JOURNAL OF BACTERIOLOGY, May 1971, p. 331-338

Vol. 106, No. 2
Printed in U.S.A.

New Regulatory Mutation Affecting Some of the Tryptophan Genes in *Pseudomonas putida*[1]

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Received for publication 7 December 1970

Three indole analogues, 5-methylindole, 5-fluoroindole, and 7-methylindole, and the tryptophan analogue 5-fluorotryptophan were found to inhibit the growth of wild-type *Pseudomonas putida*. Mutants resistant to these analogues were obtained. Some of the 5-fluoroindole- and 5-fluorotryptophan-resistant strains exhibit an abnormality in the regulation of certain *trp* genes. These strains excrete anthranilate when grown in minimal medium in the presence or absence of the inhibitor. In these strains, the *trpA*, *B*, and *D* gene products, the first, second, and fourth enzymes of the tryptophan pathway, are produced in 20-fold excess over the normal wild-type level. The other enzymes of the pathway are unaffected. Exogenous tryptophan is still able to repress the expression of the *trpABD* cluster somewhat. Similarity between the 5-fluoroindole- and 5-fluorotryptophan-resistant strains suggests that the former compound becomes effective through conversion to the latter. Repression and derepression experiments with two anthranilate-excreting, 5-fluoroindole-resistant strains showed coordinate variation of the affected enzymes. The locus conferring resistance and excretion is not linked by transduction to any of the *trp* genes.

No general relationship exists between the regulation and the chromosomal organization of genes for biosynthetic or degradative pathways in microorganisms. Several types of control and several different genetic arrangements are seen in different pathways within a single species. Moreover, comparative studies of a single pathway in different microorganisms may show several variations in the mode of regulation and genetic organization.

The biosynthesis of tryptophan has been the object of intensive genetic and biochemical work in several species. A variety of gene orders and linkages was found, ranging from a single cluster in *Escherichia coli* (22) to well dispersed genes in fungi (8). End-product repression, precursor induction, and constitutive levels of enzyme production were all reported within the pathway (13, 7, 21). The structural genes for the enzymes of the tryptophan pathway in *Pseudomonas putida* were mapped (12), and the molecular weights and aggregation of the gene products were studied (reference 10; Fig. 1). Three linkage groups were found by transductional analysis. The first cluster, consisting of genes *trpA*, *trpB*, and *trpD*, contains the information for anthranilate synthetase (AS), phosphoribosyl transferase (PRT), and indoleglycerol phosphate synthetase (InGPS). *TrpC*, the gene for phosphoribosylanthranilate isomerase (PRAI), is unlinked to the first cluster of genes. The genes coding for the tryptophan synthetase (EC 4.2.1.20) A and B components (TS-A and TS-B) are designated *trpE* and *trpF*. They are closely linked to each other but unlinked to the other structural genes in the pathway. It was previously reported that the degree of clustering of functionally related genes for several pathways is less in the genus *Pseudomonas* than among the enteric bacteria (11).

Tryptophan, the end product of the pathway, may act as a repressor for the expression of the *trpABD* cluster, but does not affect the level of the *trpC* gene product nor the amount of TS-A and TS-B proteins formed (7). In earlier work, it was not clearly established that the *trpABD* cluster represents a typical operon. The *trpB* and *trpD* gene products appeared to vary coordinately in response to tryptophan deprivation, but AS could be derepressed somewhat more than these two.

Biochemical analysis has given evidence for

[1] In partial fulfillment of the requirements for the Ph.D. degree (R. M.) at the University of Bern, Switzerland. A preliminary report of this work was presented at the 38th meeting of the Genetics Society of America in Madison, Wis., August 1969.

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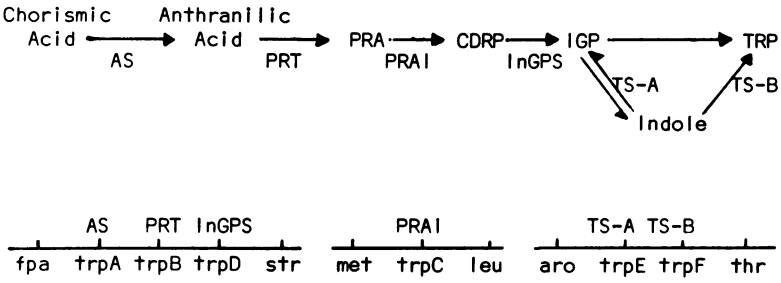

FIG. 1. *Gene-enzyme relationships in the tryptophan pathway of Pseudomonas putida. Abbreviations used: AS, anthranilate synthetase; PRT, phosphoribosyl transferase; PRAI, phosphoribosyl-anthranilate isomerase; InGPS, indoleglycerol phosphate synthetase; TS-A, tryptophan synthetase A component; TS-B, tryptophan synthetase B component; PRA, N-5'-phosphoribosylanthranilate; CDRP, 1-(o-carboxyphenylamino)-1-deoxyribulose-5-phosphate; IGP, indole-3-glycerolphosphate; TRP, L-tryptophan; fpa, p-fluorophenylalanine resistance locus; str, streptomycin resistance locus; met, methionine locus; leu, leucine locus; aro, locus for an enzyme in the common aromatic acid pathway; thr, threonine locus. It is not yet clear whether the order in the third cluster is aro trpE trpF thr or thr trpE trpF aro.*

the presence of two nonidentical subunits in the AS of pseudomonads (18). These two components were found to vary in concert. All mutants lacking AS isolated thus far, however, are defective in only the larger subunit. Without mutants of the small component, the location of its gene remains enigmatic. The amount of the *trpC* gene product, PRAI, seems to be nearly constant under all growth conditions. In contrast, the level of TS-A and TS-B is controlled by the intermediate indoleglycerol phosphate which acts as an inducer.

This study was undertaken to obtain more detailed information on the regulation of the tryptophan pathway in *P. putida*. Our particular interest was to attempt to learn whether the entire *trpABD* cluster is controlled as a single, coordinate unit.

MATERIALS AND METHODS

Organisms and media. *P. putida* Clop, originally isolated from a camphor enrichment culture (4), was used throughout this study. This strain is sensitive to the transducing phage pf16h$_0$ (5). A temperature-sensitive mutant of this phage was isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (75 μg/ml) treatment of phage-infected wild-type cells at 30 C for 30 min. A large number of progeny phage plaques were screened for growth at 30 and 37 C. One out of several mutant strains isolated (ts7h$_0$) showing a very low (10^{-8}) reversion frequency was used in transduction experiments. The soft agar overlayer technique was used for the preparation of phage stocks (1). An overnight culture of cells (0.5 ml) in L broth (16) and the same amount of a lysate diluted to 10^7 plaque-forming units (PFU) per ml were added to 3 ml of soft agar and layered over an L agar plate. Plates were incubated 18 hr at 30 C. The soft agar layer was triturated in 3 ml of PM medium (12), shaken with 0.5 ml of chloroform, and centrifuged. For transductions, stock phage solutions were diluted to 2×10^9 to 5×10^9 PFU/ml in PM medium and mixed with an equal number of cells grown overnight in L broth. After 20 min at room temperature, the transduction mixture was centrifuged and suspended in L broth to the original cell density. Cells were spread on Vogel-Bonner minimal medium E (20) plates and incubated at 37 C.

Isolation of mutants. Most analogue-resistant mutants were obtained after the wild-type strain was mutagenized with 20 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml for 1 hr at 30 C (2) and then spread on minimal agar plates containing the growth inhibitor at 200 μg/ml. (Untreated wild-type cells, 2×10^9, from an overnight L-broth culture were used for the isolation of 5-fluorotryptophan-resistant mutants on plates supplemented with this analogue.) After 3 to 5 days of incubation at 30 C, a number of the small colonies appearing were isolated and purified by two streakings on minimal agar plates. All other mutant strains were described earlier (12).

Accumulation tests. Anthranilate was quantitated after extraction into ethyl acetate from acidified culture supernatants. The ethyl acetate was evaporated at 37 C, and fluorescence of the residue was measured in solution in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 7.8). The two fluorescent intermediates, anthranilate and CDRP, were distinguished by paper chromatography in methanol-*n*-butanol-benzene-water (2:1:1:1). All other accumulation tests were performed qualitatively as described elsewhere (7).

Chemicals. 2-Methylindole, 5-methylindole, and 7-methylindole, 7-azaindole, and 4- and 6-fluorotryptophan were purchased from Sigma Chemical Co.; 4- and 6-methyltryptophan were obtained from Mann Research; and 5-methyltryptophan and *N*-acetyl-L-tryptophan were obtained from Calbiochem. K & K Laboratories was the source of 5-fluoroindole and 3- and 5-methylanthranilic acid. 7-Azatryptophan was obtained from Nutritional Biochemical Corp.

Enzyme and protein assays. Linear reaction rates were obtained in the AS assay by using potassium phosphate instead of Tris buffer; all other assay conditions were as described earlier (7). Protein concentration was determined colorimetrically by using the

method of Lowry et al. (17). One enzyme unit is defined as the production or consumption of 0.1 μmole of product or substrate in 20 min at 37 C.

Column chromatography of AS. A tryptophan auxotroph lacking PRAI, *trpC621*, was grown with limiting tryptophan (4 μg/ml) in Vogel-Bonner medium E plus 0.2% glucose to early stationary phase. Cells were centrifuged, washed, and suspended in 0.1 M potassium phosphate buffer (*p*H 7.5) containing 1 mM 2-mercaptoethanol. Extracts were prepared by disruption with a Branson sonic oscillator. The extract was partially purified after addition of protamine sulfate (5 ml of a 5% solution per 100 ml extract), a heat step (6 min at 55 C), and ammonium sulfate fractionation. The protein precipitating between 30 and 50% saturation with ammonium sulfate was considerably enriched for the two components of AS. A portion of this fraction containing about 30 mg of protein was dialyzed against the mercaptoethanol-supplemented potassium phosphate buffer and applied to a Sephadex G150 column (2.5 by 27 cm). Fractions were assayed in the standard AS reaction as described in the legend to Fig. 4. The same methods were used for extract preparation and column chromatography with the 5-fluoroindole-resistant strain (5FIR-17).

Derepression experiments. Strain 5FIR-17 was harvested in the exponential-growth phase in 1 liter of minimal medium plus 0.2% glucose and 50 μg of L-tryptophan per ml. Cells were centrifuged, washed twice with cold minimal medium, and resuspended in the original volume of prewarmed minimal medium containing 0.2% glucose and 0.02% acid-hydrolyzed casein. Portions (100 ml) were aerated vigorously in 250-ml flasks on a reciprocal shaker at 30 C. After 20 min of derepression, successive flasks received excess tryptophan (50 μg/ml) at 10-min intervals. Each flask was agitated for an additional 10 min at 30 C, 100 μg of chloramphenicol per ml was then added, and the culture was chilled in an ice-water bath. Cells were harvested and washed in 0.1 M potassium phosphate buffer (*p*H 7.0), supplemented with 1 mM 2-mercaptoethanol. A dense suspension of the cells in the same phosphate buffer was exposed for 30 sec to a Branson sonic oscillator, and the extracts were clarified by centrifugation for 15 min at 40,000 × *g*.

Mapping of the 5FIR marker. It proved difficult to select fluoroindole-resistant transductants on analogue-supplemented plates, but the 5FIR marker could be scored nonselectively by its accumulation of anthranilate during early stationary phase. The use of the temperature-sensitive phage mutant $ts7h_0$ made the addition of antiphage serum unnecessary if the plates were incubated at 37 C.

RESULTS

Effect of analogues on growth. Many different analogues of tryptophan and its pathway intermediates were tested for their ability to inhibit the growth of wild-type *P. putida* (Table 1). The two analogues most commonly used in studies with other organisms, 4- and 5-methyl-tryptophan, had little or no inhibitory effect on *P. putida*. Indoles substituted at the five or seven position and 5-fluorotryptophan (5FT), however, were strong inhibitors at relatively low concentrations. (*Pseudomonas* strains often tolerate levels of metabolic inhibitors that are toxic to enteric bacteria, thus 200 μg/ml is not an unusually high level for this organism.) *P. putida* mutants resistant to any one of the four most effective analogues can be obtained in a single step, either spontaneously or after mutagenesis, by spreading wild-type cells on plates containing the inhibitor at an appropriate concentration. In general, a higher analogue concentration is needed to give an inhibition on plates equal to that seen in liquid media. A number of resistant strains were tested for accumulation of intermediates in tryptophan biosynthesis during growth in minimal medium. A majority of the 5-fluorotryptophan-resistant (5FTR) strains and some of the 5-methylindole- and 5-fluoroindole-resistant (5MIR and 5FIR) strains were found to accumulate anthranilate. No other tryptophan precursors such as indoleglycerol phosphate or indole were excreted by any of the strains. Most of the mutants isolated, including all of the 7-methylindole-resistant strains, failed to excrete anthranilate. Since the anthranilate-excreting strains seemed to be affected somehow in the regulation of the tryptophan pathway, we studied more closely one mutant resistant to each analogue. These were designated 5MIR-14, 5FIR-17, and 5FTR-2.

TABLE 1. *Effect of tryptophan analogues on the growth of Pseudomonas putida*[a]

Analogue	Concn (μg/ml)	Growth
DL-4-Methyltryptophan	500	++
DL-5-Methyltryptophan	500	+++
DL-6-Methyltryptophan	500	+++
DL-4-Fluorotryptophan	400	++
DL-5-Fluorotryptophan	200	0
DL-6-Fluorotryptophan	500	+++
DL-7-Azatryptophan	500	+++
N-acetyl-L-tryptophan	500	+++
2-Methylindole	200	+
5-Methylindole	200	0
7-Methylindole	200	0
7-Azaindole	500	+
5-Fluoroindole	200	0
3-Methylanthranilate	400	++
5-Methylanthranilate	400	++

[a] Overnight culture (0.1 ml) of the wild type growing in minimal medium plus 0.2% glucose was used to inoculate 9.9 ml of the same medium containing the indicated amount of analogue. Analogues were autoclaved 15 min in the medium without glucose. Cultures shaken at 30 C for 18 hr were recorded as (+++) normal, (++) medium, (+) slight, and (0) no growth.

The growth of wild type and 5FIR-17, with and without added inhibitor, is shown in Fig. 2. 5FI (50 μg/ml) inhibits growth of the parent strain completely. After an initial lag, however, the resistant mutant grows to the maximum turbidity in the presence of the analogue. Addition of tryptophan in a twofold excess over the analogue concentration did not reverse the inhibition of the wild type.

Cross resistance. Both 5FIR and 5FTR strains showed resistance to 5FI (75 μg/ml), 5FT (200 μg/ml), and 5 MI (100 μg/ml) (Table 2). On the other hand, growth of the 5MIR strains was inhibited appreciably by the 5-fluoro-substituted analogues. It is clear that the mutant strains resistant to 5FI and 5FT are similar and show some cross-resistance to all three analogues, but the 5MI-resistant strains appear to fall into a different category.

Excretion of anthranilate. Those resistant mutants that excrete anthranilate into the medium do not do so at all stages of growth. Maximal accumulation is observed near the end of the exponential growth phase (Fig. 3). AS was assayed at different points in the growth curve and found to have a constant specific activity throughout.

Specific activity of the trp enzymes. We determined the specific activity of all of the enzymes of the pathway (except TS-A) in cultures grown to early stationary phase in unsupplemented minimal medium (Table 3). The values reported were normalized to the level found in an extract of wild-type cells. The first two enzymes of the pathway, AS and PRT, are slightly lower in the 5MIR strain than in wild type, but these enzymes and InGPS are overproduced in the 5FIR and 5FTR strains. The amount of overproduction seems greater for AS than for PRT and InGPS. The other enzyme activities measured were not affected in any of the strains tested. Since no abnormality of enzyme regulation was uncovered in the 5MIR strain, we concentrated on characterizing 5FIR-17 as typical of the fluoroanalogue-resistant strains with altered regulatory properties.

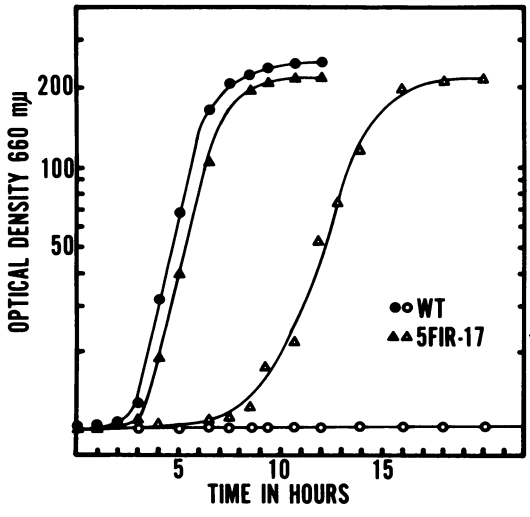

FIG. 2. *Effect of 5-fluoroindole on wild type and a 5-FIR mutant. Optical density at 660 nm was followed as a function of time. Wild-type* (○) *and 5FIR-17* (△) *cells were grown with (open symbols) and without (filled symbols) added 5-FI (50 μg/ml) in glucose-supplemented minimal medium with agitation at 30 C.*

Effect of tryptophan on the expression of the trp genes. Strain 5FIR-17 was grown to early stationary phase in minimal medium with and without added tryptophan. Extracts were prepared in the usual way and assayed for the activities indicated in Table 4. Extracts of wild-type cells grown under the same conditions were assayed at the same time. All specific activities were normalized to those in wild-type cells grown in excess tryptophan. The enzymes encoded in the *trpABD* gene cluster are overproduced 20-fold when the resistant strain is grown in minimal medium. Excess tryptophan reduces the amount of AS more than six times and decreases the PRT and InGPS level threefold. The amount of these three enzymes produced by the resistant strain under conditions of repression is still somewhat elevated over the basal wild-type level, however. No changes were observed in the activity of the third enzyme of the pathway, PRAI. TS-B activity responded similarly in the mutant and wild-type strains. Cells grown with citrate as sole carbon source were not significantly different from glucose grown cells (*unpublished data*).

Synthesis of the large and small AS components. As mentioned earlier, AS in *P. putida* contains two nonidentical subunits which can be separated by Sephadex gel filtration. A mutant lacking PRAI, *trpC621*, was used as a source of normal AS. The elution profile of the AS components is shown in Fig. 4A. A crude extract of a *trpA* mutant was added to the early fractions to assay the large subunit. This material was then used to assay the small subunit. The ratio of the amount of activity in the small and the large subunit regions was determined to be 1:2.5. An extract of a 5FIR strain grown on minimal medium was applied to the same column and assayed similarly (Fig. 4B). The ratio of activities in the two components was the same as was found with the *trpC* mutant. Therefore, the two polypeptide chains of AS appear to be overproduced coordinately in the mutant strain.

Repression and derepression studies. The 5FIR

TABLE 2. *Cross-resistance among analogue-resistant mutants of Pseudomonas putida*[a]

Strain	Analogue														
	None	5FI (μg/ml)					5MI (μg/ml)					5FT (μg/ml)				
		50	75	100	150	200	50	75	100	150	200	50	75	100	150	200
Wild type	+++	0	0	0	0	0	+	+	0	0	0	0	0	0	0	0
5FIR-17	+++	+++	++	+	0	0	+++	+++	+++	0	0	+++	+++	+++	+++	+++
5FIR-27	+++	+++	+++	+	0	0	+++	+++	+++	0	0	+++	+++	+++	+++	+++
5MIR-3	+++	0	0	0	0	0	+++	+++	+++	0	0	+	+	+	0	0
5MIR-16	+++	0	0	0	0	0	+++	+++	++	+	0	+	+	0	0	0
5FTR-2	+++	+	+	+	0	0	+++	+++	+++	+	0	+++	+++	+++	+++	+++

[a] One drop of a 1:5 dilution of an overnight culture in L broth was used to inoculate 10 ml of minimal medium containing 0.2% glucose and the indicated amount of analogue. Cultures were grown for 18 hr at 30 C with shaking. Growth was estimated as in Table 1.

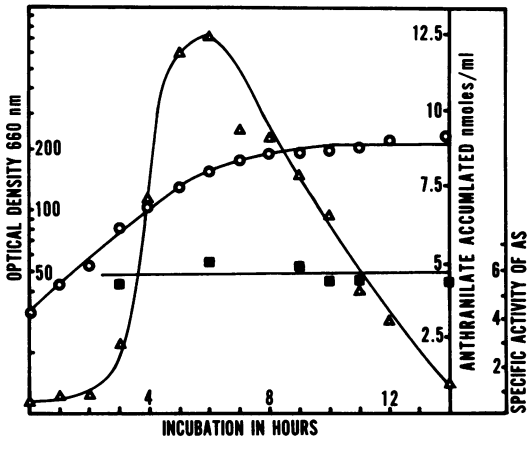

FIG. 3. *Anthranilate accumulation during the growth of strain 5FIR-17. At various times aliquots of a culture of 5FIR-17 growing in glucose-supplemented minimal medium were analyzed for anthranilate* (△). *Cells washed twice with 0.1 M potassium phosphate (pH 7.0) were disrupted by sonic oscillation and assayed for AS* (■). *The optical density of the culture was followed at 660 nm* (○).

strain was grown under repressing conditions (50 μg of tryptophan per ml) till mid-exponential phase and then allowed to derepress in minimal medium. The increase of all the activities of the pathway except TS-A was followed for 100 min. When the specific activities of AS, PRT, PRAI, and TS-B were plotted against the InGPS activity, coordinate response of those activities controlled by the *trpABD* cluster was apparent (Fig. 5A, B). No change in the specific activities of PRAI (produced by the *trpC* gene) or of tryptophan synthetase, product of the *trpEF* gene cluster, was observed during the first 100 min. of derepression.

Location of the 5FIR mutation. Attempts to transfer the anthranilate excreting property of several 5FIR strains along with the wild type

TABLE 3. *Enzyme levels in three anthranilate-excreting mutants*

Strain	Specific activity[a]				
	AS	PRT	PRAI	InGPS	TS-B
Wild type	1.0	1.0	1.0	1.0	1.0
5MIR-14	0.85	0.37	1.04	—[b]	1.0
5FIR-17	19.3	11.6	1.04	9.9	0.93
5FIR-2	24.7	7.6	1.56	9.5	1.12

[a] Cells were grown to the early stationary phase in glucose-supplemented minimal medium without added tryptophan. Extracts obtained by sonic oscillation were assayed as described in the text. Specific activities (units per milligram of protein) for each activity were normalized to the wild-type value. Abbreviations: AS, anthranilate synthetase; PRT, phosphoribosyl transferase; PRAI, phosphoribosyl-anthranilate isomerase; InGPS, indoleglycerol phosphate synthetase; TS-B, tryptophan synthetase B component.

[b] Not determined.

TABLE 4. *Repression of enzyme levels by exogenous tryptophan*

Strain	Specific activity[a]									
	AS		PRT		PRAI		InGPS		TS-B	
	+	0	+	0	+	0	+	0	+	0
Wild type	1.0	1.5	1.0	1.8	1.0	1.03	1.8	1.2	1.0	5.0
5FIR-17	3.0	19.6	5.5	16.0	1.08	1.14	4.4	12.6	1.9	4.7

[a] Cells were grown in glucose-containing minimal medium with (+) and without (0) a supplement of 50 μg of tryptophan per ml. Extracts obtained by sonic oscillation were assayed as described in the text. Specific activities (units per milligram of protein) for each enzyme were normalized to the level in wild type grown on excess tryptophan. Abbreviations: AS, anthranilate synthetase; PRT, phosphoribosyl transferase; PRAI, phosphoribosyl-anthranilate isomerase; InGPS, indoleglycerol phosphate synthetase; TS-B, tryptophan synthetase B component.

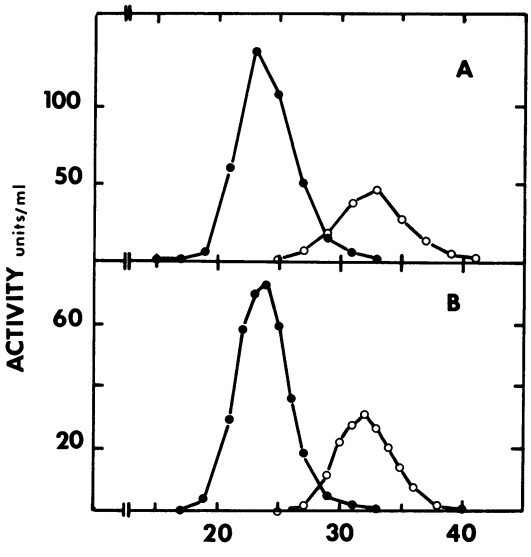

FIG. 4. *Dissociation of AS on a column of Sephadex G150. A partially purified extract of trpC621 cells was applied to a column (2.5 by 27 cm) of Sephadex G 150 equilibrated with 0.02 M Tris-hydrochloride (pH 7.5) containing 10 mM $MgCl_2$ and 13 mM 2-mercaptoethanol (A). The activity of the large component (●) was determined with the aid of a crude extract of trpA601, a mutant lacking this protein. The small component (○) was determined in an excess of the large component from the early fractions. A similar experiment employed a partially purified extract of 5FIR-17 (B).*

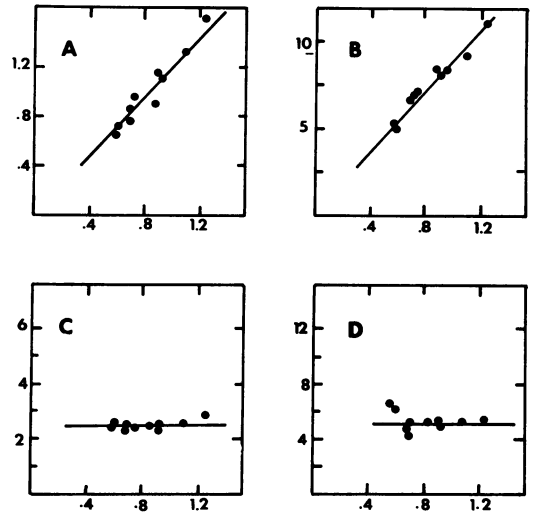

FIG. 5. *Derepression kinetics of the 5-fluoroindole-resistant mutant 5FIR-17. At different times during derepression by tryptophan deprivation, cells were harvested and assayed for five activities of the tryptophan pathway. The abscissa of each graph represents the specific activity of InGPS. The ordinates are specific activities of: A, AS; B, PRT; C, PRAI; and D, TS-B.*

form of each of the *trp* genes were unsuccessful. Strains *trpA602*, *trpC621*, *trpD631*, *trpF651* and *trpE642* were used as recipients in these transduction experiments. In addition, cotransfer could not be observed with *met-601* and *leu-501*, markers linked to *trpC* (12), or with a *thr* marker linked to *trpE* and *trpF* (C. F. Gunsalus and the authors' unpublished observations).

DISCUSSION

Biochemical and genetic analyses have established that *P. putida* employs a unique genetic and regulatory arrangment for the tryptophan pathway. Unlike the well studied enteric organisms *E. coli* and *Salmonella typhimurium*, the genes coding for the tryptophan enzymes in *P. putida* do not constitute a single operon, as defined by Jacob and Monod (15). There has even been some doubt whether the *trpABD* cluster functions coordinately (12); thus, we investigated a number of analogues for their ability to alter the expression of this gene cluster.

Indole analogues containing a methyl group at the five or seven position and indole or tryptophan molecules substituted at the five position with a fluorine atom inhibit the growth of *P. putida* completely. Surprisingly, 4- and 5-methyltryptophan, excellent growth inhibitors in other bacteria, have either a very slight effect on *P. putida* or show no inhibition at all. There may be a permeability barrier for 4- and 5-methyltryptophan, a demethylating enzyme may change the analogues to the regular end product, or the target site may be insensitive to these compounds. These two analogues were also found rather ineffective as growth inhibitors for *Neurospora crassa* and *Saccharomyces cerevisiae*.

A number of mutants resistant to the four effective growth inhibiting analogues were isolated. In some of these, an imbalance in the regulation of specific segments of the pathway was observable by accumulation tests. A number of 5MIR and 5FIR and the majority of the 5FTR strains excreted anthranilate, but none of the other pathway intermediates could be detected. The 7-methylindole-resistant strains formed a separate class showing resistance chiefly to that analogue and unaffected in pathway regulation. This type was not further investigated.

Assays for most of the enzymes of the tryptophan pathway were performed with several of the resistant and anthranilate-excreting types of mutants. If the resistant strain did not excrete anthranilate, no differnce between it and the wild

type was found. The level of AS, PRT, and InGPS in the excreting 5FIR and 5FTR mutants, however, was considerably higher than in the parent strain. The other enzymes assayed, PRAI and TS-B, were unaffected. Surprisingly, one anthranilate-excreting 5MIR mutant was no different from wild type in enzyme levels. Although the findings with this 5MIR mutant are unexplained at the present time and need further investigation, the rest of the results clearly show an alteration in the regulation of the *trpABD* cluster in the excreting 5FIR and 5FTR strains.

Cross-resistance experiments among the 5FIR, 5FTR, and 5MIR strains confirmed the enzymatic results and suggested that the excreting 5FIR and 5FTR mutants were very similar. *P. putida* tryptophan auxotrophs blocked early in the pathway grow very poorly on indole (6). Thus, it is not too surprising that the 5FTR and 5FIR mutants were not completely identical in their response to 5FI. We feel there is nothing in our experiments to contradict the logical hypothesis that the cell metabolizes 5FI to 5FT, the real growth inhibitor.

When a 5FIR mutant is derepressed by removal of exogenous tryptophan, the products of the first, second, and fourth genes of the pathway increase coordinately and independently of the rest of the pathway. The finding that the small component of AS is overproduced also gives strength to the presumed location of its gene within the *trpABD* cluster (18). The tryptophan pathway of *P. putida* thus contains several small operon-like structures, one being the *trpABD* cluster. The expression of the *trpC* gene seems to be almost constant under all growth conditions (7), whereas the *trpE* and *trpF* genes must form another small operon under induction control. The fact that the level of the *trpC* gene product is almost invariant and that the TS enzyme is not overproduced in the anthranilate-excreting 5FIR and 5FTR strains suggests that the third step in the pathway limits the production of indoleglycerol phosphate, the inducer of TS. If overproduced AS and PRT synthesize more intermediates in these mutants than can be metabolized by the *trpC* gene product, anthranilate would be the sole product excreted, since PRA decomposes to this compound spontaneously (9). The disappearance of anthranilate during the stationary phase may most probably be ascribed to the appearance of an inducible degradation system.

Since the mutation causing the overproduction of the enzymes of the *trpABD* cluster is not cotransducible with any of the *trp* genes, it may be the structural gene for the aporepressor of the *trpABD* operon. Two kinds of 5-methyltryptophan-resistant mutants were obtained and mapped in *E. coli* (6, 19). One locus, *trpR*, is unlinked to the *trp* operon, whereas the other maps within the gene coding for AS. The excreting mutants in *P. putida* then resemble the *trpR* mutant type in *E. coli*. Until dominance tests can be performed in this system, however, an exact comparison with the regulatory genes in other organisms will not be possible.

It is tempting to propose that the site of action of the 5-fluoroanalogues we studied is the repressor molecule for the *trpABD* operon. False repression by the analogue leading to a tryptophan deficit could be overcome by a mutation altering the response of the aporepressor to both the normal and abnormal effector molecules. Hypotheses similar to this were presented in connection with studies of 5-methyltryptophan resistance in enteric bacteria (3, 6, 14). At present, we feel that the amount of information available in *Pseudomonas* is insufficient to furnish strong support for this mechanism. The similarities between the regulation of the *trpABD* cluster in pseudomonads and the entire *trp* operon in enteric bacteria should be emphasized, however, because of their implications for the understanding of natural evolutionary relationships between these groups.

ACKNOWLEDGMENT

This work was supported by National Science Foundation grant GB 6841.

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