$ (KY7321) cells carrying pYT3. The cells carrying the plasmid were grown to log phase, and mitomycin C was added at 1 μg/ml. The experimental procedures were as described in the text. Catechol 2,3-dioxygenase (C230) activity was expressed as milliunits per milligram of protein of the crude extract.

Induction of catechol 2,3-dioxygenase from the ColE1-*xylE* fusion plasmid. To examine the regulation of *xylE* gene expression from the promoter-operator of the colicin E1 gene, we introduced pYT3 into *recA*$^+$ and *recA*$^-$ cells. Catechol 2,3-dioxygenase synthesis from pYT3 was repressed under noninduced conditions, but induced by mitomycin C in *recA*$^+$ cells (Fig. 3). No enzyme synthesis was observed in the *recA*$^-$ cells under either induced or noninduced conditions. The induction of the dioxygenase synthesis from pYT3 by mitomycin C was delayed in the *uvrB*$^-$, but not in the *uvrA*$^-$ cells (data not shown). The same response of colicin E1 synthesis from ColE1 in the *uvrA* and *uvrB* mutants was reported previously (17). We also cloned the *Sst*II fragment carrying the *xylE* gene into the unique *Sst*II site of ColE1. Catechol 2,3-dioxygenase synthesis from this recombinant plasmid was regulated in the same way as from pYT3 (data not shown). These results showed that sequences other than those in the *Hae*III-*Sst*II region on ColE1 were not essential to the controlled expression of the colicin E1 gene.

We have obtained evidence that the *lexA* protein has a high affinity for the operator on the *Hae*III-*Sst*II fragment and strongly represses colicin E1 mRNA synthesis in vitro at relatively low concentrations of the protein (Y. Ebina et al., in press). Spontaneous catechol 2,3-dioxygenase synthesis from pYT3 was derepressed in DM1187 [*lexA*(Def)] and NS872 [*recA lexA*(Def)] cells (Table 2). Therefore, the *lexA* protein is the proper repressor of the colicin E1 gene.

cAMP dependency of colicin E1 synthesis. The induced synthesis of colicin E1 from ColE1 was dependent on cAMP in the adenylate cyclase-defective cells (Fig. 4a), as previously reported (12). The transcription in vivo of the colicin E1 gene was shown to be dependent on cAMP (6). Sequence analysis revealed that the operator region has the sequence homologous to other cAMP-CRP binding sites (18). However, in the in vitro experiments using the DNA fragment carrying the colicin E1 promoter region, transcription from the promoter occurred even in the absence of cAMP and CRP and was not significantly stimulated by these factors (4). Therefore, some factor responsible for the cAMP dependency of the colicin E1 gene expression should be missing in the in vitro transcription system.

To search for the plasmid-encoded factor, we examined colicin E1 induction in the adenylate cyclase-defective cells carrying a deletion variant of the ColE1 plasmid. ColE1 was cleaved into three fragments (4.8, 1.5, and 0.5 kilobase pairs) with *Pvu*II (Fig. 5). The largest fragment was rejoined, and pYE21 was obtained. In the adenylate cyclase-defective cells carrying pYE21, the induced synthesis of colicin E1 was independent of cAMP (Fig. 4b). Next, ColE1 was cleaved into two fragments (3.1 and 3.7 kb) with *Tth*111I (Fig. 5). The 3.1-kilobase-pair fragment contained neither the colicin E1 structural gene nor the replication origin, but had the region of the deleted fragments of *Pvu*II described above. The *Tth*111I fragment was ligated to pACYC177 at the unique *Sma*I site after converting the 3'-protruding ends to blunt ends by the action of T4 DNA polymerase, and pKS1 was obtained. The induced synthesis of colicin E1 was dependent on cAMP in the adenylate cyclase-defective cells carrying both pYE21 and pKS1 (Fig. 4c). These results indicated that the *Pvu*II fragments provided a *trans*-acting material

TABLE 2. Catechol 2,3-dioxygenase synthesis by pYT3 in *lexA* and *recA* mutants

Strain	Dioxygenase synthesis in mutant		Sp act (mU/mg)[a]	
	recA	*lexA*	Overnight culture[b]	Induced culture[c]
W3110(pYT3)	+	+	2.3	1,647
DM1187(pYT3)	+	−	275	ND[d]
NS872(pYT3)	−	−	1,250	ND

[a] One milliunit corresponds to the formation of 1 nmol of the product per min at 27°C.
[b] Cells grown in L-broth overnight.
[c] After incubation with mitomycin C (1 μg/ml) in L-broth for 4 h.
[d] ND, Not determined.

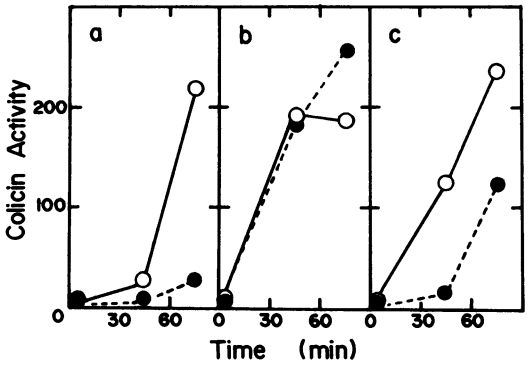

FIG. 4. Effect of cAMP on colicin E1 induction in the adenylate cyclase-defective cells (KH720) carrying ColE1 (a), pYE21 (b), and both pYE21 and pKS1 (c). Induction with mitomycin C (1 μg/ml) was done in the presence of 1 mM cAMP (○) and in its absence (●). Colicin E1 activity was expressed as units synthesized per cell culture turbidity (absorbance at 660 nm).

that confers cAMP dependency on the plasmid.

The region deleted in pYE21 is thought to synthesize components of the relaxation complex (3), which has the ability to nick ColE1 at the site near the replication origin (9) and participates in plasmid transfer (19). It has been reported that tryptophanase and β-galactosidase inductions are stimulated by cAMP in wild-type cells, but are not affected by the nucleotide in the topoisomerase I mutant strains (16). As

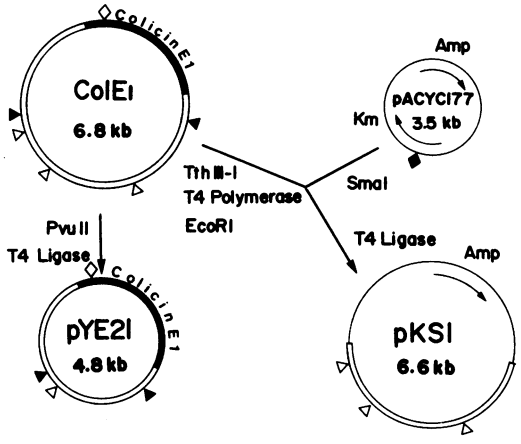

FIG. 5. Construction of the deletion and hybrid plasmids of ColE1. The filled portion represents the colicin E1 structural gene. Symbols: ▽, *Pvu*II; ▼, *Tth*111I; ◇, *Eco*RI; and ◆, *Sma*I. Amp and Km represent the genes that confer ampicillin and kanamycin resistance, respectively. Cells bearing pYE21 were selected by colicin E1 immunity. Cells harboring pKS1 were isolated by ampicillin resistance and kanamycin sensitivity. The enzymes used at each step of the construction of the deletion and hybrid plasmids are indicated.

topoisomerase I catalyzes the relaxation reaction of negatively supercoiled DNA, the negative superhelicity increases in the mutant strain (15). Genes on DNA in such a condition are well transcribed (16). In the case of pYE21, a similar event might occur with respect to the superhelicity of the plasmid. From preliminary observations with electron microscopy, pYE21 showed increased superhelicity as compared with parental ColE1. The increased superhelicity might bring about a change in the local DNA structure of the promoter region so that RNA polymerase can bind more easily to the promoter even without cAMP-CRP.

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