TRYPTOPHANYL TRANSFER RNA SYNTHETASE AND EXPRESSION OF THE TRYPTOPHAN OPERON IN THE *trpS* MUTANTS OF *ESCHERICHIA COLI*

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IN addition to their role in protein synthesis, aminoacyl tRNA synthetases in bacteria have been shown to have a regulatory role in the formation of enzymes involved in the synthesis of corresponding amino acids. This has been demonstrated at least in the case of isoleucine and valine (EIDLIC and NEIDHARDT 1965; YANIV, JACOB and GROS 1965) and histidine (SCHLESINGER and MAGASANIK 1964; ROTH, ANTON and HARTMAN 1966; ROTH and AMES 1966). Transfer RNA (tRNA) also appears to be involved in the regulation of these systems (FREUNDLICH 1967; SILBERT, FINK and AMES 1966).

The expression of the genes determining the tryptophan biosynthetic enzymes, the tryptophan operon in *Escherichia coli*, is subject to repression by excess L-tryptophan, the end product of the pathway. The mutants of a regulatory gene, *trpR*, located outside of this operon, exhibit constitutive synthesis of the biosynthetic enzymes (COHEN and JACOB 1959). In addition, we have previously reported tryptophan auxotrophic mutants in which the known structural genes of the tryptophan operon remain intact. One of these mutants, designated *trpS5*, has been mapped near *strA* on the chromosome (HIRAGA, ITO, HAMADA and YURA 1967a). In the present paper, we report on further characterization of the *trpS5* mutant and discuss the possible role of the *trpS* gene in the regulation of the tryptophan operon. The results suggest that *trpS*, located between *strA* and *malA*, is the structural gene for tryptophanyl tRNA synthetase, and that tryptophanyl tRNA synthetase is somehow involved in the repression of the tryptophan operon. An abstract of a part of this study has appeared elsewhere (HIRAGA, ITO and YURA 1967).

MATERIALS AND METHODS

Bacterial and phage strains: Strains of *Escherichia coli* K-12 used in this study are listed in Table 1. The *trpS5* mutant requiring tryptophan for growth had been isolated from an F′ strain carrying the episome *Ftrp* after treatment with nitrosoguanidine followed by penicillin selection. The mutant grows with either tryptophan or indole in the minimal medium (HIRAGA *et al.* 1967a). Strains KY960 and KY995 are described elsewhere (HIRAGA 1969). KY970 was isolated as a mutant resistant to 5-methyltryptophan. Phage P1 (IKEDA and TOMIZAWA 1965) was used for transduction experiments and phage λ *vir* (JACOB and WOLLMAN 1954) for isolation of the *malA* strains.

Chemicals: H^3-L-tryptophan, H^3-uridine (generally labeled) and P^{32}-sodium pyrophosphate were obtained from the Radiochemical Center. C^{14}-L-serine and C^{14}-L-valine (uniformly labeled)

TABLE 1

Bacterial strains used

Strain		Genetic characters	Origin
Y-mel	F+	wild type	
A2	F+	*trpA2*	Y-mel
KY4029	F−	*cysB malA*	Y-mel
KY131	F−	*thr leu his met thi lac gal malA xyl mtl ara tonA azi strA*	PA678
KY960	F−	*trpO1*†	KY886—×KY833
KY995	F+	*trpE5927–1*‡	E5927
KY970	HfrH	*trpR970*§ *metA strA*	HfrH
KY4040	F−	*trpS5*	W3102—×KY825
KY4041	F−	*trpS5 malA*	KY4040
KY825	F−	*trpS5 cysB*	KY485
KY873	F−	*trpS5 cysB thr strA*	KY825
KY4008	F−	*trpS5 cysB thr malA strA*	KY873

Gene symbols: genes determining biosynthesis; *cys*, cysteine; *his*, histidine; *trp*, tryptophan; *thr*, threonine; *leu*, leucine; *met*, methionine; *thi*, thiamine. Genes determining sugar utilization: *gal*, galactose; *lac*, lactose; *mal*, maltose; *xyl*, xylose; *mtl*, mannitol; *ara*, arabinose. *str* (genes determining response to streptomycin); *azi* (genes determining response to azide); *tonA* (genes determining response to phage T1 and T5). Transductional cross by phage P1 is represented as donor—×recipient.

† Operator constitutive mutation.

‡ Feedback resistant anthranilate synthetase mutation.

§ Regulator constitutive mutation.

were the products of the New England Nuclear Corp. N-methyl-N′-nitro-N-nitrosoguanidine was obtained from Aldrich Chemical Co. and 5-methyl-DL-tryptophan from Sigma Chemical Co.

Media: A minimal salt medium, medium E (VOGEL and BONNER 1956), was used with 0.5% glucose. Medium EA was an enriched minimal medium containing 0.2% Difco casamino acids. 5-Methyltryptophan medium was medium EA supplemented with 10 μg/ml 5-methyl-DL-tryptophan and 50 μg/ml L-cysteine.

Growth of bacteria: Growth of bacteria was followed by measuring turbidity of a culture with a Klett-Summerson colorimeter with a filter No. 54.

Transduction: Transduction with phage P1 was carried out by a modification (HIRAGA 1969) of the method described by LENNOX (1955).

Assay of tryptophan synthetase and anthranilate synthetase activity: Cells were grown in medium EA with (repression) or without (derepression) 20 μg/ml L-tryptophan unless otherwise noted, harvested, washed and resuspended in 0.05 M Tris-HCl pH 7.8. Cell suspension or sonicated extract was used for enzyme assay. Assay for anthranilate synthetase was carried out according to the modified procedure (HIRAGA 1969) of GIBSON and GIBSON (1964). Tryptophan synthetase activity was measured by the standard method (SMITH and YANOFSKY 1962).

Preparation of aminoacyl tRNA synthetase: Cells were grown in 250 ml of medium EA supplemented with 50 μg/ml L-tryptophan and other amino acids as required to the turbidity of 150 in a Klett-Summerson colorimeter, harvested in the cold, washed with 30 ml of 0.01 M Tris-HCl (pH 7.5) containing 0.01 M $MgCl_2$ and 7 mM β-mercaptoethanol, and sonicated in 5 ml of the same buffer for 5 min by a Raytheon sonic oscillator (10 kc). After removal of cell debris by centrifugation, the extract was spun at 105,000 × *g* for 120 min, and the resulting supernatant dialysed against the same buffer at 4°C for 12 hr and used for assay of aminoacyl tRNA synthetases unless otherwise stated. In some experiments, the 105,000 × *g* supernatant was adsorbed on a DEAE-cellulose column and the materials eluted with 0.3 M KCl was dialysed. Protein was measured by the method of LOWRY *et al.* (1951).

Preparation of tRNA: Transfer RNA was prepared by phenol extraction followed by DEAE-cellulose column chromatography as described by SILBERT, FINK and AMES (1966). Amino acids attached to tRNA were stripped off by incubation in 0.1 M Tris-HCl (pH 8.8) for 1 hr at 37°C. The transfer RNA preparation was finally dissolved in 0.001 M Tris-HCl (pH 7.5) containing 0.01 M Mg-acetate and dialysed overnight against the same buffer at 4°C.

Assay of tryptophanyl tRNA synthetase: (1) ATP-pyrophosphate exchange assay. The procedure of BERG (1956) was followed with minor modifications. The reaction mixture (1 ml) contained the following (in μmoles): potassium phosphate buffer pH 7.5, 100; $MgCl_2$, 10; KF, 2; ATP, 3; L-tryptophan, 1; and P^{32}-sodium pyrophosphate, 1 (0.1–0.3 μc). The reaction was started by addition of extract. After incubation at 37°C for 10 to 20 min, 0.2 ml of cold 25% trichloroacetic acid and 0.2 ml of 15% Norit A were added, the preparation was centrifuged, the precipitate was washed three times with water and suspended in 2 ml of 0.3 M NH_4OH in 50% ethanol. An aliquot of the suspension was dried on a planchet and the radioactivity counted by a Nuclear Chicago gas-flow counter. A reaction mixture containing no tryptophan served as a control for each assay.

(2) Tryptophanyl tRNA formation assay. The reaction mixture (0.6 ml) contained (in μmoles): Tris-HCl (pH 7.5), 15; $MgCl_2$, 9.6; ATP, 3; CTP, 0.01; tRNA, 10 optical density (260 mμ) units, and H^3-L-tryptophan, 0.001 (0.02 μc). The reaction was started by addition of an extract and the incubation continued for 3 min at 37°C. After the reaction was stopped by adding 2 ml of cold 5% trichloroacetic acid and 1.2 mg of bovine serum albumin, the precipitate was collected by centrifugation, washed 3 times with cold 5% trichloroacetic acid, dissolved in 0.3 ml of 2N NH_4OH, and the radioactivity counted by a Nuclear Chicago liquid scintillation counter in the Bray's solution. Extract was omitted from the control tube. H^3-L-tryptophan had been treated with 0.1 N HCl in the presence of carrier tRNA, centrifuged, and the supernatant neutralized with NaOH (IMAI, personal communication). This effectively removed the contaminating acid-insoluble materials that would cause high background radioactivity in the assay. For measuring other aminoacyl tRNA synthetase activity, H^3-L-tryptophan was replaced by 5 mμmoles of another C^{14}-L-amino acid under otherwise the same conditions.

Assay of the charge level of $tRNA^{trp}$: The charge level of $tRNA^{trp}$ was measured by the extent of inactivation of tryptophan acceptor activity of tRNA by periodate oxidation as described by BÖCK, FAIMAN and NEIDHARDT (1966). Tryptophan acceptor activity of an RNA preparation was determined by the same procedure used in the tryptophanyl tRNA formation assay described above, except that an excess amount of extract was used and the reaction was run for 20 min.

Determination of messenger RNA specific for the tryptophan operon: RNA was pulse labeled with H^3-uridine, extracted by the hot phenol method and hybridized with denatured DNA of phage ϕ80 and $\phi 80pt_{190}$. Messenger RNA (mRNA) specific for the tryptophan operon was determined by the difference in radioactivity retained on a pair of membrane filters carrying $\phi 80pt_{190}$ DNA or ϕ80 DNA. As a control of bulk mRNA synthesis, a portion of the RNA sample was hybridized with denatured *E. coli* DNA. The detailed procedure of RNA extraction and DNA-RNA hybridization has been described elsewhere (YURA, IMAI, OKAMOTO and HIRAGA 1968).

RESULTS

Growth characteristics of the mutant: The growth of the *trpS5* mutant with various concentrations of L-tryptophan was followed in a liquid medium and the results are presented in Figure 1a. It is seen that the growth rate depends on the concentration of tryptophan within the range tested. In contrast, with the auxotrophic mutant *trpA2* deficient in the tryptophan synthetase A protein, it was the final yield of cells and not the growth rate that depends on the concentration of added tryptophan (Figure 1b).

Transduction mapping of the mutant: Previous results of mating experiments suggested that *trpS5* is linked to *strA* (HIRAGA *et al.* 1967a). The possibility of

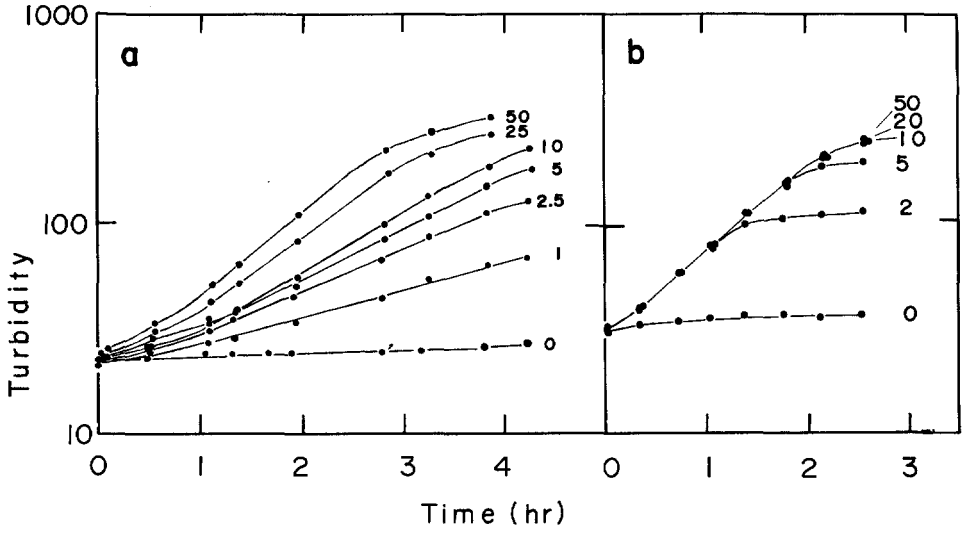


FIGURE 1.—Effect of tryptophan concentration on the growth of *trpS5* and *trpA2* mutants.—Log-phase culture was harvested, washed and cells were inoculated into medium EA supplemented with L-cystein (50 μg/ml) and with various concentrations as indicated (μg/ml) of L-tryptophan and the turbidity was followed during shaking at 37°C. (a) *trpS5* (KY825) (b) *trpA2* (A2).

cotransduction of *trpS* with *strA* or other genes was then examined by phage P1. The results presented in Table 2 revealed that *trpS* can be cotransduced with *strA* at a frequency of about 7% and with *malA* at 10 to 20%. Cotransduction of *strA* and *malA* was not observed in this experiment, where 1% cotransduction would have been detected. Thus it is concluded that *trpS* is located between *strA* and *malA*, being slightly closer to the latter, on the chromosome (Figure 2).

Transduction of the *trpR* gene was also carried out and the results show that *trpR* is cotransducible with *thr* at a frequency of about 70%.

TABLE 2

Mapping of trpS5 *by transduction with phage P1*

Donor	Recipient	Selected marker	Unselected marker*				Number of transductants
			malA	*trpS*	*strA*		
KY4008	Y-mel	*strA*	0	0	.		89
			0	1	.		7
						Total	96
Y-mel	KY4008	*malA*+	.	0	0		36
			.	1	0		11
						Total	47
KY825	KY4029	*malA*+	.	0	.		175
			.	1	.		20
						Total	195

* 1 represents markers from the donor strain and 0 represents markers from the recipient strain.

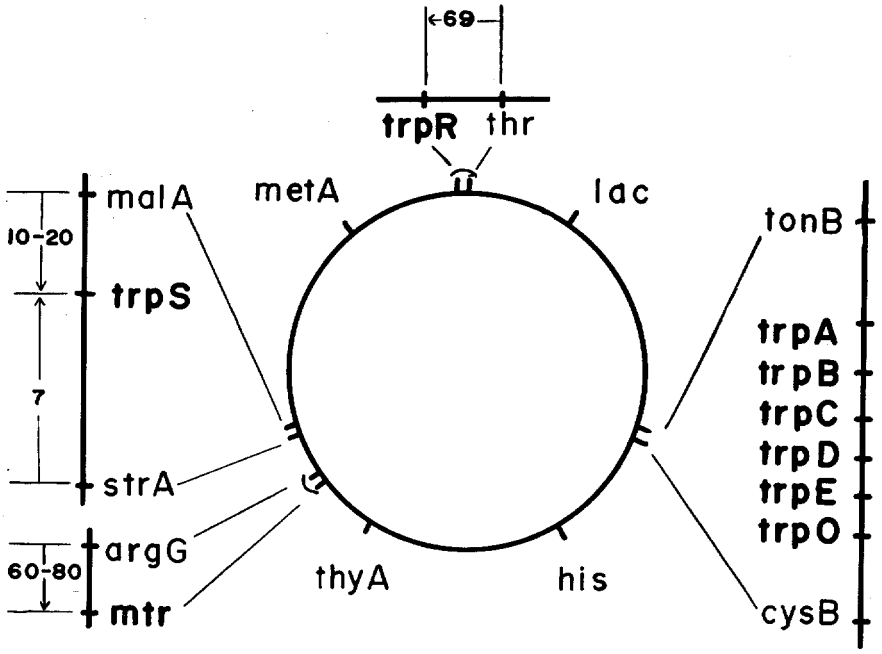


FIGURE 2.—Location of *trpS* and *trpR* on the *E. coli* chromosome.—Figures represent cotransduction frequencies in per cent. *mtr* is a gene or genes determining sensitivity to 5-methyltryptophan (HIRAGA *et al.*, 1968).

Tryptophanyl tRNA synthetase of the mutant: Activity of tryptophanyl tRNA synthetase was measured in the dialyzed extract of *trpS5* and no activity was detected either by tryptophanyl tRNA formation assay (Table 3) or by ATP-pyrophosphate exchange assay (Table 7). When equal amounts of the wild type and *trpS5* extracts were mixed, the specific enzyme activity was reduced to half that of the wild-type extract (Table 3), suggesting the absence of any inhibitor of the enzyme in the *trpS5* extract or any activator in the wild-type extract. Activities of valyl and seryl tRNA synthetases were not affected by the *trpS5* mutation.

Stability of tryptophanyl tRNA synthetase: Absence of tryptophanyl tRNA synthetase activity in the extract of *trpS5* mutant raised at least two possibilities

TABLE 3

Activity of aminoacyl tRNA synthetases in the trpS5 *mutant**

Extract	Activity of aminoacyl tRNA synthetase		
	Tryptophan	Valine	Serine
Wild type (Y-mel)	235	1270	398
trpS5 (KY4040)	1	1600	479
Wild and *trpS5*†	119	....	...

* Activity is represented as $\mu\mu$moles of amino acid incorporated into tRNA per min per mg protein.

† Equal amounts of the two extracts were mixed.

to explain the growth characteristics presented above. (1) The enyzme has an unusually low affinity to L-tryptophan; (2) The enzyme is unstable and is stabilized by L-tryptophan. To test the first possibility, the concentration of tryptophan was elevated to 10^{-4} M in the tryptophanyl tRNA formation reaction and to 10^{-2} M in the ATP-pyrophosphate exchange reaction, but still no activity was detected. On the other hand, some activity (about 10% of the wild type) was in fact observed when fresh extract was assayed immediately before dialysis. The activity observed was lost completely by dialyzing the extract for 12 hr at 4°C.

The following experiments were carried out to find conditions to stabilize the enzyme by the substrates. Fresh extracts (omitting 105,000 × *g* centrifugation and dialysis) of *trpS5* and of wild type were divided into several portions, to which tryptophan, ATP, indole and anthranilate were added separately or in combinations. They were either treated at 37°C for 10 min or dialyzed against buffer supplemented with the same compounds. The results are shown in Table 4. The mutant enzyme was completely inactivated by the treatment at 37°C in the unsupplemented buffer and was partially protected by ATP and more effectively by the combination of ATP and tryptophan, whereas the wild-type enzyme underwent no severe inactivation in any of the buffers used. Similarly, dialysis inactivated the mutant enzyme in the unsupplemented buffer, but not in the presence of both ATP and tryptophan. ATP or tryptophan alone had little, if any, stabiliz-

TABLE 4

*Stability of tryptophanyl tRNA synthetase**

Strain	Supplements to the buffer†	Enzyme activity		
		Control	Treated at 37°C‡	Dialyzed§
Wild (Y-mel)	None	449	389	616
	Trp, ATP	537	870	970
	ATP	519	489	681
	Trp	630	N.T.¶	554
	Ind, ATP	439	340	698
	Anth, ATP	559	489	681
trpS5 (KY4040)	None	676	0	37
	Trp, ATP	1,210	625	1,295
	ATP	1,017	283	119
	Trp	904	N.T.	0
	Ind, ATP	1,214	450	233
	Anth, ATP	890	201	298

* Sonicated extracts were centrifuged at 10,000 × *g* for 30 min and the resulting supernatants used for enzyme assay measuring tryptophanyl tRNA formation as described in MATERIALS AND METHODS. 21 μg of wild-type protein or 255 μg of *trpS5* protein were used. The values represent radioactivity (counts per minute) of H^3-tryptophan (3.4×10^4 counts per min/mμmole) incorporated. Corrections for isotopic dilution have been made where tryptophan was added to the buffer during the treatment at 37°C or dialysis.

† The following supplements were made to 0.01 M Tris-HCl buffer (pH 7.5) containing 0.01 M $MgCl_2$ and 7 mM β-mercaptoethanol: ATP, 10^{-3} M; Trp (L-tryptophan), 10^{-5} M; Ind (indole), 5×10^{-4} M; Anth (anthranilic acid), 5×10^{-4} M.

‡ Treatment at 37°C for 10 min.

§ Dialysis at 4°C for 12 hr.

¶ Not tested.

ing effect. Little stabilization was observed by indole or anthranilate in the presence of ATP. These results clearly show that tryptophanyl tRNA synthetase in the *trpS5* mutant is extremely unstable and can be stabilized by tryptophan in the presence of ATP.

Double mutants carrying trpS5 *and a 5-methyltryptophan-resistant mutation:* Resistance to 5-methyltryptophan can be acquired by a mutation in *trpR* (regulator constitutive; COHEN and JACOB 1959), *trpO* (operator constitutive; HIRAGA 1969), *trpE* (feedback-resistant anthranilate synthetase; MOYED 1960) or in *mtr* (linked to *argG*, mechanism unknown; HIRAGA, ITO, MATSUYAMA, OZAKI and YURA, 1968).

As *trpR* is co-transducible with *thr*, and *trpO* and *trpE* with *cysB*, double mutants carrying *trpS5* and a 5-methyltryptophan-resistant mutation were constructed by transduction with phage P1, using KY970 (*trpR970*), KY960 (*trpO1*) or KY995 (*trpE5927–1*) as donor and KY873 (*cysB thr trpS5*) as recipient. The transductants of *thr*$^+$ (when KY970 was the donor) or *cysB*$^+$ (when KY960 or KY995 was the donor) were selected and those that were shown by subsequent transduction experiments to carry one of the 5-methyltryptophan-resistant markers were used as double mutants. Table 5 shows the characteristics of these double mutants. All of them could grow in the absence of tryptophan in contrast to the *trpS5* mutant, although growth could be further stimulated by addition of L-tryptophan. The constitutivity of the tryptophan operon in the *trpR970* and *trpO1* mutants was not affected by the *trpS5* mutation. As was expected, activity of tryptophanyl tRNA synthetase was not detected in the double mutant *trpR970 trpS5* (Table 7).

Tryptophan-independent revertants of trpS5: Tryptophan-independent revertants were isolated from the *trpS5* mutant either on minimal medium or on 5-methyltryptophan medium and were examined for their genetic constitution by

TABLE 5

Double mutants carrying trpS5 *and a 5-methyltryptophan-resistant mutation*

Strain	Growth*		Doubling time (min)†		Tryptophan synthetase‡	
	−Trp	+Trp	−Trp	+Trp	Derepressed	Repressed
Wild (Y-mel)	+	+	..	..	100	3
trpS5	—	+	..	..	..	..
trpO1	+	+	..	..	165	41
trpO1 trpS5	±	+	89	54	305	98
trpR970	+	+	..	..	168	145
trpR970 trpS5	±	+	142	61	58	47
trpE5927–1	+	+	..	..	109	9
trpE5927–1 trpS5	±	+	139	85	N.T.	N.T.

* +, — or ± represents growth, no growth or slow growth, respectively, on minimal agar with or without L-tryptophan (50 μg/ml) as indicated; L-threonine or L-cysteine was added as required.

† Doubling time was measured in medium EA supplemented with or without 50 μg/ml L-tryptophan at 37°C with constant shaking.

‡ Values represent percentages of the derepressed wild-type activity using cell suspensions for enzyme assay.

TABLE 6

Revertants of the trpS5 *mutant*

Strain	Mutagen	Selective media	Genotype tested			Tryptophan synthetase*	
			trpS	*trpR*	*mtr*	Derepressed	Repressed
Wild type (Y-mel)	..	..	+	+	+	100	4.8–10
trpS5	..	..	5	+	+	..	..
Revertants							
#9	Spont†	ME	5–9	..	..	110	4.5
#31	NG	ME	5–31	..	..	110	9.7
#32	NG	ME	5	32	..	182	172
#34	NG	ME	5–34	..	..	125	5.7
#38	NG	ME	5–38	..	..	100	10.4
#133	NG	ME	5	133	..	152	140
#138	NG	ME	5	138	..	N.T.	160
#4010‡	NG	MT	5–4010	+	4010	27	4
#4023‡	NG	MT	5–4023	+	4023	31	4
#4024‡	NG	MT	5–4024	4024	+	40	30

* Values represent percentages of the derepressed wild-type activity using cell suspensions for enzyme assay.

† Spont, spontaneous; NG, N-methyl-N′-nitro-N-nitrosoguanidine; ME, minimal medium; MT, 5-methyltryptophan medium.

‡ These revertants were isolated on 5-methyltryptophan medium from strain KY873 (*cysB thr trpS5*) whereas others from strain KY4040 (*trpS5*). The former strains exhibited a lower growth rate and activity of tryptophan synthetase than the latter.

P1 transduction. P1 phage was grown on each of the revertants and *malA*$^+$ transductants were selected from strain KY4041 (*malA trpS5*) infected with these phages. If the reversion had occurred at or around *trpS5*, 10 to 20% of the transductants would be tryptophan independent, whereas no prototrophic transductants would be expected if the reversion had occurred outside the *trpS* region. As seen in Table 6, among 10 revertants tested, seven gave rise to prototrophic transductants. These transductants as well as the parental revertants produced at least some active tryptophanyl tRNA synthetase (Table 7). These results suggest that the *trpS* locus controls the synthesis and/or activity (stability) of tryptophanyl tRNA synthetase. Furthermore, one of the revertants (*trpS5–4024*) produced the enzyme with a Km value for L-tryptophan about 6 times higher than that of the wild-type enzyme. The enzyme of another revertant tested (*trpS5–4010*) gave approximately the normal Km value under the conditions used (Table 8).

The synthesis of tryptophan synthetase was then examined with these revertants. Most of the revertants are normally repressible by tryptophan. Several revertants exhibiting constitutive synthesis of the enzyme were all shown to carry the 5-methyltryptophan-resistant marker, presumably *trpR*, that could be co-transduced with *thr* at high frequency. That is, they were in effect double mutants *trpS5 trpR*, except for one revertant (#4024) in which a third mutation occurred at or around the *trpS* locus that brought about the production of the altered tryptophanyl tRNA synthetase as described above. However, when *trpS* region of

TABLE 7

Tryptophanyl tRNA synthetase in the trpS5 *revertants and the prototrophic transductants*

Strain	Relative specific activity	
	Tryptophanyl tRNA formation	ATP-pyrophosphate exchange
Control		
Wild type (Y-mel)	100*	100†
trpS5 (KY4040)	< 0.5	< 1
trpS5 trpR970	< 0.5	< 1
Revertants		
trpS5–4010	139	150
trpS5–4023	9	3
trpS5–4024	5	3
trpS5–9	34	N.T.
trpS5–38	46	N.T.
Transductants from donor‡		
Wild type (Y-mel)	114	N.T.
trpS5–4024	10	N.T.
trpS5–9	35	N.T.
trpS5–38	36	N.T.

* 240 μμmoles L-tryptophan incorporated per min per mg protein.
† 15 mμmoles pyrophosphate exchanged per min per mg protein.
‡ See the text.

the latter revertant (#4024) was transduced into strain KY4041, the resulting transductant exhibited a normal regulation in the synthesis of tryptophan synthetase. Other revertants (#4010 and #4023) that had been isolated on the 5-methyltryptophan medium were found to carry another 5-methyltryptophan-resistant marker, designated *mtr*, linked closely to *argG* (HIRAGA *et al.* 1968). Thus no constitutive mutation has been found at the *trpS* region among the revertants so far studied.

Expression of the tryptophan operon in the trpS5 *mutant:* Earlier results indicated that the derepressed level of the tryptophan biosynthetic enzymes in the mutant *trpS5* is somewhat lower than that of the wild-type strain, whereas the

TABLE 8

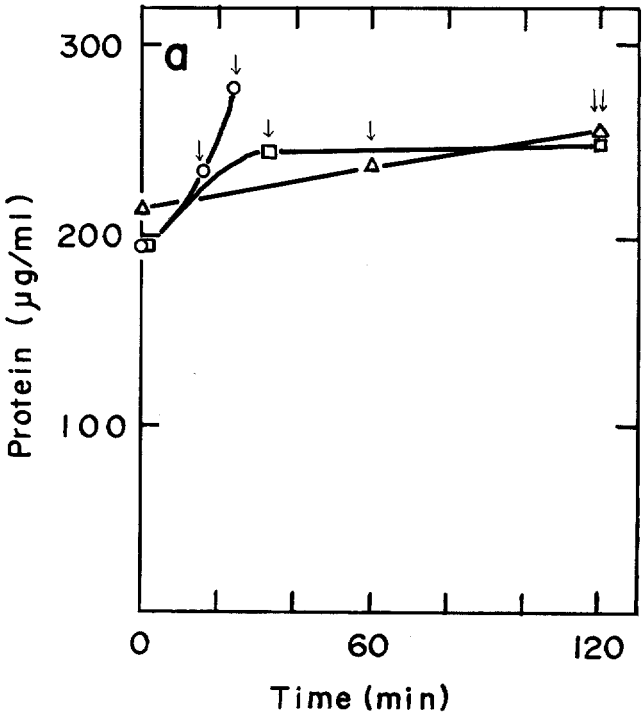
Km of tryptophanyl tRNA synthetase for L-*tryptophan with* trpS5 *revertants**

Strain	Tryptophanyl tRNA formation	ATP-pyrophosphate exchange
Wild (Y-mel)	6.5×10^{-7} M	7.5×10^{-5} M
trpS5–4010	1.0×10^{-6} M	8.8×10^{-5} M
trpS5–4024	4.2×10^{-6} M	

* Eluates from DEAE-cellulose column were used as enzyme preparation (see MATERIALS AND METHODS). In tryptophanyl tRNA formation assay, various concentrations (10^{-7} M to 2×10^{-5} M) of H^3-L-tryptophan (specific activity, 1.2×10^5 counts per min/mμmole) were added to the reaction mixture and the rate of reaction (counts per min of H^3-tryptophan incorporated) was plotted by the method of LINEWEAVER-BURK. In the case of ATP-pyrophosphate exchange reaction, 2×10^{-5} M to 4×10^{-4} M L-tryptophan was used in otherwise the standard reaction mixture.

repressed level remains unaltered by this mutation (HIRAGA *et al.* 1967a). To examine the enzyme derepression in the *trpS* mutant more quantitatively, formation of anthranilate synthetase and tryptophan synthetase were followed upon transfer of the repressed *trpS5* culture to medium containing 1 μg/ml L-tryptophan and the result was plotted against the increase in total protein content (Figure 3b). It is seen that the enzyme formed per total protein synthesized is higher in the *trpS5* than in the *trpA2* or wild-type strain at least up to about 40 μg/ml increase in total protein, although a striking derepression occurs with the *trpA2* culture (Figure 3b) after tryptophan is exhausted as indicated by the cessation of bulk protein synthesis beyond 30 min incubation under these conditions (Figure 3a). Similar results were also obtained with respect to the synthesis of mRNA specific for the tryptophan operon (tryptophan mRNA). As shown in Table 9, at 10–13 min after transfer of the repressed cells to the medium with 1 μg/ml L-tryptophan, tryptophan mRNA synthesis was derepressed in the *trpS5* mutant but not in the wild-type strain. Both the mutant and the wild-type cultures were derepressed at 30–33 min under these conditions.

Tryptophan mRNA synthesis was then studied in the absence of exogenous tryptophan with *trpS5*, *trpA2* and the wild-type strains. RNA was pulse-labeled with H^3-uridine at 10, 30 and 60 min after removal of tryptophan from the medium and the tryptophan mRNA was determined. Figure 4 indicates that the rate of tryptophan mRNA synthesis (normalized with bulk mRNA synthesis) at 10 min after tryptophan deprivation with *trpS5* and *trpA2* is about twice as high



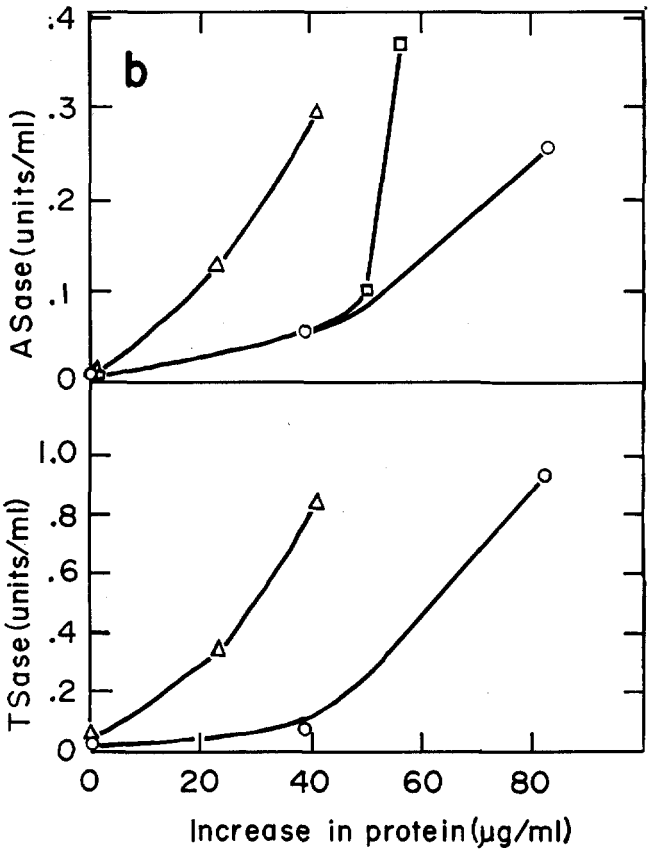


FIGURE 3.—Formation of anthranilate synthetase (ASase) and tryptophan synthetase (TSase) in *trpS5* mutant.—Cells were grown in medium EA with 50 μg/ml L-tryptophan to about 120 Klett-units, centrifuged, rinsed and resuspended in medium EA with 1 μg/ml L-tryptophan. After shaking at 37°C, aliquots were removed at times indicated and were assayed for ASase and TSase activity using sonicated extracts. For measuring the protein content of the culture, aliquots of the culture were treated with cold 0.5 N perchloric acid, precipitates were collected by centrifugation, washed with 0.25 N perchloric acid, suspended in 0.5 N perchloric acid, and heated at 90°C for 15 min. Hot perchloric acid insoluble material was dissolved in 1 N NaOH and the protein content measured. A solution of bovine serum albumin treated by the same procedure was used as the standard. (a) Time course of increase in total protein of the culture. (b) Amount of enzyme formed as a function of increase in total protein. One enzyme unit is expressed as 0.1 μmole of substrate consumption or product formation during 20 min incubation at 37°C. ○, wild type (Y-mel); □, *trpA2* (A2); △, *trpS5* (KY4040).

as that with the wild-type strain and remains almost constant at least for 60 min, whereas mRNA synthesis in the wild-type strain is repressed markedly at 30 min and completely at 60 min under the conditions employed. When L-tryptophan (50 μg/ml) is present, synthesis of tryptophan mRNA is repressed completely with *trpS5* as well as with the wild-type strain. Thus it is clear that the extent of derepression of the tryptophan operon in *trpS5* with respect to the mRNA

TABLE 9

*Tryptophan mRNA synthesis in tryptophan-limited medium**

Strain	Pulse-labeling (min)	Radioactivity retained on filter with			Percent tryptophan mRNA
		$\phi 80pt_{190}$DNA	$\phi 80$DNA	Difference	
Wild type (Y-mel)	10–13	25	28	—3	0
	30–33	450	33	417	.208
trpS5 (KY4040)	10–13	431	139	292	.146
	30–33	339	127	212	.106

* Cells were grown in medium EA with 50 μg/ml L-tryptophan to turbidity of 160 Klett units, filtered through a Millipore filter (HA, 0.45 μ; 47 mm), washed in medium E (minus glucose) and resuspended in ½ vol of medium EA with 1 μg/ml L-tryptophan. After shaking at 37°C, RNA was pulse-labeled with H^3-uridine for the indicated period, extracted and hybridized with denatured DNA of $\phi 80pt_{190}$ and $\phi 80$. Values represent the radioactivities (average of duplicates) found when 200,000 counts/min of H^3-RNA was used for hybridization.

synthesis is at least comparable with that in *trpA2* defective in tryptophan biosynthesis.

The amount of charged $tRNA^{trp}$ in total $tRNA^{trp}$ was then measured with the

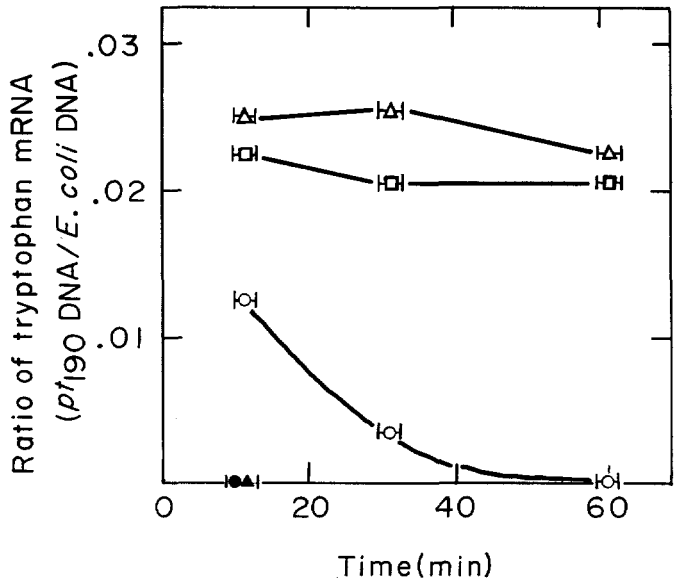


FIGURE 4.—Tryptophan mRNA synthesis in the absence of tryptophan.—Cells were grown, washed as described in the footnote to Table 9 and resuspended in medium EA with no tryptophan. After shaking at 37°C for 10, 30 or 60 min, RNA was pulse-labeled with H^3-uridine for 5 min. Tryptophan mRNA synthesized is represented as the ratio of per cent RNA specifically hybridizable with $\phi 80pt_{190}$ DNA and with *E. coli* DNA. Actual values of RNA (pulsed at 10 min) specifically hybridized with $\phi 80pt_{190}$DNA were: wild type, 0.19: *trpA2*, 0.43; and *trpS5*, 0.50 (percent of total radioactivity used). RNA hybridized with *E. coli* DNA was 15.0 to 20.7%. All the hybridization was carried out in duplicate. ○, wild type (Y-mel); □, *trpA2* (A2); △, *trpS5* (KY4040); ● and ▲, wild type and *trpS5*, respectively, in the presence of 50 μg/ml L-tryptophan.

TABLE 10

Charge level of tRNA^{trp}

Strain	L-tryptophan (μg/ml)	State of growth*	Percent charged tRNA^{trp}†
Wild (Y-mel)	50	Late exponential	65
	0	Late exponential	66
trpA2(A2)	50	Late exponential	73
	0	Tryptophan starvation	< 8
trpS5 (KY4040)	50	Late exponential	76
	0	Tryptophan starvation	23

* "Late exponential" corresponds to the turbidity of 150 in Klett-Summerson colorimeter in medium EA supplemented with L-tryptophan as indicated. In the case of tryptophan starvation, cells were incubated for an additional 60 min in the tryptophan-free medium at 37°C.
† Averages of two independent determinations are presented.

above three strains. As shown in Table 10, the charged tRNA^{trp} in both *trpA2* and *trpS5* decreased markedly upon removal of tryptophan from the medium.

These results suggest that under the condition of tryptophan limitation, tryptophanyl tRNA synthetase is made inactive in the *trpS5* mutant bringing about derepression of the tryptophan operon. It may be concluded that tryptophanyl tRNA synthetase is somehow involved in repression of this operon.

Effect of 5-methyltryptophan on expression of the tryptophan operon and charging of tRNA^{trp}: Although the above results might be taken to indicate that charging of tRNA^{trp} is required for repression of the trytophan operon, 5-methyltryptophan that represses this operon does not attach to tRNA *in vitro* (DOOLITTLE and YANOFSKY 1968). We studied the effect of 5-methyltryptophan on charge level of tRNA^{trp} *in vivo*. It was first confirmed that 5-methyl-DL-tryptophan (10–100 μg/ml) indeed represses the mRNA synthesis of the tryptophan operon with the *trpA2* mutant (Figure 5). Under these conditions (50 μg/ml 5-methyl-DL-tryptophan), the charged tRNA^{trp} was found to decrease to a low level comparable to that with a tryptophan-starved culture (Table 11). Thus the charging of the bulk of tRNA^{trp} does not seem to be a prerequisite to repression of the tryptophan operon. It may be suggested that some other function(s) of trypto-

TABLE 11

Effect of 5-methyltryptophan on in vivo *charge level of tRNA^{trp}**

Supplement to medium	Percent charged tRNA^{trp}
Tryptophan	66
5-methyltryptophan	8
None	9

* The *trpA2* mutant grown in medium EA with 50 μg/ml L-tryptophan was centrifuged, washed and resuspended in medium EA supplemented with L-tryptophan (50 μg/ml) or 5-methyl-DL-tryptophan (50 μg/ml) as indicated. After shaking at 37°C for 10 min, cells were harvested and the charge level of tRNA^{trp} was determined.

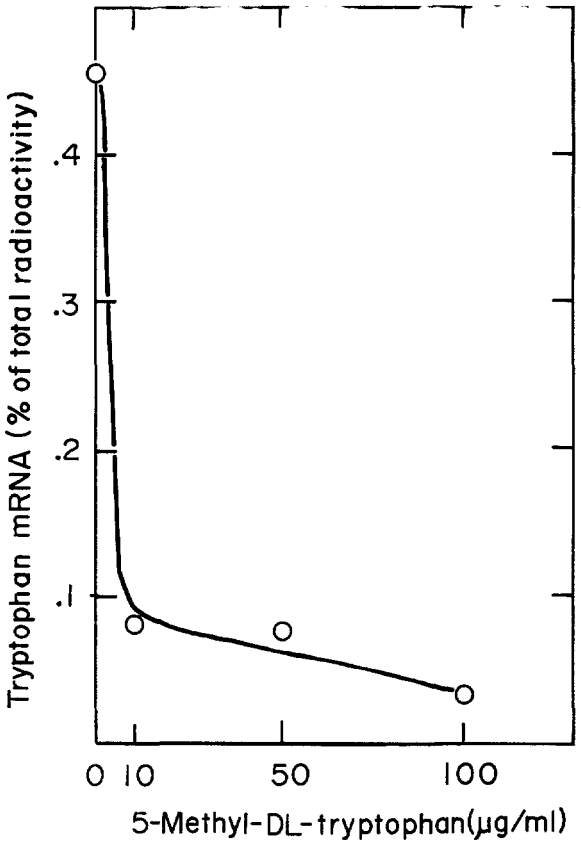


FIGURE 5.—Effect of 5-methyltryptophan on tryptophan mRNA synthesis.—The *trpA2* mutant (A2) was grown, washed as described in the foot note to Table 9 and resuspended in medium EA with 5-methyl-DL-tryptophan as indicated (μg/ml). After shaking at 37°C for 10 min, the cells were pulse-labeled with H^3-uridine for 3 min and RNA was extracted and hybridized with denatured DNA of $\phi 80pt_{190}$ and $\phi 80$. Percent of radioactivity retained on $\phi 80pt_{190}$DNA filter after subtraction of the value for $\phi 80$ DNA filter is presented.

phanyl tRNA synthetase rather than the formation of the bulk of tryptophanyl tRNA is involved in repression of the tryptophan operon.

DISCUSSION

The present study revealed that the *trpS* mutation, located between *strA* and *malA* on the *E. coli* chromosome, affects the activity and stability of tryptophanyl tRNA synthetase. Tryptophanyl tRNA synthetase of *trpS5* is very unstable and can be stabilized by tryptophan and ATP (Table 4). The enzyme activity was partially or fully recovered by prototrophic reversions of *trpS5* that occurred at or around the originally mutated locus (Table 7). The enzyme from one of the revertants shows an altered affinity to L-tryptophan (Table 8). Thus it seems reasonable to conclude that *trpS* is the structural gene for tryptophanyl tRNA

synthetase. DOOLITTLE and YANOFSKY (1968) and KANO, MATSUSHIRO and SHIMURA (1968) have independently isolated tryptophan-requiring mutants like *trpS5* reported here and reached similar conclusions. Auxotrophic mutants analogous to *trpS5* have also been isolated in the histidine (NASS and NEIDHARDT 1966) and glycine (FOLK and BERG 1968) systems of *E. coli*.

The results presented above also suggest that the tryptophan requirement in the *trypS5* mutant is due to instability of tryptophanyl tRNA synthetase in the absence of a sufficient amount of tryptophan. The growth characteristics of the *trpS5* mutant presented in Figure 1 also seem to agree with this interpretation. Although *trpS5* could grow on indole as well as on tryptophan (HIRAGA *et al.* 1967a), no significant stabilization of the mutant tryptophanyl tRNA synthetase was found by indole *in vitro* (Table 4). The following alternatives may explain the growth-stimulating effect of indole: (1) Indole stabilizes the enzyme *in vivo* but stabilization was not demonstrated *in vitro* owing to unsuitable conditions; (2) Indole does not stabilize the enzyme but it is effectively converted to tryptophan *in vivo* which in turn exerts the stabilizing effect.

The fact that the double mutants carrying *trpS5* and one of the 5-methyltryptophan-resistant mutations exhibit a less strict requirement for tryptophan (Table 5) seems to be accounted for by the increase of the internal tryptophan concentration due to the 5-methyltryptophan-resistant mutation. Among the revertants of *trpS5* isolated in earlier experiments were the 5-methyltryptophan-resistant mutations linked to *cysB* and the tryptophan cluster (HIRAGA *et al.* 1967a). These mutants (*trpO1* and *trpO2*) have been shown to be operator-constitutive mutants (HIRAGA *et al.* 1967b; HIRAGA, ITO and YURA 1968; HIRAGA, 1969).

The formation of the tryptophan biosynthetic enzymes and the tryptophan mRNA in the *trpS5* mutant was derepressed under conditions where exogenous tryptophan had not been exhausted, in contrast to the *trpS*$^+$ strains (Figure 3, Table 9), suggesting the involvement of tryptophanyl tRNA synthetase in repression. The rate of tryptophan mRNA synthesis with this mutant in a tryptophan-free medium was more than twice that for the wild-type strain and was comparable to that for strain *trpA2* defective in tryptophan biosynthesis (Figure 4). Under the latter conditions, the charged tRNA ^{trp} was found to decrease to low levels in both *trpS5* and *trpA2* (Table 10). These results also suggest that the formation of tryptophanyl tRNA or possibly some other function of tryptophanyl tRNA synthetase is involved in repression of the tryptophan operon. It is assumed here that free tryptophan in the *trpS5* mutant does not decrease as much as in the *trpA2* mutant under the condition of tryptophan deprivation. This is plausible because the *trpS5* mutant, in contrast to *trpA2* mutant, should continue to synthesize tryptophan endogenously under these conditions and the termination of growth results presumably from the inactivation of tryptophanyl tRNA synthetase. Preliminary determination of internal free tryptophan indeed showed that this assumption appears to be correct.

DOOLITTLE and YANOFSKY (1968) showed that the derepressed levels of tryptophan synthetase in their *trpS* mutants were lower than those of the usual tryptophan auxotrophs and concluded that charging of tRNA^{trp} is not involved in re-

pression. However, they did find that the *trpS* mutants have highly derepressed enzyme levels under certain conditions. Furthermore, KANO, MATSUSHIRO and SHIMURA (1968) reported that some of their *trpS* mutants can not be repressed normally by excess tryptophan. More recent studies in this laboratory revealed that when the *trpS* region of strain KY4040 was transduced into certain strains, the resulting transductants carrying *trpS5* could grow at 30°C but not at 42°C in complete medium. These transductants showed a repressed level of anthranilate synthetase several-fold higher than that of the parental *trpS*$^+$ strain at 42°C. It was also found that the repressed level of the enzyme with *trpR* mutants was higher in the *trpS5* than in the *trpS*$^+$ bcakground (ITO, HIRAGA and YURA, 1969). These results provide further evidence for the conclusion that tryptophanyl tRNA synthetase is involved in the regulation of this operon.

It has been reported that 5-methyltryptophan that represses the tryptophan operon does not attach to tRNA *in vitro* (DOOLITTLE and YANOFSKY 1968), although the situation is somewhat complicated by the observation that 5-methyltryptophan appears to be incorporated into protein under certain conditions *in vivo* (EZEKIEL personal communication). We also found that 5-methyltryptophan can not maintain the charge level of tRNA^{trp} *in vivo* under conditions where the tryptophan operon is repressed (Table 11, Figure 5). Thus it might be suggested that it is not the charging of the bulk of tRNA^{trp} but some other function of tryptophanyl tRNA synthetase that is involved in the repression of the tryptophan operon. It is conceivable, for example, that the charging of some minor species of tRNA^{trp} is involved in repression or that tryptophanyl tRNA synthetase itself constitutes a part of the repressor molecule. Further experiments are required to elucidate the mechanism of repression, and in particular, to establish the regulatory role of tryptophanyl tRNA synthetase.

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SUMMARY

A tryptophan auxotroph *trpS5* of *Escherichia coli* carrying the intact structural genes of the tryptophan operon has been studied. The gene *trpS* is mapped between *strA* and *malA* by transduction with phage P1. Tryptophanyl tRNA synthetase of this mutant is extremely unstable and can be stabilized by tryptophan and ATP. Reversions at or around the *trpS5* mutation result in partial or full recovery of the enzyme activity. The enzyme of one revertant exhibits an altered affinity to L-tryptophan, suggesting that *trpS* is the structural gene for this enzyme.—Expression of the tryptophan operon in the *trpS5* mutant is repressed normally by excess tryptophan. However, when a repressed culture of the *trpS5* mutant was transferred to a tryptophan-limited medium, derepression for the tryptophan biosynthetic enzymes as well as for tryptophan mRNA occurred under conditions where exogenous tryptophan has not been exhausted, in contrast to the *trpS*$^+$ strains. These results suggest that tryptophanyl tRNA synthetase is

somehow involved in repression of this operon. On the other hand, studies on the effect of 5-methyltryptophan on the tryptophan mRNA synthesis and on the charge level of $tRNA^{trp}$ revealed that the charging of the bulk of $tRNA^{trp}$ is not a prerequisite to repression. It is suggested that some other function of tryptophanyl tRNA synthetase than the formation of the bulk of tryptophanyl tRNA is involved in the repression of this operon.

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