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Mitomycin C-Induced Expression of *trpA* of *Salmonella typhimurium* Inserted into the Plasmid ColE1

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*Eco*RI endonuclease digestion of the deoxyribonucleic acid of a ϕ80 transducing phage carrying the entire tryptophan (*trp*) operon of *Salmonella typhimurium* (ϕ80 *S.t.trpE-A*) yielded a 4.3 × 10^6-dalton fragment containing intact *trpE*, *trpD*, and *trpC* and a 3.35 × 10^6-dalton fragment containing intact *trpA*. The *trpA* fragment inserted into *Eco*RI-cleaved plasmids ColE1 and pCR1 was expressed regardless of its orientation of insertion. Mitomycin C, a compound that induces colicin E1 production in ColE1-containing bacteria, stimulated tryptophan synthetase α production in cells containing ColE1-*trpA* plasmids with the *trpA* fragment inserted in one orientation but not the other. We conclude that in the inducible plasmids *trpA* can be expressed from the colicin E1 promoter.

Prokaryote and eukaryote deoxyribonucleic acid (DNA) segments have been introduced into and amplified on bacterial plasmids and viruses, making available for study large quantities of specific genes, gene clusters, and regulatory sequences (13, 17, 19, 24). It is not yet known whether expression of such DNA segments is at least partially dependent upon the promoters of the prokaryote vectors that have become fused to the foreign DNA. When DNA is inserted into the single *Eco*RI site of ColE1 DNA, the hybrid plasmid cannot produce colicin, suggesting that the *Eco*RI site is within the ColE1 region responsible for colicin formation (13). If a foreign DNA fragment were inserted into ColE1 at its *Eco*RI site, it is conceivable that the inserted fragment would be expressed from the colicin promoter. In the present study we introduced an *Eco*RI fragment of the tryptophan (*trp*) operon of *Salmonella typhimurium* into ColE1 and a related plasmid, pCR1 (3), in both possible orientations. The *trp* operon fragment contains *trpA* and a portion of *trpB* but lacks both the principle *trp* promoter, *p1* (1), and the low-efficiency internal promoter, *p2* (1, 15). We find that *trpA* is expressed at a low level when inserted in either orientation. However, when it is inserted into ColE1 in one orientation, *trpA* expression is increased appreciably by mitomycin C, a drug known to induce colicin production in ColE1-containing strains (14).

MATERIALS AND METHODS

Strains. All bacterial strains used in this study are derivatives of *Escherichia coli* K-12. C600/ColE1, C600/pCR1, and P162-8ts309/ColE1 were kindly provided by V. Hershfield and D. Helinski. pCR1 (3) is a derivative of the ColE1-*kan* plasmid pML2 (13) in which one of the two *Eco*RI sites has been deleted. Strain W3110 Δ[*tonB-trpA905*] has a deletion extending from the operator-proximal region of *trpA* to or beyond *tonB* (2). *E. coli* nomenclature is used throughout to designate the genes of the *trp* operon (gene order is *trp P,O EDCBA*).

Isolation of DNA. Plasmid DNA was amplified and isolated essentially as described by Hershfield et al. (13). Bacteria were grown in M9 medium (22) containing 0.2% glucose and 0.5% Casamino Acids, or in L-broth, at 37°C. Chloramphenicol (200 μg/ml) was added to early-log-phase cultures. Fourteen hours later sodium azide was added at a final concentration of 1 mM, and the cells were immediately harvested by centrifugation. Cleared lysates from lysozyme-treated cells (16) were centrifuged for 40 h at 36,000 rpm (Beckman 50 Ti rotor) and 15°C in CsCl gradients containing ethidium bromide (4). Gradient fractions containing the bottom band (visualized by ultraviolet illumination and representing covalently closed circular plasmid DNA) were collected. Ethidium bromide was extracted with isopropanol equilibrated with CsCl-saturated water. The DNA was diluted with 2 volumes of 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.0 at 4°C) containing 5 mM ethylenediaminetetraacetate (EDTA) and 50 mM NaCl and was then precipitated with 6 volumes of absolute ethanol and 0.3 volume of 3 M sodium acetate (pH 6.0). The precipitated DNA was suspended in 100 mM Tris-hydrochloride (pH 8.0 at 4°C) containing 0.75 mM EDTA and 60 mM NaCl. ColE1 DNA was isolated from C600/ColE1 or P162-8ts309/ColE1, and pCR1 DNA was prepared from C600/pCR1.

Bacteriophage DNA was prepared as described elsewhere (27).

Restriction endonuclease digestion of DNA. *Pst*I

and *Hind*III endonucleases were obtained from New England Biolabs, and *Eco*RI endonuclease was purchased from Miles Laboratories. Digestions were carried out in 20- to 200-μl reaction mixtures containing 10 mM Tris-hydrochloride (pH 7.9 at 37°C), 7 mM $MgCl_2$, 6 mM 2-mercaptoethanol, and 60 mM NaCl (18). After digestion for 1 h at 37°C, reactions were terminated and cohesive ends were dissociated by heating for 5 min at 65°C followed by rapid chilling.

Ligation of DNA fragments. Polynucleotide ligase isolated from T4-infected *E. coli* B was obtained from Miles Laboratories. The ligation method employed was that of Hershfield and Helinski (personal communication). Reactions were carried out in volumes ranging from 200 to 900 μl and containing 66 mM Tris-hydrochloride (pH 7.8 at 25°C), 6.6 mM $MgCl_2$, 0.066 mM adenosine 5'-triphosphate, 10 mM dithiothreitol, approximately 30 μg of DNA, and 0.1 to 0.3 unit of ligase. After incubation at 15°C for 4 h, EDTA was added to a final concentration of 10 mM to stop the reaction, and a sample of the DNA was examined by electrophoresis on 0.8% agarose gels.

Agarose gel electrophoresis. Intact plasmid DNA, restriction products of plasmid and bacteriophage DNA, and ligated DNA were analyzed on 0.8% agarose (Bio-Rad Laboratories) gels in Tris-EDTA-acetate-NaCl buffer (9). The digested DNA (0.2 to 0.6 μg) was applied to the sample wells in a maximum volume of 20 μl containing 1% sodium dodecyl sulfate, 5% glycerol, and 0.005% bromophenol blue. Electrophoresis was carried out at 15 V for 18 h with a slab gel apparatus (10). Gels were stained with 0.5 μg of ethidium bromide per ml for 15 min and photographed on a short-wavelength ultraviolet transilluminator.

Extraction of DNA from agarose gels. *Eco*RI endonuclease digestion products from about 150 μg of DNA were separated on a 0.8% agarose slab gel having a cross-sectional area of 4 cm^2. A strip of the gel was stained with ethidium bromide to locate the band of interest, the area corresponding to this band was cut from the unstained gel, and the DNA was eluted as follows (M. Dieckmann, personal communication). The gel fragment was suspended in 2 volumes of 10 mM Tris-hydrochloride (pH 8 at 4°C) containing 2 mM EDTA and 1 M NaCl by repeatedly forcing it through a 20-gauge needle. The resulting suspension was incubated for 16 h at 47°C. The gel was sedimented by centrifugation for 30 min at 18,000 × *g*, and the supernatant solution was extracted with 1 volume of buffer-saturated phenol. The DNA was precipitated with 3 volumes of absolute ethanol and collected by centrifugation at 4°C for 3 h at 30,000 rpm (Beckman SW30 rotor).

Transformation and plasmid stability. The procedure for transformation was essentially that of Cohen et al. (5). Bacteria were grown at 37°C in L-broth to a density of about 3×10^8 cells per ml, quickly chilled, sedimented, and washed once with 0.5 volume of 10 mM $CaCl_2$. After centrifugation, bacteria were suspended in 0.5 volume of chilled 30 mM $CaCl_2$, kept at 0°C for 20 min, sedimented, and then resuspended in 0.2 volume of 30 mM $CaCl_2$. A 0.6-ml volume of $CaCl_2$-treated cells was incubated with DNA (1 to 10 μg) for 30 min at 0°C. The bacteria-DNA mixture was then incubated for 1.5 min at 42°C, added to 5 ml of L-broth, and shaken at 37°C for 90 min. The bacteria were then sedimented, washed twice with 0.85% NaCl solution, and spread on plates containing the appropriate agar media, with or without kanamycin (25 μg/ml) as desired.

The stability of *trpA* plasmids was determined by measuring the appearance of tryptophan auxotrophs in cultures grown for at least 20 generations in L-broth. By this test all plasmids studied were >99% stable.

Mitomycin C treatment and *trp* operon enzyme assays. Concentration of mitomycin C (Sigma Chemical Co.) was determined spectrophotometrically, using a molar extinction coefficient of 21,840 at 367 nm in water (23).

Cultures to be treated with mitomycin C were grown to a density of about 6×10^8 cells per ml in a Tris-buffered medium (50 mM Tris-hydrochloride [pH 7.4], 50 mM NaCl, 40 mM KCl, 10 mM NH_4Cl, 0.6 mM KH_2PO_4, 0.16 mM Na_2SO_4, 1 μM $FeCl_3$, and 2.5 mM $CaCl_2$). The medium was supplemented with 0.3% glycerol or 0.2% glucose and contained 0.5% Casamino Acids and 200 μg of L-tryptophan per ml unless indicated otherwise. Samples of 25 to 50 ml were incubated with mitomycin C at 37°C with shaking for 3 h, and the cells harvested, washed, and disrupted by sonic oscillation. Debris was removed by centrifugation, and the supernatant solutions were assayed for tryptophan synthetase α and β_2 (EC 4.2.1.20) in the indole + L-serine → L-tryptophan reaction as described elsewhere (6). The percent survival of cells after mitomycin C treatment was determined by viable cell counts.

RESULTS

***Eco*RI endonuclease cleavage sites in the *S. typhimurium trp* operon.** DNA of φ80 and φ80 *S.t.trpE-A* (7) was digested with *Eco*RI endonuclease, and the DNA fragments produced were separated by electrophoresis on agarose slab gels. The cleaved transducing phage DNA lacked the 9.2- and 1.66-megadalton (Mdal) fragments characteristic of φ80 and instead had two distinct new fragments of 3.1 and 4.3 Mdal (Fig. 1). In addition, the 3.35-Mdal band contained a second DNA fragment as judged visually and by microdensitometry. To identify the *Eco*RI fragment(s) containing *Salmonella trp* genes, we ligated *Eco*RI-cleaved DNAs of φ80 *S.t.trpE-A* and pCR1 and transformed recipient strains containing either a *trpE* deletion (W3110 Δ*trpE5*) or a nonreverting mutation in *trpA* (W3110 *trpA33*). Several Trp^+ transformants were obtained from each strain. The $trpE^+$ transformants contained hybrid plasmids consisting of a 4.3-Mdal *Eco*RI fragment and the *Eco*RI fragment corresponding to pCR1, whereas the *trpA* transformants had plasmids with a 3.35-Mdal *Eco*RI fragment and the pCR1

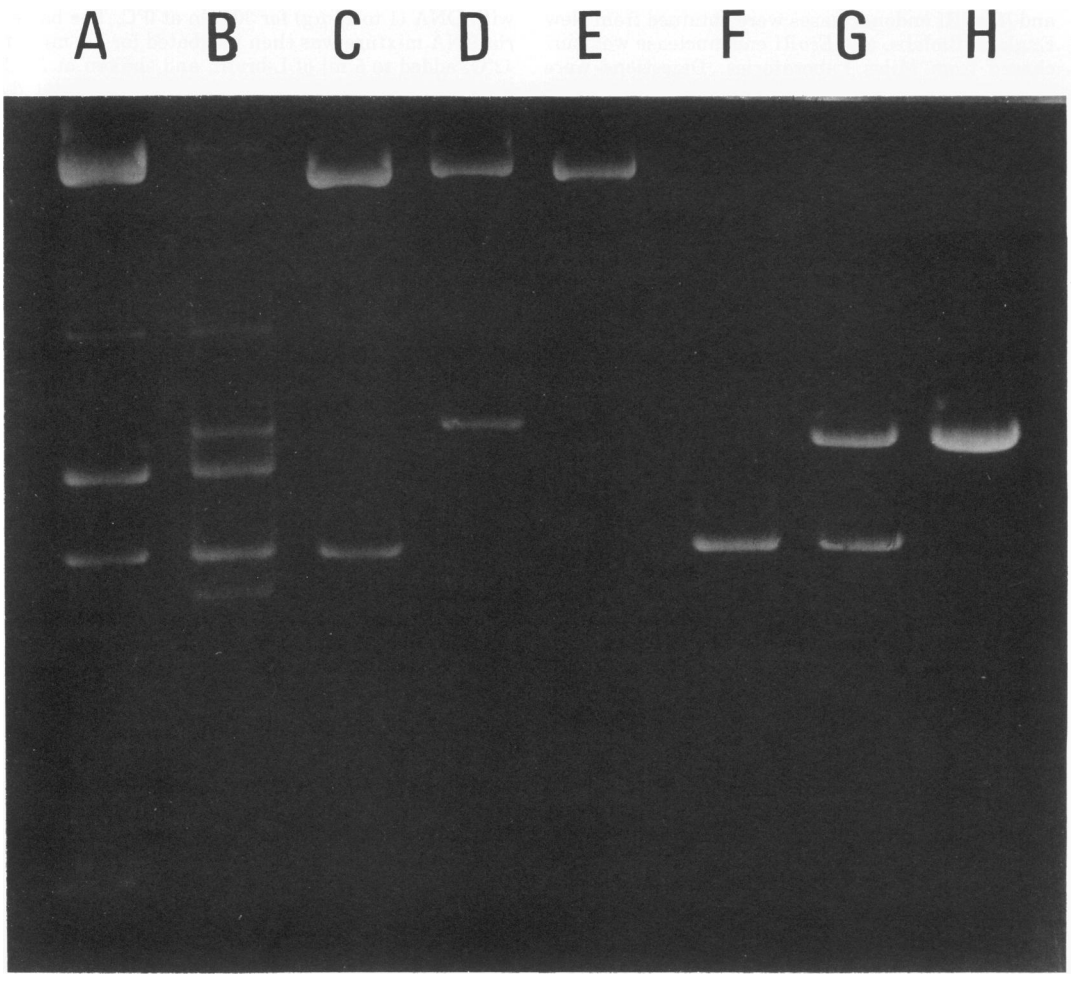

FIG. 1. *Agarose slab gel electrophoresis of DNA digested with EcoRI endonuclease. EcoRI fragments of ϕ80 DNA were used as molecular weight standards (11). (A) ϕ80 fragments: Molecular weights, 9.2 $\times$ 10^6, 5.3 $\times$ 10^6, 4.0 $\times$ 10^6 (doublet), 3.35 $\times$ 10^6, and 1.66 $\times$ 10^6. (B) ϕ80 S.t.trpE-A (7) fragments: Molecular weights, 5.3 $\times$ 10^6, 4.3 $\times$ 10^6, 4.0 $\times$ 10^6 (doublet), 3.35 $\times$ 10^6 (doublet), and 3.1 $\times$ 10^6. (C) pES9 fragments: Molecular weights, 8.7 $\times$ 10^6 and 3.35 $\times$ 10^6. (D) pES1 fragments: Molecular weights, 8.7 $\times$ 10^6 and 4.3 $\times$ 10^6. (E) pCR1: Molecular weight, 8.7 $\times$ 10^6. (F) trpA fragment: Molecular weight, 3.35 $\times$ 10^6. (G) pES11 fragments: Molecular weights, 4.2 $\times$ 10^6 and 3.35 $\times$ 10^6. (H) ColE1: Molecular weight, 4.2 $\times$ 10^6.*

fragment (Fig. 1). The 3.35- and 4.3-Mdal fragments correspond to two of the *Eco*RI fragments of ϕ80 *S.t.trpE-A* DNA. The fact that single, distinct ϕ80 *S.t.trpE-A* fragments were sufficient to complement *trpE* and *trpA* mutants demonstrates that neither *trpE* nor *trpA* from *Salmonella* contains an *Eco*RI-sensitive sequence, but that such a site exists in the *trp* operon, between these genes. To localize this site, a number of *trp* operon mutants were transformed, selecting for kanamycin resistance, with plasmids containing *trpE* (pES1) or *trpA* (pES9). The transformants were then tested for growth in the absence of tryptophan. We found that pES1 complemented mutations in *trpE*, *trpD*, or *trpC* but not in *trpB* or *trpA*, whereas pES9 complemented only the *trpA* mutation. This suggests that *trpB* of *Salmonella* contains a site sensitive to cleavage by *Eco*RI endonuclease. To confirm that the 3.35-Mdal *Eco*RI fragment from pES9 contains *Salmonella trpA*, this fragment was isolated from an agarose slab gel, ligated to *Eco*RI-cleaved ColE1 DNA, and used to transform a strain having a *tonB-trpA* deletion (W3110 Δ[*tonB-trpA905*]). Trp^+ derivatives were obtained. The

*Eco*RI restriction pattern of plasmid DNA isolated from a representative transformant, W3110 Δ[*tonB-trpA905*]/pES11, consisted of the expected 3.35-Mdal fragment plus the 4.2-Mdal ColE1 DNA (Fig. 1). This strain, W3110 Δ[*tonB-trpA905*]/pES11, failed to grow on an iron-free minimal medium (7, 25, 26), indicating that the 3.35-Mdal *trpA* fragment has an end point before or within *tonB* (Fig. 2). φ80 spot tests on strains carrying the pCR1-*S.t.trp* plasmids showed that the 4.3-Mdal *Eco*RI fragment (carrying *trp P,O EDC*) also contains the φ80 immunity region. This marker is present in the 1.66-Mdal *Eco*RI fragment of φ80 DNA (Fig. 2) (11). Apparently, the *Eco*RI site separating the 9.2- and 1.66-Mdal fragments in φ80 has been lost in φ80 *S.t.trpE-A*. The 3.1-Mdal fragment unique to φ80 *S.t.trpE-A* presumably extends from the *Eco*RI site left of the attachment site to the 3.35-Mdal *trpA* fragment (Fig. 2).

Detection of hybrid plasmids with *trpA* inserted in both orientations. Several ColE1-*trpA* and pCR1-*trpA* hybrid plasmids were obtained by ligating *Eco*RI digests or fragments and selecting Trp$^+$ transformants in strain W3110 Δ[*tonB-trpA905*]. Plasmids with opposite fragment orientations were distinguished by treating isolated plasmid DNAs with *Hind*III endonuclease or a mixture of *Hind*III and *Pst*I endonucleases. These enzymes generated two patterns of nonidentical DNA fragments from the treated plasmid DNAs, indicating that *trpA* was inserted with opposite orientations in both ColE1 (Fig. 3 and 4) and pCR1 (Fig. 3). Since the strains bearing these plasmids are Trp$^+$, *trpA* of the plasmids must be expressed regardless of its orientation. Strains with ColE1-*trpA* plasmids with orientation I (Fig. 4) grew more rapidly on media lacking tryptophan than strains with the three other plasmid types.

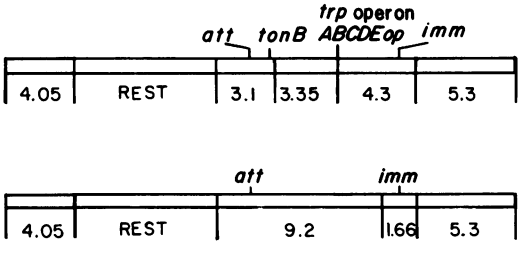

FIG. 2. *EcoRI restriction map of φ80 (top) and φ80 S.t.trpE-A (bottom). These maps are based on the φ80 restriction map of Helling et al. (11) and on restriction data reported in Fig. 1 and in the text. The locations of EcoRI cleavage sites and the molecular weights (in megadaltons) of the resulting fragments are indicated. The DNA region designated REST includes EcoRI fragments of 3.95, 3.35, 0.39, 0.38, 0.32, and 0.13 Mdal (11).*

Measurement of *trpA* expression. In all of the *trpA* transformants, *trpA* of the plasmid is separated from the *trp* operon promoter-operator region as well as the internal promoter, *p2*, which is located before *trpC* (1, 15). In plasmids with one *trpA* orientation, the *trp* gene should be transcribed in the same direction as the colicin structural gene (Fig. 4). If in such plasmids *trpA* is fused to the colicin operon, *trpA* expression might be increased by treatment with mitomycin C or ultraviolet light, agents that are known to induce production of colicin by colicinogenic bacteria (8, 21). As can be seen in Table 1, mitomycin C induces expression of *trpA* in ColE1-*trpA* plasmids with orientation I (pES33 and pES11; also see Fig. 4) but not those with orientation II (pES40). Formation of tryptophan synthetase β_2 (Table 1) and anthranilate synthetase (data not shown) was unaffected by mitomycin C. The genes specifying the proteins responsible for these activities reside on the chromosome of the host strain. Induction was greater in glycerol-supplemented medium than in glucose-supplemented medium, in agreement with the finding that induction of colicin is sensitive to catabolite repression (16). Mitomycin induction was found to be dependent upon mitomycin concentration, exhibiting a maximum at about 0.9 μg/ml under the conditions used. This concentration killed approximately 95% of the cells in the 3-h test period. The maximum tryptophan synthetase α specific activity observed upon induction was about 36, a value twice that of *trpR* cultures (15) and corresponding to 0.7% of the cellular protein as tryptophan synthetase α (12). In other studies with a strain containing pES11 it was shown that ultraviolet irradiation also induces tryptophan synthetase α production.

Neither of the pCR1-*trpA* plasmid-containing strains responded to mitomycin C treatment by producing elevated levels of tryptophan synthetase α. This finding suggests that, in the pCR1-*trpA* plasmids pES9 and pES10, *trpA* is not fused to the colicin promoter in a manner permitting its expression from this promoter. In addition, the observation that mitomycin C treatment does not induce tryptophan synthetase α production in strains containing plasmids other than those of the pES33 and pES11 type indicates that plasmid replication is not responsible for the increased *trpA* expression in strains with pES33 and pES11. This conclusion is supported by the finding that a ColE1-*trp* plasmid-containing strain in which the *trp* op-

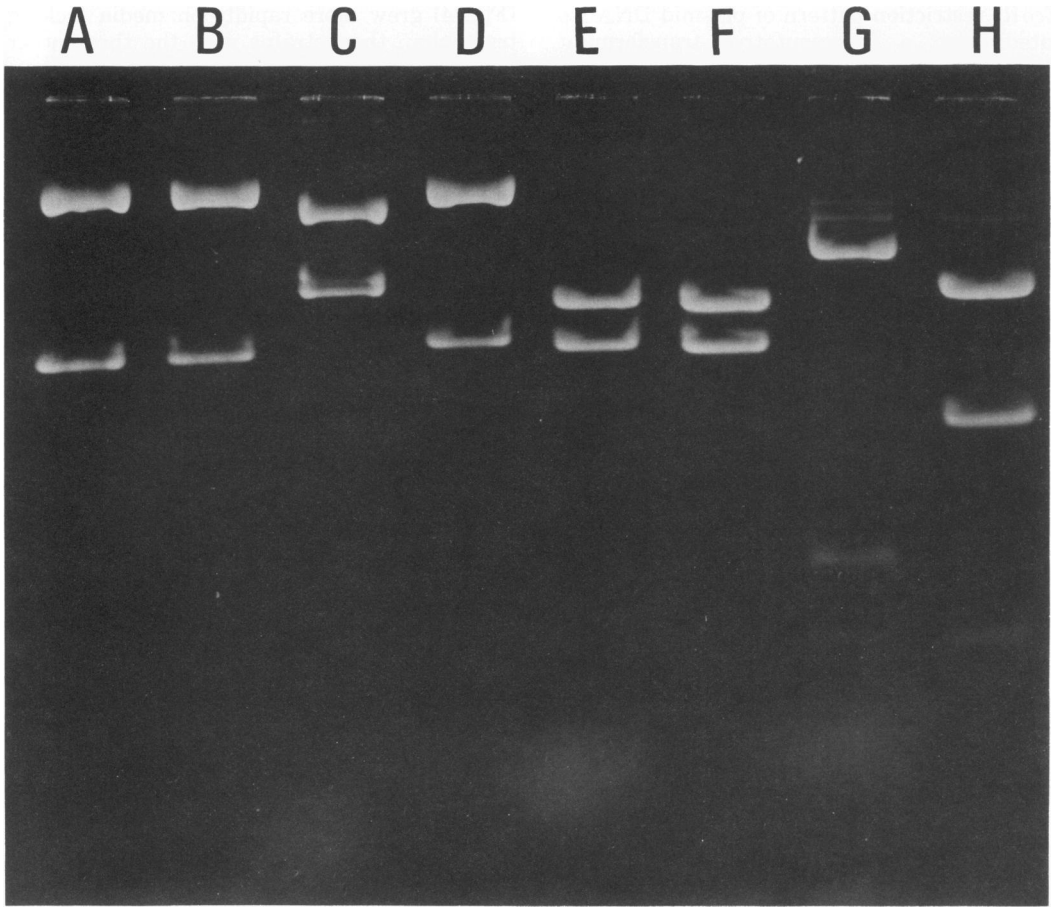

FIG. 3. *Agarose slab gel electrophoresis of restricted DNA. (A) EcoRI digest of pES9 (pCR1-trpA). (B) EcoRI digest of pES10 (pCR1-trpA). (C) HindIII digest of pES9. (D) HindIII digest of pES10. (E) EcoRI digest of pES33 (ColE1-trpA). (F) EcoRI digest of pES40 (ColE1-trpA). (G) HindIII-PstI digest of pES33. (H) HindIII-PstI digest of pES40.*

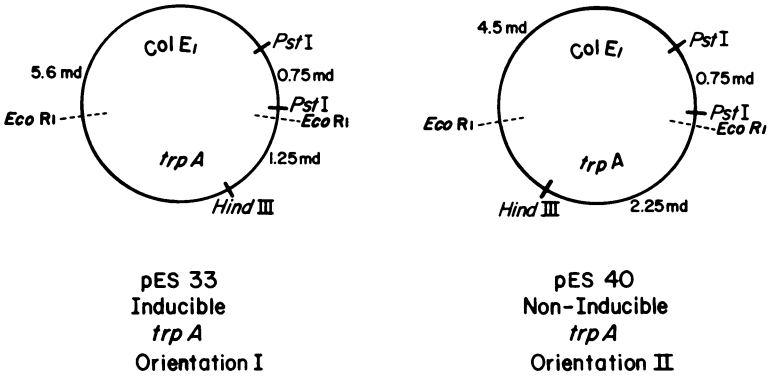

FIG. 4. *Orientation of trpA inserted in plasmids pES33 and pES40. The positions of the EcoRI sites (dotted lines) relative to PstI and HindIII sites are shown (unpublished data). All other data are derived from Fig. 3. Molecular weights of the PstI-HindIII fragments were determined from their electrophoretic mobilities relative to EcoRI fragments of phage λ DNA (11).*

TABLE 1. *Mitomycin C treatment of strains with and without trpA plasmids*[a]

trpA plasmid	Plasmid vehicle	Carbon supplement	Mitomycin[b]	Tryptophan synthetase sp act[c]	
				α	β_2
None		Glucose	+	0.05 (<0.03)	0.10
None		Glucose	−	0.08 (<0.03)	0.18
None		Glycerol	+	0.23 (0.21)	0.34
None		Glycerol	−	0.20 (0.25)	0.27
pES11	ColE1	Glucose	+	8.5 (2.3)	0.25
pES11	ColE1	Glucose	−	0.9 (0.5)	0.28
pES11	ColE1	Glycerol	+	24 (36)	0.44
pES11	ColE1	Glycerol	−	1.1 (0.5)	0.40
pES33	ColE1	Glycerol	+	12	
pES33	ColE1	Glycerol	−	0.4	
pES40	ColE1	Glycerol	+	0.29	
pES40	ColE1	Glycerol	−	0.30	
pES9	pCR1	Glycerol	+	0.59 (0.51)	0.48
pES9	pCR1	Glycerol	−	0.52 (0.39)	0.43
pES10	pCR1	Glycerol	+	0.21 (0.23)	0.35
pES10	pCR1	Glycerol	−	0.17 (0.27)	0.27

[a] All plasmids were present in strain W3110 Δ[*tonB-trpA905*], which also served as the control.
[b] Mitomycin C concentration was 0.9 μg/ml except for pES33 and pES40 where the concentration was 0.75 μg/ml.
[c] Values shown are for cultures grown with 200 μg of tryptophan per ml except for those in parentheses where cultures were grown with 20 μg of tryptophan per ml. The low levels of tryptophan synthetase α and β_2 activity seen are probably due to low levels of tryptophanase (20) in the cell extracts. Note the tryptophan synthetase α levels of the control strain, which lacks *trpA*.

eron fragment has the *trp* promoter and operator does not respond to mitomycin by producing increased amounts of *trp* operon enzymes (unpublished data). However, we have not directly excluded the possibility that mitomycin treatment greatly increases the rate of replication of ColE1-*trpA* plasmids with orientation I.

DISCUSSION

A fragment of the *S. typhimurium trp* operon containing only *trpA* intact was inserted at the *Eco*RI sites of ColE1 and a related ColE1-*kan* plasmid, pCR1. *trpA* was expressed in both plasmids regardless of its orientation of insertion. In one orientation, in ColE1 only, *trpA* expression was increased appreciably by treatment with mitomycin C, an agent known to induce colicin production. Since the *Eco*RI site of ColE1 may be within the colicin operon (13), it is likely that in this case *trpA* can be transcribed from the colicin promoter. This finding offers the possibility of obtaining high-level expression of other foreign genes inserted with the proper orientation at the *Eco*RI site of ColE1. In addition, since tryptophan synthetase α production can be quantitated readily, ColE1-*trpA* plasmids with orientation I may be used to study expression from the colicin promoter.

In both plasmid vehicles employed, ColE1 and pCR1, *trpA* is expressed regardless of its orientation. This observation implies either that there is transcription initiation at some site(s) within the residual segment of *trpB* or that transcription can proceed in either direction across the *Eco*RI sites of ColE1 and pCR1. A third possibility, that *trpA* expression results from the integration of the *trpA* segment of the plasmid into the host chromosome, seems unlikely since all of the plasmid DNAs transform with high efficiencies.

The finding that mitomycin C does not induce tryptophan synthetase α production with either type of pCR1-*trpA* plasmid suggests that in pCR1, as opposed to ColE1, the colicin promoter is not available for direct in-phase fusion to the *trpA* fragment. This implies that the *kan* region of the pCR1-*trpA* plasmids separates *trpA* from the colicin promoter and that the *Eco*RI-site deletion of pCR1 (3) eliminates the possibility of fusion of *trpA* to the colicin promoter.

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