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In Vitro Repression of Transcription of the Tryptophan Operon by *trp* Repressor

(tryptophan analogues/*trp* transducing phages/affinity chromatography)

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ABSTRACT **The *in vitro* repression of transcription of the tryptophan operon by the *trp* repressor from *Escherichia coli* was studied. By measuring the inhibitory effect for *trp*-specific RNA synthesis in an *in vitro* transcription system directed by DNA of *trp*-transducing phage, we have detected and concentrated a *trp* repressor in an eluate of a Φ80 ptED native DNA-cellulose column. The repression of transcription of *trp* operon required the addition of L-tryptophan in the system, and when several tryptophan analogues were added, the repression or derepression was similar to that observed *in vivo*. The repressor fraction was separated from the majority of tryptophanyl-tRNA synthetase activity by Bio-gel P60 column chromatography.**

The tryptophan operon of *Escherichia coli* consists of a cluster of five structural genes (*trp* E, D, C, B, and A) for the tryptophan biosynthetic enzymes (1, 2). Regulation of expression of the *trp* operon is affected by mutations in two genetic regions, designated as *trp* O (3, 4) and *trp* R (5, 6). The operator locus, *trp* O, is located immediately before the first structural gene (*trp* E) of the operon and is presumed to be the site of action of the *trp* R product (3–5). *Trp* R is located far from the *trp* operon and is believed to be the structural gene for an aporepressor, a cytoplasmic protein produced in limited quantity within the cell (5–7). Since these regulatory elements function in response to the intracellular tryptophan concentration, it seems likely that L-tryptophan or a metabolic derivative of it serves as a corepressor and activates the aporepressor (5). The presumed activated repressor then can bind to *trp* O and prevent initiation of transcription at the promoter site on the operon (4, 5, 8–11). Furthermore, it has been proposed that the *trp* S gene, a structural gene for tryptophanyl-tRNA synthetase (TRSase) also located far from the *trp* operon (12–15), somehow participates in repression of the *trp* operon (14–16). However, it seems unlikely that the enzyme plays its regulatory role by charging $tRNA^{Trp}$, which in turn acts as a corepressor (13, 17). Evidence has been presented that TRSase may actually constitute a part of the repressor molecule (18). At the molecular level, the mechanisms for control of the *trp* operon, not only the repression mechanism itself but also the nature of the corepressor, remain to be elucidated.

Recently, Zubay *et al.* (19) have detected the repressing activity of the *trp* R gene product in *E. coli* extracts from a *trp* R^+ strain but not from a *trp* R^- (repressor negative) strain. They measured the inhibition of β-galactosidase synthesis by a crude extract from *trp* R^+ cells when phage DNA carrying a fusion of the *trp* and *lac* operons, in which *lac* expression was controlled by *trp* O, was used as the template in a transcription and translation coupled system. They also partially concentrated the repressing activity by phosphocellulose chromatography. This pioneering work enabled us to further purify the *trp* repressor. Our preliminary attempts to detect *trp* repressor by using convenient DNA-binding techniques (20) have failed to give any consistent results under several experimental conditions. Since *trp* repression is believed to occur at the transcription level (4, 5, 8, 21), we used an *in vitro* transcription system directed by DNA of λ pt60-3 to assay the *trp* repressor. The λ pt60-3 phage genome contains a complete *trp* operon, including the *trp* operator and promoter (21). In this transcription system, *trp* RNA is transcribed asymmetrically from the *l*-strand of DNA as would be expected from *in vivo* observations (Shimizu and Hayashi, in press). By measuring the inhibition of *trp*-specific RNA synthesis *in vitro*, we have detected and isolated a protein fraction responsible for repression of *trp* operon transcription.

Abbrevation: TRSase, tryptophanyl tRNA synthetase.

MATERIALS AND METHOD

Bacterial and Viral Strains. E. coli W3110 *trp*A46 *trp* R^+ rel^+ were grown to late log phase in M9 medium (22) supplemented with 0.1 mg/ml of L-tryptophan, 0.1% casamino acids, 0.4% glucose, 0.1 mM $CaCl_2$, and 1 mM $MgSO_4$ and kept frozen at −90°. The following phage strains were used: Φ80 wild type, Φ80 ptEA (Φ80 pt190h), Φ80 ptED (Φ80 pto), Φ80 ptCA (Φ80 pt77), λ pt60-3, and λ C_I857S7. Details of the preparation of these phages, DNA isolation, and strand separation procedures are described elsewhere (Shimizu and Hayashi, in press).

Native DNA Cellulose was prepared by the method of Alberts and Herrick (23). Cellulose powder (5 g, Whatman CF-11) was kneaded with 15 ml of Φ80 ptED DNA solution [150 μg/ml in 10 mM Tris·HCl (pH 7.4)–1 mM EDTA] and dried.

Tryptophan–Sepharose was prepared by the method of Cuatrecasas and Anfinsen (24). Sepharose 4B (Pharmacia), activated with cyanogen bromide (250 mg/ml of Sepharose), was incubated with ethylenediamine (2 mmol/ml of Sepharose) to convert it to aminoethyl-Sepharose. Tryptophan (200 mg in 15 ml of 66% dimethylformamide) was

TABLE 1. *Effect of various fractions (Exp. I) on trp RNA synthesis with and without* L-*tryptophan*

Fraction added to reaction mixture*	Addition of L-Trp	% Net RNA synthesis†	Hybridization‡			Repressible activity	Trp-tRNA synthetase activity§
			% *l*-strand RNA in total RNA	% *trp* ED RNA in *l*-strand RNA	% *trp* EA RNA in *l*-strand RNA		
No addition	−	100	57	30.9	35.7		—
(control)	+	95	52	29.2	NM¶		
Phosphocellulose	−	48	33	24.5	52.4	Yes	238
	+	50	41	8.5	29.9		
Tryptophan–	−	62	40	27.7	60.4	No	163
Sepharose	+	.66	44	31.5	59.7		
DNA–cellulose	−	52	39	32.1	40.2	Yes	0.83
	+	48	35	14.8	16.4		
Two-column	−	79	54	25.0	NM	No	3.61
flow-through	+	72	52	31.0	NM		

* Protein concentration of each fraction is as follows: phosphocellulose, 150 mg in 10 ml; tryptophan–Sepharose, 18.0 mg in 4.5 ml; DNA–cellulose, 1.75 mg in 2.0 ml; flow-through, 120 mg in 6.0 ml. These fractions, except phosphocellulose, were extensively dialyzed against buffer A (without L-tryptophan) containing 60% glycerol and kept at −20°.

† ^{3}H-Labeled RNA was synthesized with λ pt60-3 DNA as a template with and without L-tryptophan (0.1 mg/ml). 25 μl of each fraction were added before RNA polymerase was added to the reaction mixture. 5% trichloroacetic acid-insoluble counts were compared with that of standard condition (no addition, 8.23×10^4 cpm).

‡ A constant amount of [^{3}H]RNA was hybridized to separated strands of DNA from λ pt60-3, Φ80 ptED, Φ80ptEA, and Φ80 wild type, respectively. Sum of cpm hybridized to *l*-strand and *r*-strand of λ pt60-3 DNA was taken as a total RNA. The difference between hybridization values for Φ80 pt and Φ80 pt wild-type *l*-strands was taken as *trp*-specific RNA.

§ pmol of [^{14}C]Trp incorporation by addition of 25 μl of each fraction.

¶ *NM* = not measured.

coupled to the aminoethyl-Sepharose in the presence of the coupling reagent, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (500 mg for 15 ml of activated Sepharose).

Preparation of Crude Extracts and Fractionation of trp Repressor. The crude *trp* repressor fraction (phosphocellulose fraction) was prepared from *E. coli* W3110 *trp*A46 *trp* R^+ rel^+ cells (200 g) by the procedure of Zubay *et al.* (19). The 0.1–0.25 M KCl eluate from the phosphocellulose column was precipitated with ammonium sulfate (70% saturation). The precipitate was dissolved in 10 ml of buffer A [20 mM Tris·HCl (pH 7.4)–10 mM 2-mercaptoethanol–1 mM EDTA–50 mM NaCl–5% glycerol] and dialyzed against buffer A. The repressor was further purified by affinity chromatography on Φ80 ptED DNA–cellulose and tryptophan–Sepharose (see *Results*).

RNA Polymerase was purified from *E. coli* W3110 *trp* R^- *trp* AE1 cells by the method of Berg *et al.* (25).

In vitro Transcription and Assay of trp Repressor. A reaction mixture (100 μl) contained: 2.3 μg of RNA polymerase, 1.8 μg of DNA, 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM dithiothreitol, 8 mM EDTA, 50 mM Tris·HCl (pH 7.8), 0.1 mM nucleoside triphosphates (ATP, GTP, [^{3}H]UTP, and [^{3}H]-CTP:1.0 mCi/μmol, New England Nuclear Corp.), and 100 μg/ml of bovine-serum albumin. The reaction mixture was preincubated for 5 min at 25°, and the reaction was started by addition of 1/10 volume of a mixture of 100 μg/ml of rifampicin (Lepetit) and 0.2 M $MgCl_2$. After 10 min incubation at 34° the reaction was stopped by heating at 65° for 2 min and chilling to 0°. The DNA template was digested with electrophoretically purified DNase (Worthington, 100 μg/ml, 34° for 5 min) and RNA was extracted with phenol. Carrier tRNA (100 μg) was added, and the RNA was precipitated with four volumes of cold ethanol. The precipitate was dissolved in 100 μl of 0.2% sodium dodecyl sulfate. Protein fractions to be tested for repressing activity, L-tryptophan (Calbiochem), or its analogues (5-methyl-DL-tryptophan, 3-indoleacrylic acid; K and K Laboratories) were added before RNA polymerase was added to the reaction mixture.

DNA–RNA Hybridization. ^{3}H-Labeled RNA was incubated with an excess of separated strands of phage DNAs (about 1.0 μg) in 0.15 ml of 0.30 M NaCl–0.03 M Na citrate at 67° for 3 hr. After RNase treatment (6 μg/ml of RNase A and 3 units/ml of RNase T1 at 25° for 30 min), the RNase-resistant RNA–DNA complex was collected on a membrane filter (Schleicher and Schuell Co., Bac-T-Flex type B6, 27 mm diameter, presoaked in saline–citrate) and washed with 50 ml of cold saline–citrate and counted.

Trp-tRNA Synthetase Activity. A reaction mixture (50 μl) contained: 20 μM L-[^{14}C] tryptophan (50 C/mol, New England Nuclear Corp.), 7.5 mM ATP, 500 μg of *E. coli* deacylated tRNA (General Biochemical Co.), 12 mM $MgCl_2$, 10 mM KCl, 3 mM 2-mercaptoethanol, 50 mM Tris·HCl (pH 7.4), and a protein fraction to be tested. After a 15-min incubation at 34°, the reaction was stopped by addition of 2 ml of cold 5% trichloroacetic acid, and the precipitates were collected on a glass fiber filter (Whatman GF/C) and counted.

Protein Concentration was measured by the method of Lowry *et al.* (26) with crystalline bovine-serum albumin (Armour Pharmaceutical) as a reference.

TABLE 2. *Effect of various fractions (Exp. II) on trp RNA synthesis with and without* L-*tryptophan*

Fraction added to reaction mixture*	Addition of L-Trp	% Net RNA synthesis†	Hybridization: % *l*-strand RNA in total RNA	Hybridization: % *trp* ED RNA in *l*-strand RNA	Repressible activity	Trp-tRNA synthetase activity
No addition (control)	+	100	49	30.6		—
Phosphocellulose	−	30	53	44.1	Yes	150
	+	36	44	6.5		
DNA–cellulose (I)	−	57	55	34.1	Yes	125
	+	49	37	18.1		
DNA–cellulose (II)	−	77	40	14.3	No	14
	+	85	43	24.1		
Two-column flow-through	−	105	51	17.7	No	NM
	+	105	54	21.0		

* Protein concentration of each fraction is as follows: phosphocellulose, 70 mg in 10 ml; DNA–cellulose I, 4.2 mg in 4.0 ml; DNA–cellulose II, 1.38 mg in 1.5 ml; flow-through, 60 mg in 6.0 ml.

† Net RNA synthesis of control is 8.01×10^4 cpm. Other conditions are the same as described in *Table 1*.

RESULTS

The ability of phosphocellulose fractions, prepared from an *E. coli* strain that is *trp* R^+, to repress *in vitro trp* RNA synthesis using λ pt60-3 DNA (*trp* O EDCBA) as template was assayed in the presence and absence of L-tryptophan. *Trp*-specific RNA, isolated from the reaction mixture, was determined by hybridization to the *l*-strand of Φ80 ptED (or Φ80 ptEA) DNA as compared with hybridization to the *l*-strand of wild-type Φ80 DNA. As shown in Table 1, a phosphocellulose fraction inhibited *trp* RNA synthesis in the presence of external L-tryptophan. The L-tryptophan-dependent inhibition of *trp* RNA synthesis strongly suggests the existence of *trp* repressor in the preparation. TRSase activity was also found in this fraction.

If L-tryptophan has a strong affinity for *trp* repressor, the repressor might bind to tryptophan that was covalently linked to Sepharose. The phosphocellulose fraction was applied to a tryptophan–Sepharose column (1.8 × 3.5 cm, flow rate 0.1 ml/min). After thorough washing with buffer A (Sepharose flow-through), the bound materials were eluted from the column with 1 M NaCl in buffer A (tryptophan–Sepharose fraction; 12% of the proteins in the phosphocellulose fraction). As can be seen in Table 1 the tryptophan–Sepharose fraction did not contain *trp* repressor activity. TRSase was concentrated in the tryptophan–Sepharose fraction. Thus, the tryptophan–Sepharose column used under these conditions was not useful for isolation of *trp* repressor, but it was useful for elimination of some tryptophan metabolic enzymes from the desired fraction. L-Tryptophan was added (0.1 mg/ml) to the Sepharose flow-through and the solution was charged onto a Φ80 ptED DNA-(containing *trp* operator) cellulose column (1.8 × 5.5 cm, flow rate 0.1 ml/min) equilibrated with buffer A containing L-tryptophan. 1% of the charged materials were bound and released (DNA–cellulose fraction) when the column was eluted with 2 M NaCl in buffer A after extensive washing with buffer A containing L-tryptophan (two-column flow-through). The DNA–cellulose fraction inhibited *trp* RNA synthesis in response to external L-tryptophan, demonstrating that *trp* repressor binds to DNA (Table 1). There was no repressor activity in the two-column flow-through. Net RNA synthesis was reduced to 50–80% of control by addition of each fraction regardless of whether or not L-tryptophan was present in the reaction mixture, and these reductions did not correlate with *trp* repression. This result might be due to the presence of DNA-binding proteins other than *trp* repressor. No nuclease activity was found in the DNA–cellulose fraction (data not shown).

In another experiment (Exp. II) the phosphocellulose fraction that contained *trp* repressor activity was applied to a Φ80 ptED DNA–cellulose column (same size as Exp. I) equilibrated with buffer A without L-tryptophan. After extensive washing with buffer A (the flow-through materials were saved), the bound materials were eluted with 2 M NaCl in the same buffer (DNA–cellulose fraction I; 6% of the charged materials). As shown in Table 2, the DNA–cellulose fraction I inhibited *trp* RNA synthesis in response to L-tryptophan, indicating that *trp* repressor binds to DNA even

TABLE 3. *Repression and derepression of trp RNA synthesis by* L-*Trp and its analogues*

Addition of analogue	% net RNA synthesis	Hybridization: % *l*-strand RNA in total RNA	Hybridization: % *trp* ED RNA in *l*-strand RNA
None	100	37	34.1
L-Tryptophan	54	36	18.4
5-Methyl-DL-tryptophan	67	44	23.0
3-Indoleacrylic acid	161	39	55.0
L-Tryptophan and 3-indoleacrylic acid	109	37	37.6

[^{3}H]RNA was synthesized with λ pt60-3 DNA as a template with tryptophan and/or its analogues in the presence of DNA–cellulose fraction I (25 μl), and hybridized to separated strands of λ pt60-3, Φ80 ptED, and Φ80 wild-type DNA. Net RNA synthesis is relative to control (5.28×10^4 cpm/100 μl of reaction mixture). The concentration used was as follows: L-tryptophan (0.1 mg/ml), 5-methyl-DL-tryptophan (0.2 mg/ml), 3-indoleacrylic acid (0.1 mg/ml).

TABLE 4. *Template specificity for repression of trp RNA synthesis*

Template	Addition of L-Trp	% *l*-strand RNA in total RNA	% *trp* RNA in *l*-strand RNA
Φ80 ptEA	−	43	13.0
	+	33	0.1
Φ80 ptED	−	56	38.4
	+	43	1.3
Φ80 ptCA	−	44	7.1
	+	41	8.1

^{3}H-Labeled RNA was synthesized with Φ80 ptEA DNA (1.8 μg), Φ80 ptED DNA (2.0 μg), or Φ80 ptCA DNA (1.7 μg) as templates with and without L-tryptophan (100 μg/ml) in the presence of DNA–cellulose fraction I (25 μl) and hybridized to separated strands of Φ80 ptEA and Φ80 wild-type DNA. Sum of cpm hybridized to *l*-strand and *r*-strand of Φ80 ptEA DNA was taken as a total RNA. The difference between the two hybridization values was taken as *trp* RNA.

in the absence of L-tryptophan. The mole number of *trp* operator sites in the DNA–cellulose column was calculated as 20 pmol and considered to be much less than that of *trp* repressor molecules [1 nmol, expected from calculation by Zubay *et al.* (19)] in the preparation. L-Tryptophan was added (0.1 mg/ml) to the flow-through and it was charged to the same DNA–cellulose column equilibrated with L-tryptophan (0.1 mg/ml) in buffer A. The flow-through materials were saved, and the bound materials were eluted from the column with 2 M NaCl in buffer A (DNA–cellulose fraction II; 2% of the charged materials). If *trp* repressor binds more tightly to *trp* operator in the presence of L-tryptophan, this procedure would be useful. However, DNA–cellulose fraction II did not contain *trp* repressor activity although inhibition independent of L-tryptophan was observed. The binding of the *trp* repressor to the DNA–cellulose column is not specific to the operator site of the Φ80 ptED DNA. The two-column flow-through had a significant inhibitory effect on *trp* RNA synthesis, but no response to L-tryptophan was observed.

From the data summarized in Tables 1 and 2, we can conclude that L-tryptophan-dependent repressing activity for *trp* RNA synthesis was found in the phosphocellulose fraction and concentrated in the eluate from the DNA–cellulose column. In order to confirm that the repressing activity in the DNA–cellulose fraction was *trp* repressor, we studied the effects of tryptophan analogues on *trp* RNA synthesis and template specificity. As shown in Table 3, 5-methyltryptophan, which represses the *trp* operon *in vivo* (5), repressed *trp* RNA synthesis as well as L-tryptophan. 3-Indoleacrylic acid, which derepresses the *trp* operon *in vivo* (27), stimulated *trp* RNA synthesis, and this stimulation was eliminated by the addition of L-tryptophan.

When various Φ80 transducing phage DNAs were used as templates in the repressor assay, it was clear that only those DNAs (Φ80 ptEA and Φ80 ptED) that carried the *trp* operator could be repressed (Table 4). These results are in good agreement with those of *in vivo* observations (28).

In vivo TRSase is somehow involved in the repression of *trp* operon expression (14–17). The *trp* repressor fraction eluted from the DNA–cellulose column had TRSase activity.

TABLE 5. *In vivo trp RNA synthesis* in cells infected with λ pt60-3 under repression and derepression conditions*

mRNA	mRNA derived from *l*-strand†		mRNA derived from *r*-strand	
	− Trp	+ Trp	− Trp	+ Trp
trp EA	2.94	1.84	0	0
trp ED	2.57	1.31	0.05	0.02
trp CA	1.34	0.87	0	0.004
Φ80	0.14	0.07	0.80	0.98
λ	1.33	1.14	1.21	1.20

* *E. coli* K12 *trp* AE1 *trp* R$^+$ was infected with λ pt60-3 phage at a multiplicity of 5. The phage-adsorbed cells (1×10^{10} cells in 0.1 ml) were transferred to 5 ml of Vogel–Bonner's minimal medium (30) supplemented with 19 amino acids (each 0.5 mM) in the presence (repression) and absence (derepression) of tryptophan (50 μg/ml) and glucose (1%), and shaken vigorously at 30°. Cultures were pulse-labeled at 12 min after infection for 2 min with 100 μCi of [^{3}H]uridine (15 Ci/mol). RNA was prepared by phenol extraction and was generously given by Dr. F. Imamoto.

† Amount of mRNA was represented by percent of input counts (derepressed RNA, 27378 cpm; repressed RNA, 56236 cpm). Background (no DNA) was subtracted (derepressed RNA, 29 cpm; repressed RNA, 92 cpm).

There are two possibilities why TRSase bound to DNA–cellulose: (*i*) TRSase has a strong affinity for DNA at certain sites; (*ii*) TRSase forms a complex with *trp* repressor which binds to DNA. When the DNA–cellulose fraction (I) was chromatographed on a Bio-Gel P60 column (Fig. 1), major enzyme activity was clearly separated from the *trp* repressor activity (fractions 6 and 7 in Fig. 1).

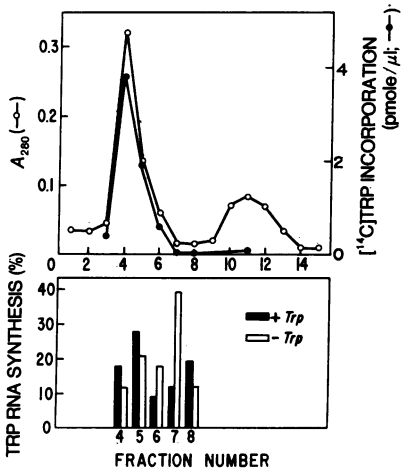

FIG. 1. Bio-Gel P60 chromatography of DNA–cellulose fraction I. 1.6 mg of DNA–cellulose fraction I in 1.5 ml of buffer A containing 60% glycerol was loaded on top of a Bio-Gel P60 column (0.7 × 35 cm) equilibrated with buffer A containing 10% glycerol and eluted with the same buffer. The absorbance at 280 nm of each fraction (1.5 ml) was measured, and 50 μl of each fraction (tubes 4–8) were used for assay of *trp* repressing activity. Trp-tRNA synthetase activity was measured with 1 μl of each fraction. When 20 μl of tube 7 was used for assay, no enzyme activity was detected.

DISCUSSION

We have isolated *trp* repressing activity from cell extracts and concentrated it by affinity chromatography on DNA–cellulose columns. The *trp* repressing activity not only depended on addition of external L-tryptophan, but also responded to tryptophan analogues as observed in repression and derepression *in vivo*. Furthermore, the *trp* repression was observed only with DNA templates containing the *trp* operator. These results strongly suggest the existence of *trp* repressor in our preparation. However, this DNA–cellulose fraction did not completely repress *trp* RNA synthesis. The partial repression can be explained in several ways: (*i*) the dissociation constant of the repressor–operator complex is high; (*ii*) *trp* RNAs synthesized *in vitro* are classified into *trp*-promoted RNA and λ *N*-promoted RNA (Shimizu and Hayashi, in press). It is likely that RNA polymerase, initiated at the λ *N*-promoter, interfered with the action of *trp* repressor on the *trp* operator. The incomplete repression of *trp* RNA synthesis in cells infected with λ pt60-3 phage was also observed *in vivo* under appropriate repression conditions (Table 5 and ref. 21).

As Cohen and Jacob proposed (5), *trp*R gene product is an aporepressor, itself inactive. Corepressor interacts with the aporepressor and converts it into active repressor. What is a corepressor is not conclusive, but several candidates have been nominated. Trp-tRNA is probably not corepressor of the *trp* operon (13, 17). Possible roles of TRSase, Trp-AMP, Trp-ATP, and tryptophan on *trp* regulation have not been clearly excluded.

TRSase activity was found in the DNA–cellulose fraction, and a majority of the activity was separated from the *trp* repressing activity on a Bio-Gel column. Thus it is unlikely that there is a stable complex between TRSase and *trp* repressor. We cannot rule out completely the possibility that *trp* repression by the *trp* repressor fraction occurs with the aid of trace amounts of TRSase. On the other hand, only a subunit of TRSase (29) may participate in part of the *trp* repressor molecule. Then Trp-tRNA formation activity could not be measured. The molecular weight of the repressor has been tentatively estimated from its position of elution from the Bio-Gel column to be 60,000. By acrylamide gel electrophoresis in the presence of Na dodecyl sulfate, two bands were found, and their molecular weights were estimated to be 30,000 and 26,000. In order to clarify the *trp* repression mechanism, especially the relationship between the *trp*R gene product and core-pressor, such as *trp*S gene product, large-scale purification of *trp* repressor is in progress.

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