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Isolation and Characterization of a Mutant ColE1 Plasmid That Allows Constitutive Colicin E1 Synthesis

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It has been possible to isolate a ColE1 mutant which synthesizes colicin E1 constitutively. This result shows that there must be a gene(s) responsible for the regulation of colicin E1 synthesis.

Colicin E1 is an antibiotic protein produced by an *Escherichia coli* strain carrying colicinogenic factor E1 (ColE1). Under normal growth conditions, only a small proportion of the colicinogenic cells produce colicin spontaneously (20). However, a significant fraction of colicinogenic cells are readily induced to synthesize colicin by treatment with mitomycin C (9) or by incubating the cells at 43°C (12).

Recently, Maeda et al. (17) isolated a colicinogenic mutant of the ColE1 plasmid that fails to confer immunity to colicin E1. This mutant plasmid (pKY2289) consists of whole ColE1 DNA and an ampicillin transposon (Tn*3*) (17). *E. coli* cells harboring pKY2289 form normal colonies on ampicillin-containing nutrient agar at 37°C. However, they form very tiny colonies on nutrient agar at 43°C (17) or on agar supplemented with a low concentration of mitomycin C at 37°C (16).

Maeda et al. (16), during the process of characterizing this mutant plasmid, noticed that colicin E1-tolerant *E. coli* cells harboring pKY2289 can form colonies on nutrient agar even at 43°C or on nutrient agar supplemented with a low concentration of mitomycin C at 37°C. This observation strongly suggests that the induction of colicin E1 is not necessarily a lethal event to colicin E1-tolerant colicinogenic cells. Therefore, we attempted to isolate ColE1 mutants that produce high levels of colicin E1 constitutively. In this communication, we describe the isolation of such a mutant and the properties of the *E. coli* cells harboring this mutant plasmid.

The bacterial strains and the plasmids used in this study are listed in Table 1. The parental ColE1::Tn*3* plasmid used in this study is called pKY2283 and consists of whole ColE1 DNA and an ampicillin transposon (Tn*3*). This plasmid was constructed from pKY2113 (23) by in vitro genetic manipulations as described by Maeda et al. (17). *E. coli* KS1616 cells harboring pKY2283 are resistant to ampicillin and immune to colicin E1 and are induced to synthesize colicin E1 in a manner similar to that used to induce colicin E1 synthesis by those harboring the wild-type ColE1 plasmid.

E. coli KS1616 cells harboring pKY2283 were incubated in polypeptone-bonito extract broth medium (5, 16) in the presence of 100 μg of chloramphenicol per ml. Extrachromosomal DNA was then extracted from these cells and purified as described previously (18). These purified pKY2283 DNAs were introduced into $CaCl_2$-treated colicin E1-tolerant *E. coli recA* cells by selecting ampicillin-resistant (Amp^r) transformants. We confirmed that *E. coli recA* strain AB2463 harboring pKY2283 plasmids produced no detectable colicin at 37°C. This result is consistent with that of Kennedy (12). However, we found that 1 out of the 50 Amp^r transformants isolated after introducing pKY2283 DNAs into AB2463 cells showed constitutive colicin E1 production at 37°C. When the plasmid DNAs extracted from this mutant transformant were returned to $CaCl_2$-treated AB2463 cells, each of the transformants conferred a phenotype similar to that of the original mutant transformant. These results demonstrate that the mutation(s) responsible for constitutive colicin E1 production is located on the plasmid DNA.

Colicin production of *E. coli recA* cells harboring parental pKY2283 and that of cells harboring the mutant plasmid were compared by the double-layer method (13). The results observed at 30 and 43°C are shown in Fig. 1. *E. coli recA* cells harboring the parental pKY2283 plasmid showed no inhibition zone at 30°C but slightly visible inhibition zones at 43°C. On the other hand, the *recA* cells harboring the mutant plasmid showed clear inhibition zones not only at 43 but also at 30°C. This mutant plasmid was named pKY2463.

To make sure that the colicin E1 synthesis of the cells harboring pKY2463 is fully derepressed, AB1157(pKY2463) cells were grown in polypep-

TABLE 1. *Bacterial strains and plasmids*[a]

Strain	Description	Source or reference
Bacteria		
KS1616	HfrH Δ(*gal-attλ-bio*) Δ(*guaA-guaB*)	Fukumaki et al. (5)
AB1157	F⁻ *rec*⁺ *thr leu pro his thi arg tsx tolC rpsL*	Howard-Flanders and Theriot (8)
AB2463	AB1157 *recA tolC*	Howard-Flanders and Theriot (8)
J53(RP4)	F⁻ *pro met*(λ) (RP4)	Barth and Grinter (2)
C600S	*thi lac suII*	Sato et al. (22)
Plasmids	(Former name)	
pML21		Hershfield et al. (7)
pKY96	(ColE1-*cosλ-guaA*)	Fukumaki et al. (5)
pKY2113	(ColE1-*amp2113*)	Shimada et al. (23)
pKY2283	(ColE1-*amp2283*)	This work
pKY2289	(ColE1-*amp2289*)	Maeda et al. (16, 17)
pKY2463	(ColE1-*amp2463*)	This work
pAO2		Oka (19)
pAO43		Oka (personal communication)

[a] AB1157 and AB2463 cells used in this experiment carry a *tolC* mutation. *tolC* mutants were isolated as colicin E1-resistant cells, and they were sensitive to sodium deoxycholate and mitomycin C. Abbreviations: *attλ*, attachment site for λ; Δ, deletion; *suII*, carrying suppressor of amber mutations (*supE*). The other genetic symbols are those used by Bachmann et al. (1). All of the plasmids listed are ColE1 derivatives.

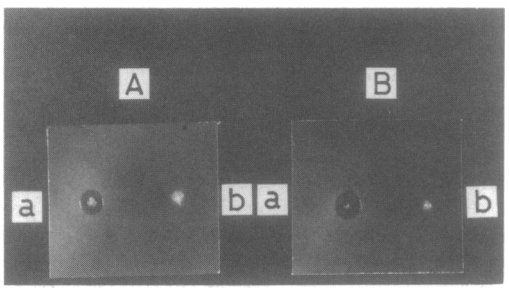

FIG. 1. *Stab test haloes of wild-type AB2463-(pKY2283) (b) and mutant AB2463(pKY2463) (a) colicinogenic cells at 30°C (A) and 43°C (B). The colonies of the colicinogenic strains, grown overnight at 30°C on polypeptone-bonito extract agar plates containing ampicillin, were picked with toothpicks onto two polypeptone-bonito extract agar plates containing ampicillin. After overnight growth, one at 30 and the other at 43°C, respectively, both of the plates were treated with chloroform vapors and then covered with colicin-sensitive and ampicillin-resistant indicator E. coli J53(RP4) cells suspended in minimal soft agar (0.35% agar containing 0.5% NaCl) and incubated at 37°C for 6 h as described previously (13).*

tone-bonito extract broth medium with or without mitomycin C treatment (5). The amount of synthesized colicin E1 was measured by the spot test as described previously (3). Cells harboring the pKY2463 plasmid synthesized colicin E1 even in the absence of mitomycin C, and the amount of synthesized colicin E1 was approximately the same as that of mitomycin C-treated *rec*⁺ cells harboring the parental pKY2283 plasmid (Table 2). These results indicate that production of colicin E1 is fully constitutive both in the *rec*⁺ AB1157(pKY2463) cells and in the *recA* AB2463(pKY2463) cells.

TABLE 2. *Induction of colicin E1 production by mitomycin C*[a]

Plasmid	Colicin E1 titer[b]			
	AB1157 *rec*⁺		AB2463 *recA*	
	Control	+Mitomycin C	Control	+Mitomycin C
ColE1	1	64–256	NT[c]	NT
pKY2283	1	64–256	1	1
pKY2463	16–64	64–256	64–256	4–16

[a] Mitomycin C induction and titration of colicin E1 of the colicinogenic cultures were performed as follows (5). A 5-ml amount of colicinogenic culture was treated with 0.5 ml of chloroform and thoroughly mixed, using a Vortex mixer. After the chloroform had settled, a sample of the culture was diluted serially. From each dilution, 5 μl was spotted onto the surface of a polypeptone-bonito extract agar plate freshly preseeded with J53(RP4) cells suspended in 2.5 ml of minimal soft agar. The plates were incubated for 6 h at 37°C, and the growth inhibition of indicator cells was examined. The colicin titer (i.e., the number of killing units per milliliter) was determined arbitrarily as the highest dilution factor at which killing of the bacterial lawn was still visible. Each successive dilution was fourfold greater than the previous dilution. Growth of colicinogenic cells was performed at 37°C.

[b] +Mitomycin C, 0.5 μg of mitomycin C per ml was added to the culture at log phase; control, mitomycin C was not added. The colicin E1 titer of AB1157(ColE1) cells was taken as 1.

[c] NT, Not tested.

The growth rate of cells harboring pKY2463 did not differ from that of cells harboring paren-

tal pKY2283; thus, the presence of the pKY2463 plasmid in a cell does not affect its growth.

The molecular weights and the structures of the mutant pKY2463 plasmid DNAs and the parental pKY2283 DNAs were compared by analyzing *Eco*RI and/or *Sma*R digests with agarose gel electrophoresis as described elsewhere (23). The electrophoretic patterns of these DNA fragments in agarose gels showed no marked difference in structure between these two kinds of DNA molecules, suggesting that the pKY2463 DNA molecule does not carry a detectable deletion or insertion mutation. At present, we cannot eliminate a possibility that the Tn*3* on pKY2283 DNA might have moved to a new place and caused the mutation.

The number of plasmid copies per chromosome was estimated by the following two methods.

(i) The percentage of covalently closed circular DNAs relative to chromosomal DNAs was measured by CsCl-ethidium bromide density gradient centrifugation of Sarkosyl lysates. The results showed that there was no significant difference in the number of covalently closed circular DNA copies per chromosome between cells harboring the pKY2283 and pKY2463 plasmids (Table 3).

(ii) The β-lactamase activity levels of the above two kinds of cells were compared. Using copy mutants of the R plasmid R1*drd-19*, Uhlin and Nordström (25) found that there was a proportional increase in the specific activity of the R plasmid-mediated β-lactamase with increasing plasmid copy number. Accordingly, the gene dosage effect on β-lactamase activity can be used to compare the approximate number of plasmid copies in the cells harboring pKY2283 with that in the cells harboring pKY2463. The results obtained showed no significant difference in the β-lactamase activity between these two kinds of cells (Table 3).

The results obtained by the above two methods suggest that there is no significant increase in the number of plasmid copies of pKY2463 compared with that of parental pKY2283.

To examine the effect of coexistence of other ColE1 plasmids on constitutive colicin E1 production, KS1616 cells harboring a non-colicinogenic hybrid ColE1 plasmid, pKY96 (15), were transformed with pKY2283 or pKY2463 DNAs to Amp^r, $GuaA^+$, and colicinogenic cells. The presence of pKY96 considerably repressed the colicin E1 synthesis of the mutant plasmid. We obtained similar results with the following non-colicinogenic ColE1 derivatives: (i) pML21 (7), a small colE1 plasmid carrying a kanamycin transposon; (ii) pAO2 (14), a small ColE1 plasmid that contains about one-fourth of the original ColE1 DNA; and (iii) pAO43, a pAO2 plasmid carrying a kanamycin transposon (A. Oka, personal communication). Typical results are shown in Fig. 2. Thus, it is most likely that the gene product(s) responsible for the regulation of colicin E1 production is coded by a region carried with pAO2.

TABLE 3. *Plasmid copy numbers and β-lactamase activities of colicinogenic cells grown at 37°C*

Strain	Copy no.[a]	β-Lactamase activity[b]
AB2463(pKY2283)	11.2	1.0
AB2463(pKY2463)	11.9	1.2

[a] Copy number was estimated by the amount of covalently closed circular DNA molecules and by the amount of chromosomal DNA molecules in CsCl-ethidium bromide isopycnic centrifugation. Experimental procedures were as described previously (18). Values were calculated by assuming that the molecular weights of the ColE1::Tn*3* monomer and the *E. coli* chromosome are 7.2×10^6 and 2×10^9, respectively.

[b] β-Lactamase activity was assayed as follows. Cells carrying ColE1::Tn*3* plasmids were grown to log phase in 80 ml of polypeptone-bonito extract broth; then they were collected by centrifugation, washed thoroughly, and suspended in 5 ml of 0.1 M potassium phosphate buffer (pH 6.5). Cell-free extracts were prepared by sonic oscillation, and cell debris was removed by centrifugation at 10,000 rpm for 10 min. The enzyme activity of these extracts was measured by the rapid fixed-time assay of Sargent (21). The protein concentration of cell-free extracts was adjusted to 50 μg/ml in 2.0 ml of 0.1 M potassium phosphate buffer (pH 6.5). The optical density at 490 nm was measured, and the enzyme activity was expressed arbitrarily in terms of optical density change per milligram of protein of the enzyme preparation. The protein content was determined by the method of Lowry et al. (14). The specific activity found in extracts prepared from cells harboring the parental plasmid pKY2283 is set to 1.0 for comparison. In this experiment, 1.0 corresponds to a change of 6.3 in optical density at 490 nm per mg of protein of extracts after incubation at 30°C for 30 min.

In conclusion, it has been possible to isolate a ColE1 mutant that produces colicin E1 constitutively. Properties of this mutant were examined, and the results indicate that ColE1 contains a gene(s) responsible for the regulation of colicin E1 synthesis and that this gene(s) probably is located around the replication origin of ColE1. Kennedy (12) suggested that production of colicin E1 is under the control of a temperature-sensitive regulatory mechanism, such as a repressor protein, and our present observations support this hypothesis.

Recently, a genetic analysis of ColE1 plasmids has been carried on extensively with Tn*A* (am-

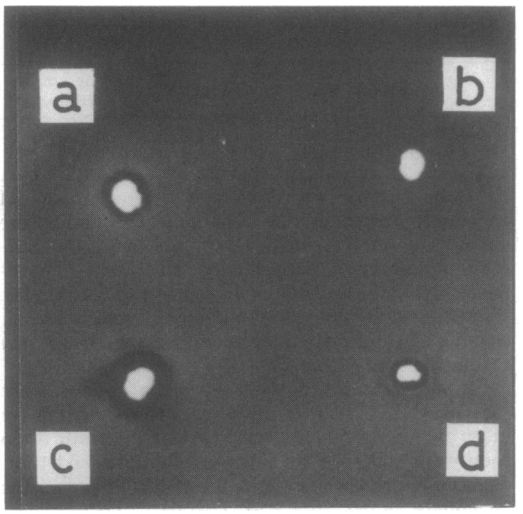

FIG. 2. *Effect of a coexisting pAO2 plasmid on colicin E1 production of E. coli cells harboring the mutant or parental plasmid. E. coli cells harboring the resident pAO2 plasmid were transformed with pKY2283 or pKY2463 plasmid DNAs to Amp^r and colicin E1-immune (Ie1$^+$) cells. Stab test haloes of them were compared with those of E. coli cells harboring pKY2283 or pKY2463 plasmid DNAs. Stab tests were performed at 37°C. Strains: a, C600S-(pKY2283); b, C600S(pKY2283,pAO2); c, C600S-(pKY-2463); d, C600S(pKY2463, pAO2).*

picillin transposon) as a mutagen (4, 10, 11, 17, 24), and the Tn*A*-induced mutations which affect colicin production, colicin immunity, relaxation of plasmid DNA, and plasmid incompatibility functions have been isolated. However, ColE1 mutants concerned with the regulatory mechanism of colicin E1 production have not yet been isolated.

Kool and Nijkamp (13) isolated a mutant of the bacteriocinogenic plasmid CloDF13 that showed enhanced bacteriocin production. This phenotype is probably due to the increased number of plasmid copies, and the mutation is probably at the level of plasmid DNA replication (13).

Furthermore, we found that colicin E1-tolerant *E. coli* cells harboring pKY2463 grow at 37°C as normally as do those harboring the parental pKY2283 plasmid, in spite of their constitutive colicin E1 production. Although it has been thought that the induction of colicin E1 synthesis in *E. coli* cells is lethal to the host cells (6, 20), our results suggest that colicin E1-tolerant colicinogenic *E. coli* cells can survive in spite of their constitutive colicin E1 production. The mode of action of colicin E1 is not clear at present (15). However, the isolation of such a mutant plasmid as pKY2463 suggests that the target of colicin E1 does not exist within the *E. coli* cell.

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