Studies on the Regulation of Colicin Ib Synthesis: Replication of the Col Ib-P9 Plasmid During Colicin Induction

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The Col Ib-P9 plasmid content of colicinogenic cells has been measured by both sedimentation analysis of cleared lysates and by deoxyribonucleic acid hybridization to purified Col Ib-P9 factor deoxyribonucleic acid. The results show an independence between mitomycin C-induced colicin synthesis and Col Ib-P9 replication. In addition, the ratio of the various molecular configurations (i.e., covalently closed circles or open circles) of the Col Ib-P9 plasmid does not change upon induction of colicin synthesis. Heat treatment of a colicinogenic strain carrying a thermosensitive *dnaB* mutation on the bacterial chromosome leads to complete inhibition of Col Ib-P9 plasmid replication and the simultaneous induction of colicin Ib synthesis in all cells of the colicinogenic population. These studies rule out mechanisms or regulation of colicin Ib synthesis based on the induced replication of the Col Ib-P9 plasmid. Our studies show that in a Col Ib-P9 colicinogenic population there is, on the average, one plasmid per cell.

The presence of the colicinogenic plasmid Col Ib-P9 confers upon the cell containing the plasmid the ability to synthesize colicin Ib. The synthesis of colicin Ib in colicinogenic strains of *Escherichia coli* K-12 appears to be a regulated process. For example, the addition of mitomycin C (MTC) to a normally repressed colicinogenic population causes a large increase in the amount of colicin synthesized (4, 7). We have recently presented evidence that the mechanism of regulation is at the level of ribonucleic acid transcription (9). In addition, we were able to demonstrate that both Col Ib-P9 factor transcription and colicin synthesis requires cyclic adenosine 5'-monophosphate and cyclic adenosine 5'-monophosphate receptor protein. Monk and Clowes (12) have presented indirect evidence that there exists a repressor involved in the regulation of colicin Ib synthesis. These results lead to models of regulation analogous to those controlling the lactose operon (10). It should be pointed out, however, that the induction of colicin synthesis need not occur as a direct result of repressor inactivation by inducer, but may occur by the titration of repressor by operator as a result of increased Col Ib-P9 plasmid replication. Alternatively, induction may simply be attributed to an increase in constitutive colicin synthesis due to an increase in gene dosage.

In this paper, the role of Col Ib-P9 plasmid replication in colicin Ib induction is investi-

gated. The approach used has been to measure the content of cellular Col factor before and after MTC-induced colicin synthesis. The results demonstrate that there is no correlation between colicin induction and plasmid content.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 W3110 F⁻, *thy*, λ ⁻ and *E. coli* K-12 W3110 F⁻, *dnaB*_u (306), *leu*, *thy*, *str-r* were obtained from P. Carl. Colicinogenic derivatives were constructed by conjugal transfer of the Col Ib-P9 plasmid from *E. coli* K-12 W3110 F⁻, λ ⁻, *str-r* (Col Ib-P9) by the method of Ozeki et al. (14).

Media and growth conditions. Cells were grown in tryptone broth containing 1.3% tryptone (Difco) and 0.7% NaCl, at 37°C with shaking. When it was desired to induce for colicin synthesis, MTC (0.5 µg/ml, final concentration, Calbiochem) was added to a logarithmic phase culture (approximately 2×10^8 cells per ml).

Assay of colicin and colicin-induced cells. Both the number of cells producing colicin, as determined by lacuna counting (13), and total colicin units, as determined by the end point dilution technique, were assayed as previously described (9). The percent induction is defined as the number of lacunae per number of viable cells $\times 100$. In all cases, it was found that the number of viable cells, as determined by plating, was the same as the number of total cells, as determined by direct counting under the microscope.

Preparation of cleared lysates and sedimentation analysis. Cleared lysates were prepared by the method of Clewell and Helinski (1) using cells which had been labeled with 5 µCi of [*methyl*-³H]thymine

per ml (20 mCi/ μ M, 12 μ g/ml, Schwartz-Mann). The exact labeling conditions for each experiment are described in the text. The Col Ib-P9 plasmid was identified by sedimentation through 15 to 50% (wt/vol) sucrose density gradients (containing 0.01 M ethylenediaminetetraacetate, 0.06 M KCl, and 0.03 M tris(hydroxymethyl)aminomethane, pH 8.0). Thirty-milliliter gradients were centrifuged for 14 h at 22,500 rpm at 4 C in a Beckman SW25.1 rotor. Gradients were fractionated using an ISCO model 640 density gradient fractionator. A portion of each fraction was precipitated by the addition of an equal volume of ice cold 10% trichloroacetic acid and 1 mg of bovine serum albumin (Nutritional Biochemicals). The precipitate was collected by suction filtration on glass fiber filters (Reeve Angel 934AH) and subsequently washed three times with 5 ml of ice cold 5% trichloroacetic acid. Filters were air dried, immersed in 10 ml of scintillation fluid (4 g of 2,5-diphenyloxazole per liter of toluene), and counted in a Beckman LS-100 liquid scintillation spectrometer.

To determine the amount of covalently closed circular deoxyribonucleic acid (DNA), Col Ib-P9 plasmid DNA isolated from the appropriate fractions of sucrose density gradients was centrifuged to equilibrium in cesium chloride-ethidium bromide. Plasmid DNA was diluted to 4.0 ml in $0.1 \times$ SSC (0.015 M NaCl, 0.0015 M sodium citrate, pH 7.0) and mixed with 6 g of solid CsCl (Penn Rare Metals) and 1.8 ml of ethidium bromide (1 mg/ml, Sigma). The remainder of the tube space was filled with light mineral oil. The tubes were then centrifuged at 50,000 rpm for 24 h at 20 C in a Beckman type 65 rotor. The gradients were fractionated, and radioactivity in each fraction was measured as above with the exception that the filters were washed with ice cold isopropanol after the trichloroacetic acid washes to remove ethidium bromide.

DNA-DNA hybridization. DNA-DNA hybridizations were performed as previously described using purified Col Ib-P9 DNA immobilized on nitrocellulose filters (8). [3 H]DNA for the hybridization studies was extracted as follows: cleared lysates were prepared as described above. The resulting pellet was washed with 1.0 ml of buffer (0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, 0.005 M ethylenediaminetetraacetate, 0.5 M NaCl, pH 8.0) and collected by centrifugation. The supernatant fraction was discarded and to the pellet was added 5 ml of the same buffer containing 0.25% Sarkosyl and 1 mg of Pronase per ml (Calbiochem). The pellet was then incubated at 37 C for 5 h followed by resuspension by gentle agitation. The remaining steps were performed on both the cleared lysate and the treated pellet. An equal volume of water-saturated redistilled phenol was added and mixed for 30 min at room temperature. The emulsion was broken by low speed centrifugation. Ribonuclease (50 μ g/ml) was added to the upper aqueous phase and the mixture was incubated at 37 C for 4 h. The aqueous phase was then twice extracted with phenol. The final aqueous phase was dialyzed against $0.1 \times$ SSC at 4 C for 16 h, passed through a 26-gauge syringe ten times, and then alkali

denatured (8) for use in DNA-DNA hybridization. Approximately 99% of the total cellular radioactive DNA is recovered by the extraction procedures outlined above. Of this, 5.5% is found in the cleared lysate fraction and 93.5% found is the extracted pellet fraction.

RESULTS AND DISCUSSION

Col Ib-P9 DNA synthesis during MTC-induced colicin Ib synthesis. The induction of Col Ib-P9 colicinogenic cells by MTC has no detectable effect on total cellular DNA synthesis (R. Isaacson, Ph. D. thesis, University of Illinois, Urbana, 1974). However, since the Col Ib-P9 plasmid represents only a few percent of the total cellular DNA (2; see below), increased or decreased synthesis of the plasmid itself would be difficult to detect by measurement of total DNA synthesis. Therefore, a series of experiments were performed to measure changes in plasmid content specifically.

When a cleared lysate is prepared from a Col Ib-P9 colicinogenic strain and the lysate subsequently sedimented through a 15 to 50% sucrose density gradient, the Col Ib-P9 plasmid is usually found at two positions in the gradient: at a position corresponding to 73S (super-coiled plasmid) and at a position corresponding to 45S (relaxed complex plasmid). For a description of the two plasmid forms see Clewell and Helinski (2). In the following experiments, we have considered the sum of the radioactive materials found at 45S and 73S to represent the total amount of Col Ib-P9 plasmid found in such cells. In a total of four experiments, the amount of measured radioactive Col Ib-P9 DNA in uninduced and induced cells (MTC treated) was $2.78 \pm 0.13\%$ and $2.75 \pm 0.15\%$, respectively, of the total radioactive cellular DNA. Assuming the molecular weight of the plasmid to be 61.5×10^6 (2), this is equivalent to approximately one copy of the plasmid per cell. The data presented in Table 1 clearly demonstrated that there is no correlation between the degree of colicin induction and the amount of total Col factor present. It can also be seen that the time of isotope addition does not affect the results.

Since it was considered possible that colicin induction might lead to an alteration of the Col Ib-P9 plasmid structure (i.e., more or less covalently closed circular DNA), a series of dye-buoyant density gradient centrifugation experiments were performed. Total Col factor was isolated by sucrose density gradient centrifugation and then centrifuged to equilibrium in self-generating cesium chloride-ethidium bro-

TABLE 1. *Col Ib-P9* plasmid content in cleared lysates prepared from cultures of *E. coli* K-12 W3110 F⁻, thy, λ⁻, (*Col Ib-P9*) at various levels of colicin induction^a

Expt	-MTC		+MTC		-MTC/ +MTC
	% induction (lacunae per cells × 100)	% Col factor	% induction (lacunae per cells × 100)	% Col factor	
1	0.01 ^b	2.6	10	2.9	0.90
2	0.06 ^b	2.9	55	2.5	1.16
3	0.03 ^c	2.7	83	2.7	1.00
4	0.02 ^c	2.9	75	2.9	1.00

^a Cells were grown to 2×10^8 per ml. The culture was then split and to one portion was added MTC (0.5 μg/ml). Both portions were then incubated 2 additional h. Cleared lysates were then prepared as described. The amount of *Col Ib-P9* plasmid was determined as described in the text.

^b Isotope added at the time of MTC addition.

^c Isotope added at the time of culture inoculation.

mid gradients to determine the amount of covalently closed circular DNA and open or linear DNA. A summary of the results are presented in Table 2. It can be seen that the amount of covalently closed circular DNA does not appreciably change upon MTC induction in any given experiment. However, it should be noted that the amount of covalently closed circular DNA may vary significantly from experiment to experiment ranging from 8 to 35% of the total Col factor. We have not investigated the underlying cause of such variations.

TABLE 2. Effect of MTC-induced colicin *Ib* synthesis on the amount of CCC *Col Ib-P9* plasmid^a

Expt	-MTC		+MTC		-MTC/ +MTC
	% induction (lacunae per cells × 100)	% CCC Col <i>Ib-P9</i>	% induction (lacunae per cells × 100)	% CCC Col <i>Ib-P9</i>	
1	0.07	8.5	83	7.6	1.12
2	0.05	24.0	58	26.0	0.92
3	0.02	35.0	66	34.4	1.02

^a *E. coli* K-12 W3110 F⁻, thy, λ⁻ (*Col Ib-P9*) cells were grown, and cleared lysates were prepared as described in Materials and Methods and Table 1. Percent induction was determined by counting lacunae as described. Total col factor was prepared as described in Results and centrifuged to equilibrium in cesium chloride-ethidium bromide gradients as described in Materials and Methods. CCC, Covalently closed circular DNA.

DNA-DNA hybridization. An alternate method for the measurement of cellular *Col Ib-P9* plasmid is by DNA-DNA hybridization (8). [³H]DNA was extracted from cleared lysates and from the Sarkosyl-treated pellet obtained in the preparation of the cleared lysate. The presence of ³H-labeled *Col Ib-P9* DNA was then determined in each fraction by hybridization to column purified *Col Ib-P9* DNA (8). Figure 1 shows the results of such an experiment using DNA that had been extracted from either uninduced or from MTC-induced colicinogenic cells. It is clear that the same amount of *Col Ib-P9* DNA is found in the cleared lysates (about 1.5% of total radioactivity found in lysed cells) regardless of whether MTC-induced colicin synthesis had occurred. Assuming a 50% efficiency of hybridization (8), the amount of Col factor in cleared lysates is approximately 3% of the total cellular DNA. This is in good agreement with the data obtained from sedimentation experiments.

It can be seen that a small fraction (0.006%) of the labeled DNA found in the membrane fraction does hybridize to *Col Ib-P9* DNA. Since we have previously shown that DNA prepared

FIG. 1. DNA-DNA hybridization of [³H]DNA isolated from cleared lysates and resulting pellets to purified *Col Ib-P9* DNA. DNA was extracted from *E. coli* K-12 W3110 F⁻, thy, λ⁻ (*Col Ib-P9*) and hybridization was performed as described. Symbols: (O), cleared lysate prepared from uninduced cells (0.02% induction); (●), cleared lysate prepared from MTC-induced cells (53% induction); (□), pellet from uninduced cells; (■), pellet from MTC-induced cells. The ordinate represents the percent of total DNA found in the hybrid.

from noncolicinogenic *E. coli* K-12 *thy*⁻, *str*^{-r} does not hybridize to purified Col Ib-P9 DNA (8), we feel that the small amount of hybridizable DNA in the membrane fraction is indeed Col Ib-P9 plasmid. Whether this represents contamination by soluble plasmid or represents a membrane bound replicating intermediate is unknown. It is interesting in this regard to note that Sherratt and Helinski (15) have presented evidence that newly replicated colicinogenic factor E1 is membrane associated.

Col factor replication and induction of colicin synthesis in a mutant temperature sensitive for DNA synthesis. Since the primary target of MTC is DNA (16), it was of interest to examine whether total inhibition of Col Ib-P9 synthesis would lead to induction of colicin synthesis. We therefore transferred the Col Ib-P9 plasmid into a *dnaB* mutant which is defective in DNA chain elongation at 43 C (17) and examined the effects of temperature shift on plasmid replication and colicin synthesis.

When cleared lysates were prepared from [³H]thymine-labeled *dnaB* (Col Ib-P9) cells incubated at 30 or 43 C, and then sedimented through 15 to 50% sucrose density gradients, the profiles seen in Fig. 2 were obtained. It is clear

FIG. 2. Sedimentation profiles of cleared lysates of *E. coli* K-12 W3110 F⁻, *dnaB*_{ts}(306), *leu*⁻, *thy*⁻, λ ⁻, *str*^{-r} (Col Ib-P9). Cells were grown in 2×10^8 per ml and divided into two portions, and [³H]thymine (5 μ Ci/ml) was added to each portion. One portion was incubated at 30 C (○) and the other at 43 C (▲). After 2 h, cleared lysates were prepared and centrifuged through 15 to 50% sucrose density gradients as described. Sedimentation is from left to right.

TABLE 3. Effect of temperature on colicin Ib synthesis in a wild-type colicinogenic strain and a *dnaB* colicinogenic mutant^a

Strain	Temperature (C)	% induction (lacunae per cells $\times 100$)	Colicin (U/ml)
<i>E. coli</i> K-12 W3110, F ⁻ , <i>thy</i> ⁻ , λ ⁻ (Col Ib-P9)	30	0.02	<1
	43	0.02	2
<i>E. coli</i> K-12 W3110 F ⁻ , <i>dnaB</i> ts(306) <i>leu</i> ⁻ , <i>thy</i> ⁻ , λ ⁻ , Str-r (col Ib-P9)	30	0.02	<1
	43	100	16

^a Cells were grown at 30 C to 2×10^8 per ml. Each culture was divided in half and one portion of each was incubated at 30 C and the other portion was incubated at 43 C. After 2 h the percent induction and amount of synthesized colicin was determined as described.

that at 43 C no radioactive Col factor was synthesized. It is thus apparent that the replication of the Col Ib-P9 plasmid requires the *dnaB* gene product (a ribonucleotide triphosphatase [18]). Table 3 shows the effects of temperature treatment at 43 C on colicin synthesis in this strain. After 2 h of heat treatment, 100% of the mutant cells were producing colicin, as determined by lacuna counting. In addition, there is greater than a 16-fold increase in the total amount of colicin synthesized. In comparison, heat treatment of the wild-type colicinogenic strain does not lead to the same stimulatory effect on colicin synthesis. Whereas parent and mutant show the same degree of induction at 30 C, the mutant synthesizes eightfold more colicin at 43 C than does the parental strain. Thus, it is clear that Col Ib-P9 synthesis is not required for induction of colicin synthesis. On the contrary, we show here that the inhibition of plasmid DNA synthesis has resulted in the induction of the colicinogenic cell population.

In summary, the results reported here rule out models of regulation of colicin synthesis based on induced synthesis of plasmid DNA. Similar conclusions have been made in the case of induction of colicin E1 (3), colicin E2 (3, 5, 11), and colicin B (6). These results are consistent with models of regulation of colicin synthesis operating at the level of direct transcriptional control (9).

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