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Regulation of Enzyme Synthesis in the Tryptophan Pathway of *Acinetobacter calcoaceticus*

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In *Acinetobacter calcoaceticus* the seven genes coding for the enzymes responsible for tryptophan synthesis map at three chromosomal locations. Two three-gene clusters, one (*trpGDC*) specifying the small subunit of anthranilate synthase, phosphoribosyl transferase, and indoleglycerol phosphate synthase and the other (*trpFBA*) specifying phosphoribosyl anthranilate isomerase and both tryptophan synthase subunits, are not linked to each other or to the *trpE* gene specifying the large anthranilate synthase subunit. When regulation of *trp* gene expression is studied in the wild type, only the level of the *trpF* gene product decreases upon addition of tryptophan to the medium. Tryptophan starvation of tryptophan auxotrophs, however, results in increased levels of all the tryptophan enzymes; this and additional evidence suggests that the expression of all the *trp* genes is subject to repression. The *trpGDC* genes are coordinately controlled, and the *trpE* gene is regulated in parallel with them. The *trpFBA* genes are controlled neither coordinately nor in parallel with the other *trp* genes, but respond proportionally when compared with each other. So far, two types of constitutive mutants have been found. The first class of mutants apparently occurs in the structural gene for a repressor protein; this repressor locus is unlinked to any of the biosynthetic *trp* genes and affects only the expression of *trpE* and the *trpGDC* cluster. The second class contains mutants closely linked to the *trpGDC* region; they overproduce only the gene products of this cluster.

The gene-enzyme relationships in the tryptophan pathway of *Acinetobacter calcoaceticus*, a gram-negative, oxidase-negative soil bacterium (4), are shown in Fig. 1. The genes for the seven polypeptides catalyzing these reactions are found at three locations, unlinked by transformation (37). Anthranilate synthase (EC 4.1.3.27; AS) is a multimer made up of two dissimilar subunits specified by the *trpE* gene and the *trpG* gene. Phosphoribosyltransferase (EC 2.4.2.18; PRT) is the gene product of the *trpD* locus; this enzyme does not aggregate with AS. Phosphoribosyl anthranilate isomerase (PRAI) and indoleglycerol phosphate synthase (EC 4.1.1.48; InGPS) are two separate polypeptides, their respective genes, *trpF* and *trpC*, being part of different gene clusters. Two classes of mutants deficient in tryptophan synthase (EC 4.2.1.20; TS) have been isolated, suggesting that, as in other bacteria, this enzyme is composed of α and β polypeptide chains. Recently, the *trp* gene disposition in the closely related oxidase-positive organism, *Moraxella osloensis*, has been reported to resemble that in *A. calcoaceticus* (P. Buckingham and E. Juni, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, K11, p. 148), but this particular chromosomal distribution of the *trp* genes has not been observed in any other major bacterial group (5).

A. calcoaceticus trpG mutants require both tryptophan and *p*-aminobenzoic acid for growth. Apparently, the G subunit of AS has a dual function, its activity being required for both tryptophan and folate synthesis. A similar situation occurs in *Bacillus subtilis* (21). *A. calcoaceticus* AS (38), like that of all other organisms studied, catalyzes both an amidotransferase reaction converting glutamine and chorismic acid to anthranilic acid and an aminotransferase reaction producing anthranilic acid from chorismic acid and ammonia. The EG complex and the E subunit are both subject to feedback inhibition by L-tryptophan (38, 44).

In view of the unusual diversity of regulatory mechanisms for the three *trp* gene clusters in *Pseudomonas* (4, 6, 29, 34), it was of interest to know how *Acinetobacter* regulates its *trp* genes. Published results of Twarog and Liggins (44) suggested that AS is the only enzyme of this pathway that is regulated in *A. calcoaceticus*. Subsequent demonstration that AS con-

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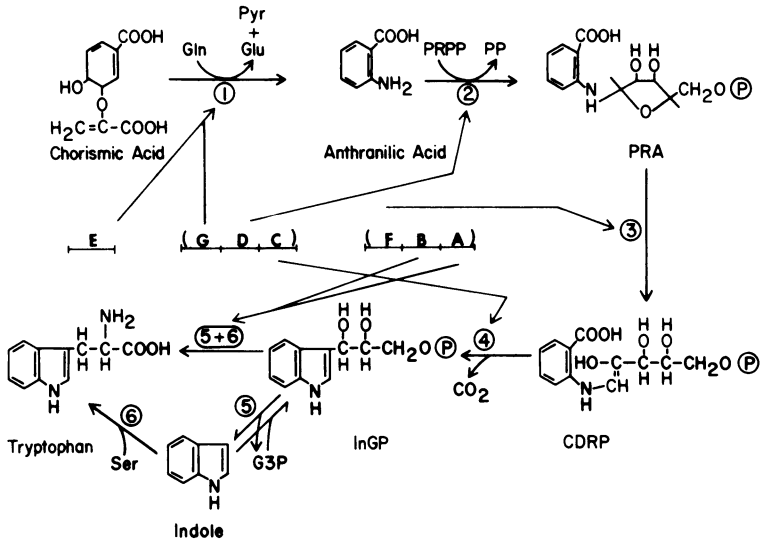

FIG. 1. *Gene-enzyme relationships in the tryptophan pathway of A. calcoaceticus. Enzymes: (1) AS; (2) PRT; (3) PRAI; (4) InGPS; (5) TS-A; (6) TS-B. The genes of the trp(GDC) cluster and the trp(FBA) cluster are represented in the most probable order. Abbreviations used: Gln, glutamine; Glu, glutamate; Pyr, pyruvate; PRPP, 1-phosphoribosyl-5-pyrophosphate; PP, inorganic pyrophosphate; PRA, N'-5-phosphoribosylanthranilate; CdRP, 1-(o-carboxyphenylamino)-1-deoxyribulose-5-phosphate; InGP, indole-3-glycerol phosphate; G3P, glyceraldehyde-3-phosphate; Ser, L-serine.*

sists of two polypeptides controlled by unlinked genes (37, 38) made it of interest to know whether both subunits are regulated in unison or whether *trpG*, in view of its dual usage, is normally either produced in excess or regulated multivalently by tryptophan and folate. Therefore, the regulation of the *trp* enzymes was reinvestigated. In contrast to the findings of Twarog and Liggins (44), we find that all the *trp* genes respond to nutritional perturbations. The regulatory pattern observed for this organism differs from that of other bacteria.

MATERIALS AND METHODS

Bacterial strains. All strains used in this study are derivatives of *A. calcoaceticus* BD413 (20), a microencapsulated prototroph. The sources or derivations of these strains are listed in Table 1. Stock cultures were kept frozen in 30% glycerol at −20 C.

Isolation of mutants. Auxotrophic mutations were induced with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) as described previously (37). *trpO*$_2$ mutants were obtained after localized mutagenesis essentially as described by others (12, 33). Strain *glt-43* (the *glt-43* marker is closely linked to the *trpGDC* cluster) was grown at 30 C with shaking in 10 ml of L-broth (23) to the early exponential growth phase. Cells were suspended in 3 ml of 0.1 M sodium citrate buffer, pH 5.0, containing NTG at a concentration of 1 mg/ml. Exposure to the mutagen was for 90 s. The cells were washed three times with cold citrate buffer before growth was continued for 4 h in prewarmed L-broth. The culture was washed and incubated overnight in minimal medium E (45) containing 0.4% L-malate. Appropriate dilutions were spread on plates containing 5-methylindole (130 μg/ml; 5MI) and anthranilate (10 μg/ml). Colonies resistant to 5MI were further characterized. *trpR* mutants were isolated as spontaneous mutants able to grow in the presence of 5-fluorotryptophan (100 or 200 μg/ml; 5FT) and shikimate (10 μg/ml).

DNA purification. Deoxyribonucleic acid (DNA) was prepared according to Sawula and Crawford (37). When a more highly purified preparation was desired, the following procedure (modified from reference 28) was used. A 200-ml overnight culture grown in minimal medium with the necessary growth supplements was centrifuged, and the cells were suspended in 7.5 ml of saline-ethylenediaminetetraacetic acid (28). Sodium dodecyl sulfate was added in 0.08 ml to a final concentration of 0.25%, and the cells were lysed by incubation for 30 min at 37 C. The lysate was mixed with 5 ml of 5 M sodium perchlorate and immediately shaken for 30 min with 18 ml of a mixture of chloroform-isoamyl alcohol (24:1). The emulsion was centrifuged to separate the layers. The aqueous layer was transferred to a beaker in which the nucleic acids were precipitated with 2 volumes of 95% ethanol and then dissolved in 2 ml of 0.15 M NaCl and 0.015 M sodium citrate (SSC), pH 7.0. After digestion with ribonuclease (50 μg/ml) for 30 min at 37 C, 2 ml of phenol, saturated with 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.5, was added and the mixture was shaken. The emulsion was centrifuged, and the aqueous layer was saved and dialyzed overnight against SSC before the ethanol precipitation was repeated. The DNA was dissolved in 2 ml of SSC and heated for 10 min at 55 C. The sterility of the

TABLE 1. *Derivation and properties of A. calcoaceticus strains used*

Genotype[a]	Phenotype[b]	Derivation or reference
BD413	Prototroph, wild type	Juni and Janik (20)
glt-43	Glutamine requiring	Sawula and Crawford (37)
trpE27	(ASN^-, ASG^-)	Sawula and Crawford (37)
trpG35	ASG^-	Sawula and Crawford (37)
trpD42	PRT^-	Sawula and Crawford (37)
trpC32	$InGPS^-$	Sawula and Crawford (37)
trpC41	InGPS bradytroph	Sawula and Crawford (37)
trpF20	$PRAI^-$	Sawula and Crawford (37)
trpB18	$TS\text{-}AB^-$	Sawula and Crawford (37)
trpA23	$TS\text{-}A^-$	Sawula and Crawford (37)
trpE27trpB62his-47	(ASN^-, ASG^-), $TS\text{-}AB^-$, His^-	*trpE27*, NTG mutagenized
trpE27trpA23	(ASN^-, ASG^-), $TS\text{-}A^-$	*trpA23* DNA × *trpE27trpB62his47*
trpB18aro-1	$TS\text{-}AB^-$, Aro^-	*trpB18*, NTG mutagenized
trpE27L-2	(ASN, ASG) bradytroph	*trpE27*, NTG mutagenized
trpG35L-4	ASG bradytroph	*trpG35*, NTG mutagenized
trpA23L-5	TS-A bradytroph	*trpA23*, NTG mutagenized
trpE27L-2aro-21	(ASN, ASG) bradytroph, Aro^-	*trpE27L-2*, NTG mutagenized
$trpO_2$*-18*	$5MI^r$	*glt-43*, NTG mutagenized
trpR39	$5FT^r$	Spontaneous, BD413 on 5FT
$trpO_2$*-18trpR18*	$5FT^r$	Spontaneous, $trpO_2$*-18* on 5FT
trpR18	$5FT^r$	$trpO_2$*-18trpR18* DNA × BD413 on 5FT

[a] Nonstandard genotype symbols: *trpE27L-2*, *trpG35L-4*, and *trpA23L-5*, bradytrophic (leaky) derivatives of *trpE27*, *trpG35*, and *trpA23*; $trpO_2$*-18*, operator-constitutive mutation for the *trpGDC* cluster.

[b] Nonstandard phenotype symbols: ASN^-, lacking the AS aminotransferase activity; ASG , lacking the AS amidotransferase activity; $TS\text{-}AB^-$, lacking both TS half-reactions; $TS\text{-}A^-$, lacking the first TS half-reaction; $5MI^r$, 5-methylindole resistant; $5FT^r$, 5-fluorotryptophan resistant.

DNA solution was tested by spreading 0.1 ml on an L-agar plate and incubating for 48 h.

Transformation. Cells were brought to competence and transformations were performed by the "plate method" as previously described (37). For linkage determinations, 0.1 ml of a suspension of competent cells in minimal medium was incubated with 0.1 ml of an appropriate dilution of purified DNA for 1 h at 30 C. To prevent further transformation, deoxyribonuclease I was added (final concentration, 200 μg/ml), and incubation was continued for another 30 min before spreading 0.05-ml aliquots on agar plates. $trpR^-$ colonies were identified by growth on minimal plates containing 5FT (200 μg/ml) and shikimate (10 μg/ml) and by indole-3-glycerol accumulation. $trpO_2^-$ colonies were identified by growth on minimal plates containing 5MI (130 μg/ml) and anthranilate (10 μg/ml).

Crude extracts. Unless otherwise mentioned, all strains were grown in minimal medium E (44) with 0.4% L-malate and supplements as required. In some instances, a medium containing one-half the concentration of the salts of S-2 medium (30) with 0.2% L-malate and 0.2% L-malic acid was used. Cells were grown at 30 C with shaking, harvested by centrifugation, washed with 0.1 M potassium phosphate buffer, pH 7.0, and resuspended in the same buffer containing 2 mM $MgCl_2$, 0.1 mM β-mercaptoethanol, and pyridoxal-5'-phosphate (8 μg/ml). Cells were disrupted by sonication, and cell debris was removed by centrifugation at 39,000 × *g* for 30 min. Equal parts of the supernatant were dialyzed overnight against the buffer used for sonication, with or without the addition of 30% (vol/vol) glycerol. Glycerol is important for stabilization of AS (38) and PRT, whereas pyridoxal-5'-phosphate helps to maintain TS-B activity.

Enzyme assays. Both the amidotransferase (ASG) and aminotransferase (ASN) activities of AS were determined as described (38), with the spectrofluorimeter adjusted so that 5 nmol of anthranilate gave a full-scale deflection. To estimate the amount of G subunit, ASG activity was determined after 150 s at room temperature in the presence of a threefold excess of added E subunits. PRT was assayed by a published method (17), but the reaction was carried out in a volume of 1 ml. PRAI was determined as described (6). InGPS was assayed by the method of Smith and Yanofsky (41), modified by using 40 mM potassium phosphate, pH 8.0, as buffer. The TS reactions were assayed according to Smith and Yanofsky (41), the first half-reaction (TS-A) with the addition to the reaction mixture of 2 M salt-free NH_2OH (8) and the second half-reaction (TS-B) by substituting 0.1 M potassium phosphate, pH 8.0, for the NaCl-supplemented tris(hydroxymethyl)aminomethane buffer used for *Escherichia coli*. One unit of activity in these assays is the disappearance of 1 nmol of substrate or the appearance of 1 nmol of product/min at 37 C. Specific activity is defined as units per milligram of protein, assayed by the method of Lowry et al. (25).

Preparation of free G and E subunits by column chromatography. Operations were performed at 0 to 4 C. A crude extract of mutant *trpR39* containing 30% (vol/vol) glycerol was concentrated by the addition of 4.72 g of ammonium sulfate per 10 ml of extract. The precipitate was suspended in 0.1 M

potassium phosphate buffer (pH 7.5) containing 10% (vol/vol) glycerol, 2 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid, and 10 mM β-mercaptoethanol and then dialyzed overnight against the same buffer. The preparation was equilibrated for 4 h in this buffer containing 0.5 M KCl (elution buffer) before it was applied to a Sephadex G-100 column. Fractions containing only G subunits were pooled and concentrated by the addition of 4.72 g of ammonium sulfate per 10 ml of extract and then dialyzed against 0.1 M potassium phosphate buffer (pH 7.0) containing 2 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid, 10 mM β-mercaptoethanol, and 30% (vol/vol) glycerol. For complete separation of E subunits from traces of G subunits, the gel filtration on Sephadex G-100 was repeated. Fractions containing only E subunit were pooled and concentrated as described for free G subunit.

Derepression experiments. Five liters of strain *trpA23L-5* in minimal-L-malate medium supplemented with 20 μg of L-tryptophan per ml was harvested in the mid-exponential growth phase, washed with cold minimal medium, and resuspended in 10 liters of prewarmed minimal medium containing 0.4% L-malate but lacking tryptophan. After 30 min, successive flasks containing 1 liter of culture were removed at intervals for 5 h, 100 mg of chloramphenicol was added, and the culture was chilled in an ice-water bath for 5 min before the cells were centrifuged and washed in 0.1 M potassium phosphate, pH 7.0. Crude extracts were then prepared as usual.

Chemicals. 3-β-Indoleacrylic acid, 2MI, 3MI, 5MI, 7MI, 5-fluoroindole, DL-4-fluorotryptophan, 5FT, and DL-5-methyltryptophan (5MT) were purchased from Sigma Chemical Co. DL-5-Hydroxytryptophan was obtained from Nutritional Biochemicals Corp. K & K Laboratories was the source of 3-methylanthranilic acid (3MA) and 5-methylanthranilic acid (5MA). Chorismic acid (10), 1-(*o*-carboxyphenylamino)1 - deoxyribulose - 5 - phosphate (CdRP; 41), and InGP (46) were prepared as described.

RESULTS

Enzyme levels in wild type and in *trp* auxotrophs. Wild-type *A. calcoaceticus* BD413 and seven tryptophan auxotrophs were examined for their regulatory response to exogenously supplied L-tryptophan. Table 2 presents the results of enzymatic analyses of dialyzed extracts of these strains after growth in limiting and excess tryptophan. For simplicity, all activities are normalized to that of wild-type cells grown on excess tryptophan, shown in the first line. In the wild type, only PRAI activity varies significantly in response to exogenous tryptophan. The mutants each lack one enzymatic activity, except for *trpB18*, which lacks both the A and B activities of TS. In preliminary experiments, when these mutants were directly starved for tryptophan, i.e., when cultures were given 2 μg of L-tryptophan per ml and harvested 2 h after growth ceased, only small increases in PRT, InGPS, and PRAI were found, and ASG, ASN, TS-B and TS-A levels remain unchanged (data not shown). The somewhat better derepression results recorded in Table 2 were obtained after the auxotrophs had been subjected to several sequential episodes of tryptophan limitation. This was achieved by cultivating in limiting tryptophan (initial concentration, 1.5 μg/ml) until the growth rate declined in comparison with a control culture containing excess tryptophan. Growth was then periodically reinitiated during the next several hours by adding small amounts (up to a total of 0.5 μg/ml) of L-trypto-

TABLE 2. *Tryptophan enzyme levels in various A. calcoaceticus strains*

Strain	L-Tryptophan (μg/ml)	Sp act (nmol/min per mg)						
		ASN	ASG	PRT	InGPS	PRAI	TS-B	TS-A
BD413	50	2.1	2.6	0.9	5.0	3.7	2.6	2.3
BD413	50	1.0[a]	1.0	1.0	1.0	1.0	1.0	1.0
	0	1.1	1.0	0.8	0.9	1.8	1.0	1.0
trpE27	50	0.0	0.6[b]	1.0	1.3	1.0	0.7	1.7
	2	0.0	1.2[b]	3.5	3.3	2.2	1.1	2.5
trpG35	50	0.4	0.0	1.3	1.4	1.1	0.8	1.8
	2	0.5	0.0	4.2	2.7	2.4	1.2	2.2
trpD42	50	0.5	0.7	0.0	1.4	1.1	0.7	1.7
	2	0.7	1.3	0.0	2.8	2.9	1.1	2.0
trpC32	50	0.4	0.7	1.0	0.0	1.4	0.8	1.3
	2	0.6	1.1	3.5	0.0	4.0	1.4	2.8
trpF20	50	0.5	0.8	0.9	1.3	0.0	0.6	0.9
	2	1.0	1.6	4.2	3.2	0.0	1.3	1.4
trpB18	50	0.6	0.8	0.8	1.3	0.9	0.0	0.0
	2	1.0	1.5	3.7	4.2	4.3	0.0	0.0
trpA23	50	0.6	0.9	0.8	1.6	0.8	0.8	0.0
	2	1.0	1.2	3.9	6.0	3.7	1.4	0.0

[a] Specific activity normalized to BD413 grown in the presence of 50 μg of L-tryptophan per ml.
[b] Specific ASG activity, assayed in the presence of added active E subunit (see Materials and Methods).

phan at frequent intervals. Under these circumstances all enzyme levels increased in response to tryptophan deprivation, but to differing degrees. The increases found ranged from less than two- to fourfold more than the levels found in control cultures with 50 μg of L-tryptophan per ml. We conclude that regulation of *trp* gene expression in this organism is rather sluggish, that several consecutive periods of starvation are better than a single one in revealing it, and that wild-type cells maintain an internal tryptophan concentration sufficient to keep all the enzymes except PRAI at their lowest levels.

Enzyme levels in bradytrophic mutants. We next examined several leaky mutants grown under conditions of tryptophan semistarvation, where cells were harvested several hours after the exogenously supplied tryptophan (2 μg/ml) had disappeared from the medium and the growth rate had slowed. Strains *trpE27L-2*, *trpG35L-4*, and *trpA23L-5* are bradytrophic revertants of *trpE27*, *trpG35*, and *trpA23*, respectively, whereas *trpC41* was leaky as originally isolated. The reversion event leading to strains *trpE37L-2*, *trpG35L-4*, and *trpA231-5* occurred in the same region as the original mutation, as determined by co-transformation. In Table 3 the influence of tryptophan excess and deprivation on these bradytrophic mutants is presented. The increase over the basal level for ASN, ASG, PRT, and InGPS ranges from 6.5- to 16-fold and for PRAI up to 6-fold, whereas TS-A and TS-B activities are augmented only 2- to 3-fold. Thus, derepression occurs to some extent for all seven polypeptides. The response of the *trpE* gene and the *trpGDC* cluster seems significantly greater than that of the *trpFAB* cluster; note that in strain *trpG35L-4*, in contrast to mutant *trpG35*, the ASN activity catalyzed by the *trpE* gene product responds to tryptophan scarcity.

In the *trpFAB* cluster, it is clear that the synthesis of the *trpF* gene product can be derepressed to a greater extent than the synthesis of the two TS subunits. It seems unlikely that a metabolic intermediate of the tryptophan pathway between anthranilate and indole induces the formation of any of the enzymes, for mutants lacking many different enzymatic activities respond similarly to tryptophan limitation.

Derepression kinetics of *trpA23L-50*. *trpA23L-5* was grown in excess tryptophan and then resuspended in medium lacking it. Samples were removed at intervals for 5.5 h. The specific activity of each enzyme was plotted against that of the G subunit of AS according to the method of Ames and Garry (1) (Fig. 2). From the straight lines obtained, G subunit, PRT, and InGPS formation appear to be coordinately controlled. The ratio of ASN to G activity also remains constant, but the fact that G activity is invariably higher than ASG activity shows that excess G subunits occur in the cell. Since the E and G subunits are not found in equimolar amounts, they appear to be controlled in parallel. PRAI and TS-B are controlled neither coordinately nor in parallel with the G subunit. When PRAI activity is plotted against TS-B activity, a straight line is obtained that fails to pass through the origin (suggesting a higher constitutive basal level of TS-B). When we take into consideration that the total increase found for TS-B activity is less than twofold and that there is some error in each enzyme assay, however, it seems unwise to form conclusions about PRAI and TS regulation without the benefit of regulatory mutants specific for the *trpFBA* cluster.

Selective conditions involving tryptophan analogues. To search for regulatory mutations, various analogues of tryptophan and its pathway intermediates were examined for growth-inhibitory effects upon wild-type *A. calcoaceticus* (Table 4). Except for DL-5-hydroxytryptophan, 2MI, and 3-β-indoleacrylic acid, all indole and tryptophan analogues tried are potent

TABLE 3. *Tryptophan enzyme levels of bradytrophic A. calcoaceticus strains*

Strain	L-Tryptophan (μg/ml)	Sp act[a]						
		ASN	ASG	PRT	InGPS	PRAI	TS-B	TS-A
trpE27L-2	50	<0.01	<0.01	1.0	0.8	0.8	0.7	ND
	2	<0.03	<0.06	7.0	6.4	2.3	1.1	ND
trpG35L-4	50	0.4	<0.01	1.1	1.2	0.6	0.6	0.6
	2	2.9	<0.01	10.8	7.8	2.5	1.0	0.9
trpC41	50	0.7	1.1	1.0	<0.01	2.0	0.7	0.5
	2	4.1	6.5	14.8	<0.01	4.9	1.5	0.9
trpA23L-5	50	0.6	0.8	1.2	1.1	1.0	1.4	<0.02
	2	3.9	6.7	16.4	9.0	5.8	3.0	<0.05

[a] Specific activity normalized to BD413 grown in the presence of 50 μg of L-tryptophan per ml (values are given in Table 2). ND, Not determined.

inhibitors. When wild-type cells are spread on minimal agar and crystals of 7MI, 5MI, or 5-fluoroindole are placed in the center of the plates, the colonies at the periphery of the zone of inhibition turn blue. This blue pigment, which is also observed when growth occurs in the presence of excess indole, is insoluble in water and is likely to be indigo, known to be formed from indole by certain pseudomonads (11). At extremely high concentrations of indole (300 μg/ml), growth of the wild type is inhibited. This indole toxicity affects *trpB* mutants also; no growth occurs on plates containing 300 μg of indole and 10 μg of tryptophan per ml. Therefore, high concentrations of indole analogues might cause growth inhibition in several ways.

At concentrations of 300 μg/ml, 5MA and 3MA exert only moderate growth inhibition, but in the presence of these compounds a purple, diffusable pigment is excreted. At 42 C, less pigment is formed and growth inhibition is more marked. In the presence of citrate, anthranilate can be used as a second carbon source. This implies a degradative pathway for anthranilate and its analogues, which could at least partially account for the relatively weak inhibitory effect.

TABLE 4. *Growth response of A. calcoaceticus to various analogues*

Analogue	Growth inhibition[a]	Minimal concn[b] inhibiting growth (μg/ml)
3-Methylanthranilic acid	+ (P)	>300
5-Methylanthranilic acid	+ (P)	>300
2-Methylindole	0	ND
3-Methylindole	+	>200
5-Methylindole	+ (B)	100
7-Methylindole	+ (B)	ND
5-Fluoroindole	+ (B)	100
3-β-Indoleacrylic acid	(±)	ND
DL-5-Methyltryptophan	+	50
DL-4-Fluorotryptophan	+	50
DL-5-Fluorotryptophan	+	50
DL-5-Hydroxytryptophan	0	ND

[a] An inoculum of 0.1 ml of an overnight culture of BD413 grown on minimal medium was spread on minimal agar, and the plate was allowed to dry before crystals of analogues were placed in the center. Absence of growth in zones around the crystals after 48 h indicated growth inhibition (+). Abbreviations: (P), a purple pigment is excreted by cells growing at the periphery of the inhibition zone; (B), cells growing at the periphery of the inhibition zone turn blue.

[b] A similar inoculum of BD413 was spread on minimal plates containing analogues at various concentrations (50, 100, 150, 200, and 300 μg/ml for all analogues with the exception of 3-methylindole, which was supplied at 200 μg/ml as its lowest concentration). Concentrations listed inhibited growth for at least 48 h. ND, Not determined.

Growth properties of *trpR* mutants. *trpR*$^-$ mutants of *A. calcoaceticus* were isolated by selecting for resistant variants on plates containing both 5FT and shikimic acid. Shikimic acid and, to a somewhat lesser extent, anthranilic acid were found to stimulate the growth of *trpR*$^-$ strains on media containing either 5FT or 5MI (Table 5). (On 5MI-supplemented plates blue colonies are formed.) Growth inhibition by 5MI at concentrations of 130 μg/ml is probably due to its conversion to 5MT. In the presence of 4-fluorotryptophan, all *trpR*$^-$ strains failed to grow, suggesting different modes of action for tryptophan analogues substituted in the 4- and 5-positions. *trpR*$^-$ strains are more sensitive than wild type to inhibition by 5MA and excrete less of the purple pigment. When grown on minimal medium, *trpR*$^-$ mutants accumulate indole-3-glycerol in the medium. For further characterization of the *trpR*$^-$ mutant class, experiments were done with a representative strain, *trpR39*.

Tryptophan synthetic enzymes in *trpR39*. Mutant *trpR39* shows derepressed levels of ASN, ASG, PRT, and InGPS, whether grown in the absence or presence of exogenous tryptophan (Table 6). These increased specific activities are comparable to those found in leaky mutants grown under tryptophan limitation. In contrast, the production of PRAI and TS is not influenced by this mutation. As in the wild type, PRAI formation can be repressed by exogenous tryptophan. This indicates that tryptophan uptake is not greatly diminished by the *trpR39* mutation.

Genetic studies with *trpR39*. The *trpR39* mutation affects the expression of the *trpE*, *-G*, *-D*, and *-C* genes. These genes map at two chromosomal locations. Reversion and transformation experiments were performed to be certain that strain *trpR39* is the result of a single mutational event. Wild type can be transformed with *trpR39* DNA to 5FT resistance at frequencies within the range found for single-marker transfer. The 5FT-resistant progeny accumulate indole-3-glycerol when grown on minimal medium and acquire, along with resistance to 5MI and 5MT, an increased sensitivity to 5MA. Table 7 presents results observed with one of our transformants. Transformant *trpR39T1* is constitutively derepressed for the *trpE*, *-G*, *-D*, and *-C* gene products. Thus, by transfer of a single marker, a genotype with altered expression of both the *trpE* and the *trpGDC* clusters was obtained.

TABLE 5. *Growth of analogue-resistant regulatory mutants of A. calcoaceticus*

Strain	Growth[a] on minimal medium supplemented with (μg/ml):[b]										Accumulation[c]
	–	5MT (100)	5 FT (200)	5FT+ SA (200 + 10)	5FT+ AA (200 + 10)	4FT (100)	5MI (130)	5MI+SA (130 + 10)	5MI+AA (130 + 10)	5MA (300)	
BD413	++++	0	0	0	0	0	0	0	0	+++(P)	0
trpR39	++++	+	+	+++	++	tr[d]	tr	++(B)	++(B)	+(P)	InG
trpR18	++++	++	+	+++	+++	0[d]	0	+++(B)	++(B)	+(P)	InG
trpO$_2$-18	++++	0	0	0	0	0	+(W)	++(W)	+++(W)	+(P)	0
trpO$_2$-18trpR18	++++	++	+	+++	+++	0[d]	+(W)	+++(W)	+++(W)	+(P)	InG

[a] Graded + to ++++ by colony size after 48 h at 30 C; tr, trace; (B), colonies turn blue; (W), colonies stay white; (P), purple pigment excreted by colonies.

[b] Abbreviations for medium additions: 5MT, DL-5-methyltryptophan; 5FT, DL-5-fluorotryptophan; 4FT, DL-4-fluorotryptophan; 5MI, 5-methylindole; 5MA, 5-methylanthranilic acid; SA, shikimic acid; AA, anthranilic acid.

[c] Accumulation tests for anthranilic acid, 1-(*o*-carboxyphenylamino)-1-deoxyribulose, indole-3-glycerol (InG), and indole were performed as previously described (37). Cultures were grown in minimal medium for 18 h at 30 C.

[d] Growth was not enhanced by the addition of shikimate (10 μg/ml) or anthranilate (10 μg/ml) to the medium.

TABLE 6. *Enzymatic survey of analogue-resistant mutants of A. calcoaceticus*

Strain	L-Tryptophan (μg/ml)	Sp act (nmol/min per mg)							
		ASN	ASG	G activity[a]	PRT	InGPS	PRAI	TS-B	TS-A
BD413	50	1.5	2.1	2.6	0.9	4.6	3.9	2.5	3.3
BD413	50	1.0[b]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	0	1.0	0.9	1.1	1.2	1.1	2.1	0.9	1.0
trpR39	50	4.7	7.5	ND	11.8	6.1	1.9	0.8	0.7
	0	5.3	6.8	ND	10.3	5.7	3.1	0.9	0.8
trpO$_2$-18	50	0.6	1.2	3.1	3.0	3.0	1.0	0.7	1.0
	0	0.8	1.0	3.0	3.0	2.9	2.2	0.4	0.8
trpR18	50	3.6	5.5	5.7	12.9	7.0	1.3	1.0	0.7
	0	4.4	6.1	6.3	13.5	7.2	1.8	1.0	0.8
trpR18trpO$_2$-18	50	3.5	6.2	13.9	17.3	12.9	1.3	1.2	0.9
	0	3.4	6.8	14.6	19.8	13.0	2.1	1.4	1.1

[a] G activity is the ASG activity assayed in the presence of the threefold excess of free E subunit. ND, Not determined.

[b] Specific activity normalized to BD413 grown in the presence of 50 μg of L-tryptophan per ml.

Growth of mutant *trpR39* is inhibited by 5MA for at least 72 h at 42 C (Table 7). Therefore, the few colonies appearing after 24 h on 5MA plates were presumed to be revertants of *trpR39* to wild type. Ten such colonies were further characterized. None of them grew on 5FT or excreted indole-3-glycerol. Enzyme levels found with the revertants *trpR39R-3* and *trpR39R-4* are presented in Table 7. Both strains show levels of ASN, ASG, PRT, and InGPS comparable to those found in wild type but different from *trpR39*. Therefore, it appears that *trpR39* reverts in a single step and is not a deletion. The reversion frequency is enhanced by NTG but not by ICR-191. This implies that *trpR39* is a point mutation. Using DNA from *trpR39*, no co-transformation was obtained with any of five tryptophan auxotrophic markers, *trpE27*, *trpG35*, *trpD42*, *trpB18*, and *trpA23* (100 colonies were tested in each case). Since the *trpR39* marker is not linked to the *trpE*, *-G*, *-D*, *-B*, or *-A* genes, there is no linkage to any of the three *trp* gene clusters.

Isolation of *trpO$_2$* mutants. The results presented so far suggest the hypothesis that the *trpGDC* cluster constitutes a single regulatory unit, i.e., an operon. Further support for this hypothesis could be obtained from mutations in an operator region, which we will designate *trpO$_2$*; these mutations should be linked to the *trpGDC* cluster and overproduce only the gene products of this cluster. NTG is known to induce closely spaced mutations (12, 33). We attempted to mutagenize this operator region by selecting NTG-induced revertants of *glt-43*, for

TABLE 7. *Properties of derivatives of strain trpR39*

Strain	Growth on			InG excretion	Enzyme levels[d]						
	5MA[a] (30 C)	5MA[b] (42 C)	5FT[c] (30 C)		ASN	ASG	PRT	InGPS	PRAI	TS-B	TS-A
BD413	+++	+++	0	0	0.7	0.9	1.1	1.5	2.2	1.0	0.6
trpR39	+	0	+++	+	6.3[e]	7.0	11.3	8.5	2.1	1.1	0.7
trpR39R-3	+++	+++	0	0	0.6[e]	0.3	1.2	1.3	2.1	0.9	0.6
trpR39R-4	+++	+++	0	0	0.5[e]	1.0	1.3	1.5	2.1	1.0	0.6
trpR39T-1	+	0	+++	+	3.9[e]	5.4	10.18	5.6	1.8	ND	ND
trpR39T-1					4.1[f]	4.6	8.6	6.2	1.2	ND	ND

[a] Growth at 30 C on plates of minimal medium containing 300 μg of 5MA per ml. Amount of growth estimated by comparison with BD413 growing on minimal medium without analogue (++++).

[b] Growth at 42 C on plates of minimal medium containing 300 μg of 5MA per ml. Amount of growth estimated by comparison with BD413 growing on minimal medium without analogue (++++).

[c] Growth at 30 C on plates of minimal medium containing 200 μg of 5FT per ml and 10 μg of shikimate per ml. Amount of growth estimated by comparison with BD413 growing on minimal medium without analogue.

[d] Specific activities normalized to BD413 grown in the presence of 50 μg of L-tryptophan per ml. ND, Not determined.

[e] Strain was grown in minimal medium.

[f] Strain was grown in minimal medium supplemented with 50 μg of L-tryptophan per ml.

this marker is closely linked to the *trpGDC* cluster (36). We selected these regulatory mutants on a medium containing 5MI (130 μg/ml) and anthranilate (10 μg/ml). ($trpO_2^-$ mutants would be expected to produce increased amounts of InGP if anthranilate were supplied; higher internal concentrations of InGP could compete with 5MI for TS-mediated conversion to tryptophan or 5MT, respectively.) This procedure yielded many 5MI-resistant mutants able to accumulate indole-3-glycerol when anthranilate was added to minimal medium. Most of the further characterization was performed with one of these, $trpO_2$-*18*.

Characterization of *trpO*$_2$*-18*. A strain containing this mutation grows poorly on 5MI-supplemented medium, but growth can be stimulated by the addition of shikimate, or even better by anthranilate (Table 5). In contrast to $trpR^-$ mutants, colonies grown on 5MI remain white during long periods of incubation, and $trpO_2$-*18* does not confer cross-resistance to any other tryptophan analogues. When grown in minimal medium, no pathway intermediates accumulate. As with $trpR^-$ mutants, $trpO_2$-*18* manifests increased sensitivity to 5MA. The $trpO_2$-*18* marker can be transformed as a single marker and is linked to the *trpGDC* cluster. $trpO_2$-*18* was co-transformed at frequencies of 70 and 67% with *trpG35* and *trpD42* (100 colonies tested in each case). The enzyme levels evoked by $trpO_2$-*18* are presented in Table 6. The first line of this table gives the specific activities of wild type grown in excess tryptophan. As with mutant *trpA23L-5*, the levels of ASG activity are enhanced when assayed in the presence of added E subunit. This indicates that free G subunit occurs in wild-type cells. An increase of available G subunit does not cause further stimulation of the ASG activity, suggesting that in wild type all of the E subunit is saturated with G subunit. In $trpO_2$-*18*, the enzymes specified by the *trpGDC* cluster are constitutive and are overproduced about threefold when compared with wild type. The expression of the remaining *trp* genes is not affected.

Synergism between *trpO*$_2$*-18* and *trpR18*. Both $trpR^-$ and $trpO_2^-$ mutations render the *trpGDC* gene cluster constitutive and derepressed. Double mutants of the $trpO_2^-trpR^-$ type were isolated by selecting for 5FT-resistant derivatives of $trpO_2$-*18*; DNA from the double mutants was used to transform the 5FT resistance character back into the wild-type background. $trpO_2$-*18trpR18* is one such double mutant, and *trpR18* was obtained from it by transformation. *trpR18* is very similar to *trpR39* in its growth properties (Table 6) and enzyme levels. Reversion and transformation studies indicate that *trpR18* is a point mutation. The double mutant $trpO_2$-*18trpR18* excretes indole-3-glycerol when grown on minimal medium, is cross-resistant to indole and tryptophan analogues substituted in the 5-position, and is very sensitive to 5MA. Growth on 5MI-containing plates does not yield blue colonies, so in this property the double mutant is like strain $trpO_2$-*18* rather than *trpR18* (Table 5). The enzymes specified by the *trpGDC* cluster are overproduced 1.5- to 2-fold when com-

pared with *trpR18*. The ratio of the ASG activity assayed with or without excess E subunit clearly demonstrates this additional derepression of the *trpG* product. Therefore, the *trpO$_2$-18* and the *trpR18* mutations are synergistic and not epistatic; that is, the *trpR18* mutation does not entirely suppress the action of the *trpO$_2$-18* mutation.

Lack of induction by pathway intermediates. The enzymes of the tryptophan pathway in *A. calcoaceticus* are not all regulated in unison. Non-coordinate regulation has also been observed in the tryptophan pathway of *Pseudomonas putida* and *Pseudomonas aeruginosa*; in both pseudomonads TS is induced by the pathway intermediate InGP (4, 6). To decide whether the expression of any of the *Acinetobacter trp* genes is regulated similarly, a double mutant, *trpE27trpA23*, was constructed. Since this strain lacks ASG, ASN, and TS-A activities, no pathway intermediates between anthranilate and InGP are synthesized unless anthranilate is supplied. When small amounts of anthranilate (3 to 5 μg/ml) are repeatedly added to the medium, formation within the cell of PRA, CdRP, and InGP occurs in sequence, as determined by indole-3-glycerol excretion. (In contrast, no indole-3-glycerol accumulation was detected when anthranilate was added to the medium at an initial concentration of 30 μg/ml, despite the fact that complete disappearance of anthranilate was observed. It seems likely that anthranilate concentrations of this magnitude induce the anthranilate degradative pathway, although degradative enzyme assays were not done to confirm this). Strain *trpE27trpA23* is very sensitive to alkaline pH and lyses in Vogel and Bonner medium. Therefore, *trpE27trpA23* cultures were grown in S-2 medium containing either excess or growth-limiting amounts of tryptophan, with or without added anthranilate. The results of several experiments with mutant *trpE27trpA23* are presented in Table 8. The specific activities are normalized to those of an extract obtained after growth in S-2 medium with 50 μg of tryptophan per ml (first line). The specific activities found for PRT, InGPS, PRAI, and TS are very similar to those found in extracts of wild type grown in Vogel and Bonner medium containing 50 μg of tryptophan per ml. Tryptophan starvation causes derepression of all tryptophan enzyme activities, whether anthranilate is added or not. In the presence of 50 μg of L-tryptophan per ml, repression of all the tryptophan enzymes occurs even when anthranilate is supplied. If one of the intermediates from anthranilate to InGP functioned as an inducer, there should be at least partial relief of the tryptophan repressive effect. Growth of mutant *trpA23* in medium with limiting (3 μg/ml) or excess (50 μg/ml) indole results in tryptophan enzyme profiles (not presented) similar to those obtained when the same strain is subjected to tryptophan scarcity or excess (Table 2). Since excess indole fails to stimulate tryptophan enzyme synthesis, this intermediate also appears not to function as an inducer.

The influence of exogenous tryptophan on enzyme levels in the tryptophan synthetic pathway was also studied in mutants blocked in the common aromatic pathway and therefore unable to synthesize chorismate. This experiment was an attempt to determine whether chorismate plays a role in the regulation of *trp* enzyme synthesis. The results are given in Table 9. With an Aro$^-$ block introduced in mutant *trpB18*, derepression of the *E*, *G*, *D*, and *C* gene products occurred under conditions of tryptophan starvation. PRAI, however, was not stimulated to its normal extent. The results obtained with mutant *trpE27L-2aro-21* confirm that all the *trp* enzymes respond only to tryptophan excess or scarcity. Since none of the pathway intermediates from chorismate to indole was found to exert an inducing effect upon the formation of any of the tryptophan enzymes, it appears that *trp* gene expression in *A. calcoace-*

TABLE 8. *Enzyme levels of strain trpE27trpA23 under various growth conditions*

Supplement (μg/ml)		InG accumulation (nmol/ml)	Sp act (nmol/min per mg)						
Tryptophan	Anthranilate		ASN	ASG	PRT	InGPS	PRAI	TS-B	TS-A
50	0		<0.01	<0.01	0.9	4.9	3.6	1.8	<0.01
50	0	0	–[a]	–	1.0[b]	1.0	1.0	1.0	–
50	18	21.9	–	–	1.0	0.9	1.2	1.0	–
2	0	0	–	–	2.2	2.2	2.9	2.0	–
2	18	78.9	–	–	4.0	3.8	3.8	2.5	–

[a] –, No detectable activity.

[b] Specific activity normalized to that in *trpE27trpA23* extracts grown in the presence of 50 μg of L-tryptophan per ml.

TABLE 9. *Enzyme levels of trp⁻aro⁻ strains*

Strain	Tryptophan supplement[a] (μg/ml)	Sp act[b]						
		ASN	ASG	PRT	InGPS	PRAI	TS-B	TS-A
trpB18aro-1	50	0.5	0.8	0.8	1.0	0.8	<0.01	<0.01
	3	2.5	3.6	5.2	3.9	1.1	<0.01	<0.01
trpE27L-2aro-21	50	<0.1	ND	1.1	1.2	1.3	1.0	1.4
	3	0.1	ND	9.7	4.5	2.1	1.9	2.0

[a] Other growth supplements: 25 μg of L-phenylalanine, 25 μg of L-tyrosine, 0.5 μg of vitamin K, 0.5 μg of 2,3-dihydroxybenzoate, 0.5 μg of *p*-hydroxybenzoate, and 2 μg of *p*-aminobenzoic acid per ml.

[b] Specific activity normalized to that of a BD413 extract grown in the presence of 50 μg of L-tryptophan per ml. ND, Not determined.

ticus is regulated solely by tryptophan repression.

DISCUSSION

In contrast to the results of Twarog and Liggins (44), we found that synthesis of all the *Acinetobacter* tryptophan pathway enzymes responds to the cell's tryptophan level. Some precautions for enzyme stabilization, increased sensitivity of assay methods, and attention to techniques of tryptophan deprivation may all have contributed to the difference in our results. For some gene products it seemed essential that auxotrophs be allowed to grow a bit under conditions of tryptophan scarcity to observe derepression. We circumvented this problem either by utilizing tryptophan bradytrophs growing in the absence of tryptophan or by feeding auxotrophs small increments of tryptophan over an extended period of semistarvation. Probably growth in a chemostat would be equivalent to or better than the latter strategem.

Although *trpE* and the *trpGDC* cluster map at separate chromosomal locations, a common regulatory mechanism seems to be imposed on their expression. This mechanism differs from that governing the formation of the *trpFBA* gene products. Mutations in a regulatory gene designated *trpR*, affecting both *trpE* and *trpGDC* gene expression, can be selected by their resistance to 5FT. As the AS in these *trpR*⁻ strains is still feedback inhibitable by tryptophan (unpublished data) and PRAI and TS levels are normal, these mutants do not excrete large amounts of tryptophan. The regulatory effect exerted at the *trpE* and *trpGDC* operators by the unlinked *trpR* gene is probably due to a cytoplasmic factor. In analogy to the situation in *E. coli* and *Salmonella typhimurium* (27, 32, 39, 43), we suspect that the *trpR* gene codes for a repressor protein acting at two operator sites. These four genes, *trpE*, *-G*, *-D*, and *-C*, would then constitute a regulon, as found in arginine biosynthesis in *E. coli* (26).

The synthesis of the *trpG*, *-D*, and *-C* gene products appears to be coordinate (Fig. 2), even though the level of the *trpD* product sometimes exceeds the other two under conditions of tryptophan limitation (Tables 3, 6, 7, and 9). These differences are reproducible, and discrepancies of this magnitude involving coordinate enzymes during starvation and semistarvation have also been recorded for *E. coli* (5, 17), *Pseudomonas* (4, 35), and *Bacillus* (16). Further evidence that these three genes form a single unit of expression comes from the fact that $trpO_2$ mutations, linked to the *trpGDC* cluster, result in constitutivity for the three polypeptides specified by this cluster. Although the nature of the $trpO_2$ mutations was not completely analyzed, it is likely that they occur in a control region analogous to that of the *trp* operon in *E. coli* (3, 15). Thus, promoter, operator, or attenuator alterations might account for the increased levels of proteins specified by the *trpGDC* cluster. Compared with the enzyme levels found in *trpR*⁻ mutants, the *trpGDC* gene products seem only partially derepressed by $trpO_2^-$ mutations. Perhaps these mutants produce sufficient tryptophan to maintain the *trpGDC* operon at a slightly repressed level even when no exogenous tryptophan is added. Further derepression would then be expected when a *trpR*⁻ mutation is introduced into a $trpO_2^-$ strain. Results obtained with the double mutant $trpO_2$-*18trpR18* show that, in combination, these two mutations are synergistic and not epistatic. This favors to some extent the possibility that the $trpO_2$-*18* mutation affects the function of the promotor or a hypothetical attenuator of the *trpGDC* operon. However, no firm conclusion on the nature of the $trpO_2$-*18* mutation can be reached.

The regulation of the *trpF*, *-B*, and *-A* genes obviously differs from that of the other *trp* genes. When wild type is shifted from minimal medium without tryptophan to tryptophan excess, only the *trpF* gene responds by a decreased level of expression. Apparently *trpF* is

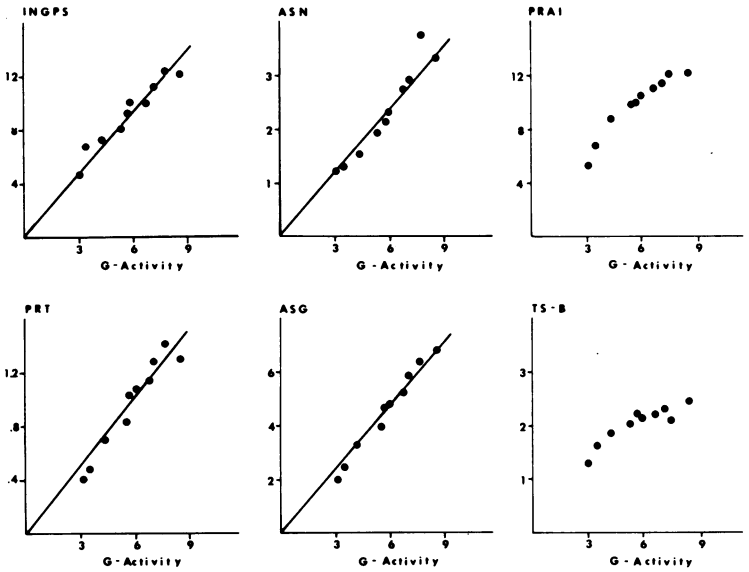

FIG. 2. *Derepression kinetics of strain trpA23L-5. At different times during derepression by tryptophan deprivation, cells were harvested and assayed for the activities of the tryptophan pathway. G activity is ASG activity assayed in the presence of an excess of E subunit. Other abbreviations are those used in Fig. 1 or explained in Materials and Methods.*

the only *trp* gene that is not kept at its maximally repressed level during growth of the wild type in our minimal medium. When a tryptophan auxotroph is starved for tryptophan, the *trpF* gene product increases up to sixfold, whereas the expression of the *trpA* and *trpB* genes is augmented only about threefold. This may imply a regulatory difference between the control of the *trpF* and the *trpA* and *-B* genes, although the *trpD* gene product often seems to show greater regulatory responsiveness than the other members of its regulon. There is some evidence suggesting a regulatory element in common for the *trpFBA* genes, since a straight line (which fails to pass through the origin) is obtained when TS and PRAI activities from a derepression experiment are plotted according to Ames and Garry (1). We must also consider other possibilities in this case, however, such as the occurrence of an internal promotor (2, 18, 31) or bidirectional transcription involving operators with different repressor binding capacities (9, 19, 22). Among other 4FT-resistant mutants, one was isolated that failed to repress PRAI formation in the presence of exogenous tryptophan; the remaining, unaffected enzymes of the pathway behaved as in wild type (data not shown). This mutation could not be cotransformed with any of the *trp* structural genes; since it appeared unlikely that failure to repress PRAI could cause resistance to 4FT, we suspected that this strain contained multiple mutations and did not analyze it further.

The formation of the *trpFBA* gene products does appear to be regulated by repression. In *Pseudomonas*, in contast, *trpF* gene expression is constitutive, whereas TS production is induced by the pathway intermediate InGP (4, 6, 34). The differences seen between pseudomonads and *A. calcoaceticus* may reflect, in part, the different chromosomal organization of the *trp* genes, since in *P. putida* and *P. aeruginosa* the *trpF* gene and the *trpAB* pair are found at different chromosomal sites (4, 13). However, in spite of a different chromosomal organization, a similar regulatory mechanism for the *trpEGDC* group of genes appears to prevail in both *Pseudomonas* and *Acinetobacter*. Moreover, for all bacteria known to regulate their *trpEGCD* genes, the available data suggest a mechanism like that for the *trp* operon of *E. coli* (5). For instance, in *B. subtilis* the *trpG* gene is not linked to the tryptophan operon (containing all the remaining *trp* genes), but all the tryptophan genes respond to tryptophan repression and are controlled by the *mtr* locus (16, 21). It is noteworthy that in *B. subtilis*, as in *A. calcoaceticus*, mutations in the *trpG* gene confer a requirement for both tryptophan and *p*-aminobenzoic acid (21). In *B. subtilis* the *trpG* gene is not part of the *trp* operon, whereas in *A. calcoaceticus* the *trpE* gene is not linked to the

trpGDC cluster. In both *B. subtilis* and *A. calcoaceticus* the AS subunits are not invariably produced in equivalent amounts. In *A. calcoaceticus* an excess of G subunit is synthesized, as might be expected because some G subunit is required for *p*-aminobenzoic acid synthesis. In *B. subtilis* the range of regulation of the G subunit seems less than that of the remaining *trp* genes. It appears, therefore, that both *B. subtilis* and *A. calcoaceticus* make use of the separate locations of the *trpG* and *trpE* genes to modulate synthesis of the two AS subunits. Although in each case the expression of the AS genes is controlled by a *trpR* gene (called *mtr* in *B. subtilis*), either the promoters, operators, or attenuaters must differ from each other, thus directing the ratio of formation of the subunits. It is clear that "split operons" can offer an evolutionary advantage when one subunit of an enzyme complex has a dual role and the genes specifying the different subunits map at separate chromosomal locations.

In *A. calcoaceticus* and *P. putida*, the *trpC* and *trpF* genes map at separate sites and are apparently regulated by different mechanisms. This is in sharp contrast to *E. coli*, where the PRAI and InGPS reactions are catalyzed by a bifunctional polypeptide specified by a single cistron, *trpC* (7, 40). An intermediate situation is known to occur in the *B. subtilis trp* operon where two adjacent genes, *trpC* and *trpF*, specify these two separate enzymes. All the available evidence from amino acid sequences indicates that the structural genes for each of the enzymes of the tryptophan pathway in the bacteria discussed are closely related (5, 24, 36). In contrast, it is quite clear that in every major bacterial group studied so far the chromosomal location of these genes, as well as the regulatory elements associated with them, differ widely.

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