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Significance of Altered Carbon Flow in Aromatic Amino Acid Synthesis: an Approach to the Isolation of Regulatory Mutants in *Pseudomonas aeruginosa*[1]

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Pseudomonas aeruginosa displays a native resistance to a variety of inhibitory compounds, including many analogues of amino acids, purines, and pyrimidines. Therefore, it has been difficult to isolate analogue-resistant regulatory mutants which have been so valuable in other microbial species for the study of enzyme control mechanisms and for the study of amino acid transport and its regulation. However, we have found that increased sensitivity to growth inhibition by analogues can be demonstrated by manipulation of the nutritional environment. When *P. aeruginosa* is grown with fructose as the nutritional source of carbon and energy, the cells become sensitive to growth inhibition by β-2-thienylalanine and *p*-amino-phenylalanine, analogues of phenylalanine and tyrosine, respectively. Thus, mutants were isolated which are resistant to growth inhibition by β-2-thienylalanine and *p*-amino-phenylalanine when fructose is the carbon source, and many of the β-2-thienylalanine-resistant mutants overproduce phenylalanine. Several lines of evidence suggest that the increased sensitivity to growth inhibition by analogues of phenylalanine and tyrosine reflects a decreased rate of synthesis of aromatic amino acids or their precursors when fructose is the carbon source. This general approach promises to be valuable in the study of regulatory phenomena in microorganisms which, like *P. aeruginosa*, are naturally resistant to many metabolite analogues.

Regulatory mutants have been extremely useful in experimental systems used to investigate the molecular control of biochemical pathways in microorganisms. Metabolite analogues have commonly been used as selective agents for the isolation of regulatory mutants which usually possess mutations to constitutivity for enzyme synthesis or to the loss of allosteric specificities for regulatory enzymes. Permeability mutants are also easily isolated by the selection of the analogue-resistant phenotype. The analogue technique has been used very effectively in studies in *Escherichia coli*, *Salmonella typhimurium*, and *Bacillus subtilis*. These bacteria are sensitive to growth inhibition by relatively low concentrations of a variety of metabolite analogues.

However, it has been our experience in the course of comparative studies of microorganisms (Jensen, *unpublished data*) that many microorganisms are quite resistant to growth inhibition by various analogues. For example, species of *Serratia*, *Streptomyces*, and *Pseudomonas* are not significantly inhibited in the presence of various analogues of aromatic amino acids. Numerous reports in the literature document the general resistance of *Pseudomonas aeruginosa* to growth inhibition by analogues of amino acids, purines, pyrimidines, and vitamins (8-10, 18; J. A. Waltho, Ph.D. thesis, University of Melbourne, Melbourne, Australia, 1968). For this reason, few, if any, regulatory mutants involving biosynthetic pathways are known in *P. aeruginosa*. As we reported in a preliminary communica-

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tion (Bacteriol. Proc., p. 158, 1971), analogue sensitivity can be achieved by the manipulation of the nutritional source of carbon and energy. Accordingly, some regulatory mutants were obtained in this manner. Our results suggest the possible general applicability of this methodology to regulatory studies in microorganisms.

MATERIALS AND METHODS

Bacteria. *P. aeruginosa* strain 1, originally obtained from B. W. Holloway (7), was used for the isolation of analogue-resistant mutants. Auxotrophic derivatives of the wild-type parent strain were isolated as previously described (1). The auxotrophic strains of *B. subtilis* used to test for excretion of amino acids and other strains used are shown in Table 1. Mutant derivatives of *P. aeruginosa* unable to utilize either tyrosine or shikimate as nutritional carbon and energy sources were isolated by plating mutagenized cells (1) to BAB plates, which contain Trypticase soy agar (BBL Division of Bioquest) and 1% yeast extract (BBL). Isolated colonies were transferred with sterile toothpicks to a BAB master plate, which was incubated overnight at 37 C and used for replica plating to media containing either tyrosine or shikimate in place of glucose as the carbon source.

Media. The minimal salts medium used was a modification of that of Spizizen (16) and contained (per liter of glass-distilled water): K_2HPO_4, 14 g; KH_2PO_4, 6 g; sodium citrate · $3H_2O$, 4 g; $(NH_4)_2$, SO_4, 2 g; and $MgSO_4 \cdot 7\ H_2O$, 0.2 g; solidified with 1.5% agar (Difco) for use as solid medium. Glucose and other carbon sources were autoclaved separately and added to give a final concentration of 0.2%. When carbon sources other than glucose were used, citrate was omitted from the medium, since citrate itself can serve as a carbon source. Stock cultures were maintained as stabs in 0.7% agar which had been incubated overnight, sealed with paraffin wax, and then kept at room temperature. Viability is retained for at least 18 months. Working cultures were kept on BAB plates.

Growth studies. Culture turbidities were determined with a Klett-Summerson colorimeter with a green filter (no. 54). Cultures of 10 ml were grown in 125-ml side-arm flasks at 37 C in a gyratory waterbath shaker (New Brunswick) at approximately 300 rev/min. Cells which had been centrifuged from cultures in the exponential phase of growth and resuspended in 1/20 the culture volume of minimal medium before storage in liquid nitrogen were used as inocula (0.03 to 0.06 ml). When the effect of amino acid supplementation upon the growth rate with glucose or fructose was tested, controls were included to test for utilization of the amino acid itself as the carbon and energy source. Under these conditions, there was no growth for at least 8 to 10 hr at the expense of aspartate, methionine, lysine, leucine, glycine, valine, serine, alanine, arginine, or cysteine. In contrast, growth with histidine and proline occurred with a lag of less than 2 hr, and therefore these two amino acids could not be tested unambiguously for their effect upon the growth rate with another carbon source.

Cross-feeding. Cells to be tested were harvested by centrifugation from exponential-phase cultures incubated with shaking at 37 C in liquid Trypticase soy broth (BBL) and diluted 1/100 in minimal medium; adjacent streaks of the resistant mutant and auxotroph were tested on minimal agar plates containing glucose as the carbon source. Cross-feeding could be detected after 18 hr. The wild type did not cross-feed significantly under these conditions. Cross-feeding could also be demonstrated by making a stab of the excretor with a toothpick to a minimal

TABLE 1. *Bacterial strains used for cross-feeding and growth experiments*

No.	Parent strain	Description[a]	Origin
BCM40	*Pseudomonas aeruginosa* 1	phe+tyr+trp	NG mutagenesis
BCM4	*Pseudomonas aeruginosa* 1	phe	NG mutagenesis
BCM354	*Pseudomonas aeruginosa* 1	trp	NG mutagenesis
TA1	*Pseudomonas aeruginosa* 1	TA^r	Spontaneous mutant
TA2	*Pseudomonas aeruginosa* 1	TA^r	Spontaneous mutant
TA3	*Pseudomonas aeruginosa* 1	TA^r	Spontaneous mutant
TA4	*Pseudomonas aeruginosa* 1	TA^r	Spontaneous mutant
4FT1	*Pseudomonas aeruginosa* 1	$4FT^r$	Spontaneous mutant
4FT2	*Pseudomonas aeruginosa* 1	$4FT^r$	Spontaneous mutant
BCM6	*Pseudomonas aeruginosa* 1	Tyrosine utilization	NG mutagenesis
BCM20	*Pseudomonas aeruginosa* 1	Shikimate utilization	NG mutagenesis
NP19	*Bacillus subtilis* 168	trp	E. W. Nester (14)
NP35	*Bacillus subtilis* 168	tyr	E. W. Nester (14)
SB163	*Bacillus subtilis* 168	phe+tyr+trp	E. W. Nester (14)
NP93	*Bacillus subtilis* 23	phe	W. S. Champney and R. A. Jensen (2)

[a] Abbreviations: growth requirements are indicated by: phe, phenylalanine; tyr, tyrosine; trp, tryptophan; and phe+tyr+trp, all three amino acids; TA^r, resistant to thienylalanine; $4FT^r$, 4-fluorotryptophan resistant; NG, nitrosoguanidine. Mutants BCM6 and BCM20 are prototrophic derivatives of the parent strain which have lost the ability to utilize the indicated compound as the sole carbon and energy source.

agar plate which had previously been spread confluently with cells of the appropriate auxotroph. Auxotrophs of *P. aeruginosa* and *B. subtilis* (Table 1) were used to detect excretion of phenylalanine, tyrosine, or tryptophan. Cross-feeding of *B. subtilis* aromatic amino acid auxotrophs by *P. aeruginosa* phenylalanine or tryptophan excretors is very efficient. Since the collection of aromatic amino acid auxotrophs in *B. subtilis* is larger than in *P. aeruginosa* and, in most cases, the specific enzymatic defect is known in *B. subtilis* but not in *P. aeruginosa*, this heterologous syntrophism procedure was used to confirm and extend the observations made with *P. aeruginosa* auxotrophs.

Isolation of analogue-resistant mutants. Wild-type cells grown overnight in liquid glucose minimal medium were diluted 10-fold, and 0.2 ml (approximately 10^9 cells) was spread to minimal agar plates containing glucose or an alternative carbon source. Crystals of the compound to be tested were placed on the surface of the agar, and the plates were scored daily for 4 days for inhibition. Resistant clones were picked and purified three or four times by single-colony isolation on plates containing sufficient analogue to inhibit growth of the wild type.

Preparation of extracts. Cell-free extracts were prepared from cells grown at 37 C in a rotary shaker in 200 ml of glucose minimal medium. The following steps were carried out at 0 to 5 C. Cells were harvested from cultures in the late exponential phase of growth, centrifuged, washed with 200 ml of 0.1 M potassium phosphate buffer (*p*H 7.0), and resuspended in 5 to 10 ml 0.1 M phosphate buffer (*p*H 7.0). The cell suspension was disrupted by two 30-sec exposures to a sonic probe (Biosonik, Bronwill Instruments), and cell debris was removed by centrifugation at 27,000 $\times$ *g* for 20 min. Nucleic acid was precipitated by adding 1.0 ml of 2% protamine sulfate (adjusted to *p*H 6.5) per 10 ml of extract. The mixture was allowed to stand for 10 min, and the precipitate was removed by centrifugation at 27,000 $\times$ *g* for 20 min and discarded. The extract was dialyzed overnight against two changes of 1,000 volumes of 0.1 M phosphate buffer (*p*H 7.0) for the assay of phosphoribosyltransferase and prephenate dehydratase, or against 0.05 M tris(hydroxymethyl)amino methane (Tris), *p*H 7.5 (at 25 C), containing 25 mM $MgCl_2$ and 13 mM 2-mercaptoethanol for the assay of anthranilate synthetase. Protein concentrations were determined by the method of Lowry et al. (11).

Prephenate dehydratase assay. Prephenate dehydratase was assayed in 0.25-ml reaction mixtures containing 1.0 mM potassium prephenate, 0.10 M potassium phosphate buffer (*p*H 7.0), and 100 to 200 μg of enzyme. A control mixture lacking enzyme was always included. After incubation for 10 min at 37 C, the reaction was terminated with 1.75 ml of 1.0 N NaOH. Phenylpyruvate concentration was determined by its absorbance at 320 nm by using a Gilford model 240 spectrophotometer and based on a molar extinction coefficient of 17,500 (4).

Anthranilate synthetase assay. The reaction mixture of 1 ml contained, in micromoles per milliliter: Tris buffer (*p*H 7.2), 50; potassium chorismate, 0.25; glutamine, 5; $MgCl_2$, 10; 2-mercaptoethanol, 13; and limiting enzyme. The reaction was initiated by the addition of chorismate to a reaction vessel previously incubated at 37 C. The rate of reaction was followed continuously in an Aminco-Bowman spectrophotofluorometer for 5 min, by observing the increase in fluorescence of anthranilate at an excitation wavelength of 313 nm and an emission wavelength of 393 nm and comparing the fluorescence to a standard curve. Authentic anthranilate showed peak fluorescence at these wavelengths.

Phosphoribosyltransferase assay. The reaction mixture of 1 ml contained, in micromoles per milliliter: Tris buffer (*p*H 7.2), 50; $MgCl_2$, 10; 2-mercaptoethanol, 13; phosphoribosyl pyrophosphate, 425; anthranilate, 0.001; and limiting enzyme. The reaction was started by the addition of enzyme to a reaction mixture previously warmed at 37 C. The rate was followed continuously as decrease in fluorescence of anthranilate at the wavelengths indicated above. The rate was constant for at least 1.5 min.

Determination of the rate of phenylalanine excretion. To determine the rate of phenylalanine excretion, samples of 10 ml were removed at intervals from a 200-ml culture growing at 37 C on a rotary shaker. The optical density at 525 nm was determined, the sample was centrifuged, and the phenylalanine concentration in the supernatant fluid was determined by (i) the fluorometric assay of McCaman and Robins (12), (ii) bioassay with *Leuconostoc mesenteroides* ATCC 8042, or (iii) bioassay with *P. aeruginosa* strain BCM4 (Table 1). Comparable results were obtained with all three methods. Phenylalanine excretion is expressed as micrograms of phenylalanine excreted per milligram (dry weight) of cells per hour. An optical density at 525 nm of 1.00 is equivalent to 0.564 mg (dry weight) of cells per ml. The wild-type organism does not excrete measurable levels of phenylalanine under these conditions.

Chemicals. Amino acids, amino acid analogues and isomers, and carbon sources were obtained from the following sources: *p*-amino-phenylalanine (PAP), 7-methyl-indole, DL-*p*-fluorophenylalanine (FPA), DL-4-fluorotryptophan (4FT), 4-methyl-DL-tryptophan, DL-α-methyl-*m*-tyrosine, β-2-thienylalanine (TA), L-phenylalanine, L-norleucine, L-norvaline, 3,5-dibromo-L-tyrosine, and pyruvic acid from Sigma Chemical Co.; 5-methyl-DL-tryptophan, L-3-methyl-histidine, D-tyrosine, L-1-methyl-histidine, D-phenylalanine, L-tyrosine, DL-4-aza-leucine, D-fructose, L-glutamic acid, and L-tryptophan from Calbiochem; L-2-thiolhistidine from Mann Research Laboratories, Inc.; α-methyl-DL-methionine and 3-methyl-phenylalanine from Nutritional Biochemical Corp.; 2-methyl-leucine from Baltimore Biological Laboratories, and glycerol from Matheson Coleman and Bell. Other chemicals were of the highest purity commercially available.

Barium chorismate and barium prephenate (85 to 95% pure) were prepared by the method of Gibson (6). In some experiments, barium chorismate and barium prephenate (70 to 80% pure) obtained from Sigma Chemical Co. were used. Barium chorismate

and barium prephenate were converted to the potassium salts by addition of a twofold molar excess of potassium sulfate.

RESULTS

Tests for growth inhibition by 20 analogues with 7 carbon sources. About 10^9 wild-type cells previously grown with glucose were spread to minimal plates containing as carbon source either glucose, glutamate, fructose, glycerol, citrate, pyruvate, or acetate. Crystals of the test inhibitors (12 analogues and isomers of aromatic amino acids and 8 analogues of nonaromatic amino acids) were placed in the center of the plate, followed by incubation at 37 C to detect growth inhibition and resistant clones which may have been present. The most potent analogues fell into two classes: (i) FPA and 4FT, which inhibit strongly on all carbon sources, and (ii) TA and PAP, which produce little or no inhibition with some carbon sources (e.g., glucose) but produce marked inhibition with other carbon sources (e.g., fructose). Some of the other analogues tested (e.g., 4-methyl-tryptophan and 7-methyl-indole) produced slight or transient inhibition on plates containing certain carbon sources, but these comparatively weak effects were not investigated further.

Isolation of mutants resistant to FPA and 4 FT. The mechanism of inhibition by FPA in *P. aeruginosa* was previously investigated (8, 18; J. A. Waltho, Ph.D. thesis, University of Melbourne, 1968). FPA apparently inhibits at the level of transfer ribonucleic acid or the ribosome, and, although more than 50 FPA-resistant mutants have previously been isolated, none of these overproduce aromatic amino acids. We isolated FPA-resistant derivatives from plates containing several different carbon sources, and none excrete aromatic amino acids. High levels of FPA (1 mg/ml) are needed to inhibit growth.

On the other hand, 4FT, which previously had not been tested in *P. aeruginosa*, inhibits markedly with all carbon sources tested, and many 4FT-resistant isolates, such as mutant 4FT1, overproduce tryptophan. Strain 4FT1 is derepressed (Table 2) about 100-fold for anthranilate synthase and phosphoribosyltransferase; the other enzymes of the tryptophan pathway are currently under investigation. Maximum growth inhibition in the wild type is achieved with as little as 10 μg of 4FT per ml. The degree of growth inhibition by 4FT in the wild type is similar with all seven of the carbon sources tested. Thus, although more than 50 metabolite analogues have previously been tested for growth inhibition in *P. aeruginosa*, this is the first report of an analogue which inhibits at low concentrations and is effective for the isolation (even with a glucose carbon source) of regulatory mutants which overproduce the corresponding end product.

TABLE 2. *Specific activity of anthranilate synthase and phosphoribosyltransferase in the wild type and strain 4FT1*[a]

Source of enzyme	Anthranilate synthase	Phosphoribosyltransferase
Wild type	0.233	1.42
4FT1	27.3	133

[a] Specific activity is expressed as nanomoles of anthranilate produced or utilized per milligram of protein per minute. Extracts from cells growing in minimal medium (with a glucose carbon source) were prepared as described in Materials and Methods.

Isolation of mutants resistant to TA and PAP. Crystals of the phenylalanine analogue, TA, and the tyrosine analogue, PAP, did not inhibit growth on plates containing a glucose carbon source, but with some other carbon sources inhibition by these compounds could be detected. The inhibition produced by TA and PAP was most pronounced on plates containing a fructose carbon source, and analogue-resistant clones were present in the area of inhibition after several days of incubation. Although TA and PAP inhibited most strongly on fructose-containing plates, some enhancement of analogue sensitivity was achieved on plates containing other carbon sources. It was qualitatively apparent from results obtained on agar plates that TA has little or no effect when the carbon source is glucose, citrate, acetate, pyruvate, or glutamate, but dramatic inhibition occurs with glycerol as well as with fructose. Similarly, PAP is a much more potent inhibitor with pyruvate, glycerol, or fructose than with glucose, citrate, acetate, or glutamate. These results suggest that this procedure may be generally useful for selecting regulatory mutants which are otherwise difficult to isolate.

Eleven PAP-resistant and four TA-resistant mutants were isolated from fructose plates and tested for aromatic amino acid excretion by cross-feeding. None of the PAP-resistant mutants excrete detectable levels of any aromatic amino acids, and these isolates were not studied further. However, all four TA-resistant derivatives excrete phenylalanine. The mutation in one of the TA-resistant phenylalanine

excretors, strain TA2, can be correlated with an alteration in prephenate dehydratase, the first enzyme in the pathway unique to phenylalanine biosynthesis. The specific activity of prephenate dehydratase in strain TA2 is similar to the wild type, but the enzyme from strain TA2 is more resistant to feedback inhibition by phenylalanine (Table 3).

Differential effect of carbon source upon growth characteristics. The influence of carbon source upon TA inhibition was confirmed more quantitatively by determination of specific growth rates in liquid medium. With a glucose carbon source, TA at 50 or 500 μg/ml produces a transient and quantitatively small inhibition followed by growth at a rate similar to that of the uninhibited control (Fig. 1). In the presence of fructose as the carbon source, TA at 50 or 500 μg/ml reduces the specific growth rate 50 and 66%, respectively (Fig. 2); the cells continue to grow at the inhibited rate for at least 10 hr. As expected, phenylalanine specifically reverses the antimetabolite action of TA upon growth.

Surprisingly, supplementation with phenylalanine alone (or phenylalanine plus TA) reproducibly increases the growth rate in fructose-containing medium relative to the unsupplemented control (Fig. 2). Fructose utilization in *P. aeruginosa* requires the induced synthesis of a specific transport system and of at least one of the enzymes required for fructose metabolism (15). A growth experiment was carried out involving a shift of carbon source from glucose to fructose. Under such conditions of nutritional shift, the growth-stimulatory effect of phenylalanine was more pronounced (Fig. 3). Also, TA increases the time required for the adaptation of glucose-grown cells to growth with fructose from about 4 hr to about 7 hr (Fig. 3). Phenylalanine reverses the effect of TA during adaptation to growth at the expense of fructose and decreases the adaptive lag observed with the uninhibited control.

The other aromatic amino acids, tyrosine and tryptophan, also stimulate growth and reverse TA growth inhibition when fructose is the carbon source, producing results similar to

TABLE 3. *Inhibition of prephenate dehydratase by phenylalanine in strain TA2 and the wild type*

Enzyme source	Phenylalanine concn	Specific activity[a]
Wild type	—	24.8
	2×10^{-5} M	13.1 (47%)
TA2	—	24.2
	2×10^{-5} M	20.2 (16%)

[a] Specific activity is expressed as nanomoles of phenylpyruvate formed per minute per milligram of protein. Figures in parentheses indicate the per cent inhibition.

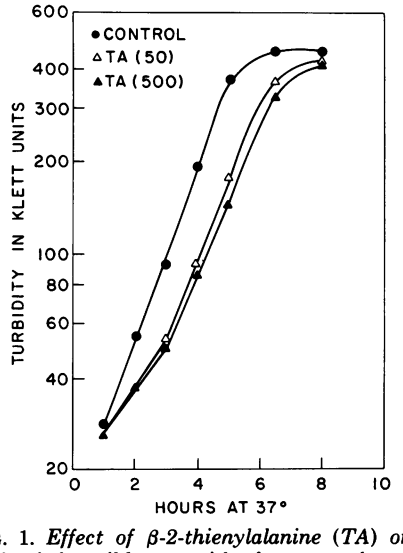

FIG. 1. *Effect of β-2-thienylalanine (TA) on the growth of the wild type with glucose as the carbon source. The inoculum was grown with a glucose carbon source. TA was present at 50 or 500 μg/ml.*

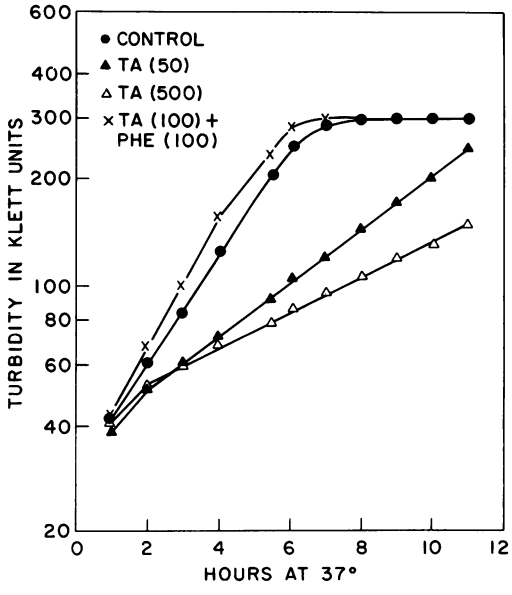

FIG. 2. *Effect of β-2-thienylalanine (TA), at 50 or 500 μg/ml, and phenylalanine (PHE) plus TA, at 100 μg/ml each, on the growth of the wild type with fructose as the carbon source. The inoculum was grown with fructose.*

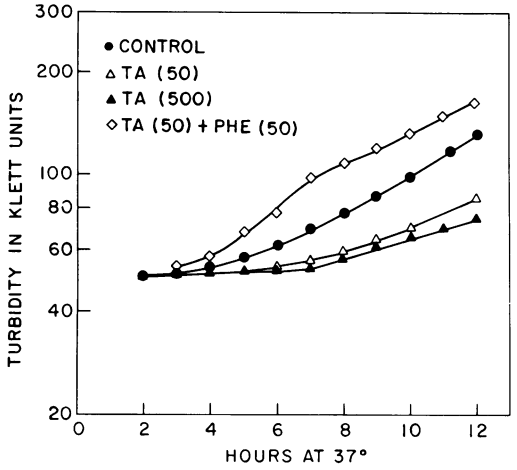

FIG. 3. *Effect of β-2-thienylalanine (TA), at 50 or 500 μg/ml, and phenylalanine (PHE) plus TA, at 50 μg/ml each, upon adaptation of wild-type cells to growth at the expense of fructose. The inoculum was grown on glucose.*

those shown for phenylalanine in Fig. 2 and Fig. 3. Furthermore, shikimic acid, a precursor in the common pathway leading to the aromatic amino acids, overcomes TA growth inhibition and stimulates the growth rate relative to the unsupplemented control in fructose-containing medium (Fig. 4). Supplementation of cultures growing in glucose with phenylalanine, tyrosine, tryptophan, or shikimic acid has no detectable effect upon the growth rate. Nonaromatic amino acids do not reverse growth inhibition by TA, and most nonaromatic amino acids do not affect the growth rate when fructose serves as the carbon source. The only nonaromatic amino acids that stimulate growth in the presence of fructose as the carbon source are alanine and arginine.

The stimulation of the growth rate in minimal-fructose media by aromatic compounds could be attributed to their utilization as carbon and energy sources. The following results tend to exclude this possibility. (i) In the minimal medium used in these experiments, this strain of *P. aeruginosa* does not utilize phenylalanine (17) or tryptophan (*unpublished data*) as sole carbon and energy sources. (ii) Growth with tyrosine or shikimic acid requires a lengthy adaptive lag of approximately 8 to 10 hr (e.g., Fig. 4). (iii) We have isolated mutants which are unable to degrade either tyrosine (strain BCM6) or shikimate (strain BCM20). These mutants, like the wild-type parent strain, are stimulated by supplementation with aromatic amino acids or shikimic acid when growing in fructose medium.

Nature of the carbon source effect. The observation that, with a fructose carbon source, the cells become sensitive to analogues of phenylalanine and tyrosine, and the finding that supplementation with aromatic amino acids or shikimic acid produces growth stimulation with fructose suggested the possibility that cells growing on fructose are relatively limited in precursors for the synthesis of aromatic amino acids. If this were the case, it might be expected that the rate of phenylalanine excretion by regulatory mutant TA2 would be greatly reduced in fructose. The data in Table 4 confirm this expectation. The phen-

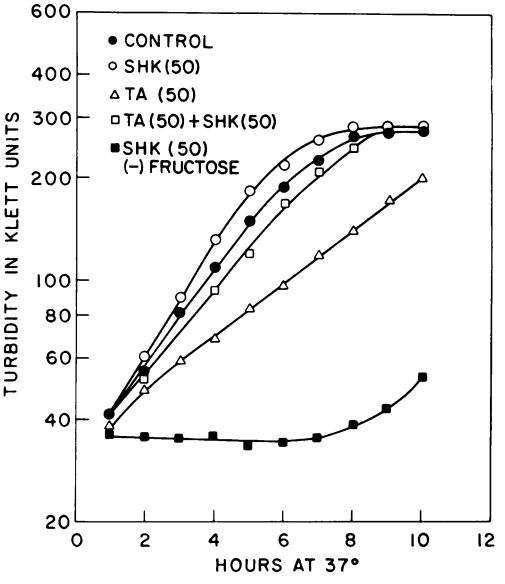

FIG. 4. *Reversal by shikimic acid of growth inhibition by β-2-thienylalanine (TA) and growth stimulation by shikimic acid (SHK) in the wild type. Supplements were present at 50 μg/ml. The inoculum was grown in fructose. The lower curve shows the time course of adaptation to growth at the expense of shikimic acid in a flask in which fructose was omitted.*

TABLE 4. *Effect of carbon source upon phenylalanine excretion by strain TA2*

Carbon source	Phenylalanine excretion[a]	
	Cells (μg/mg)	Cells/hr (μg/mg)
Glucose	10.4	1.45
Fructose	2.29	0.16

[a] Phenylalanine concentration was measured as described in Materials and Methods. The wild type does not excrete detectable levels of phenylalanine under these conditions.

ylalanine concentration in culture supernatant fluids of strain TA2 is more than fourfold greater in glucose than in fructose, when expressed as micrograms of phenylalanine excreted per milligram (dry weight) of cells. Considering the time factor, the rate of excretion with glucose (doubling time about 1.0 hr) is about ninefold greater than with fructose (doubling time about 2.3 hr). Consistent with the latter results, cross-feeding of phenylalanine auxotrophs by strain TA2 is markedly reduced on fructose-containing plates.

DISCUSSION

As part of an investigation of aromatic amino acid biosynthesis in *P. aeruginosa*, we wished to isolate amino acid analogue-resistant mutants which are altered in the regulation of the biosynthesis of aromatic amino acids. However, because of the native resistance of *P. aeruginosa* to most analogues, previous attempts to use this technique to isolate mutants with altered biosynthetic control mechanisms have been generally unsuccessful. In an attempt to reveal any inhibitory properties of amino acid analogues, twenty analogues were tested upon cells growing on seven different carbon sources. One of the analogues tested, 4FT, was found to inhibit growth strongly on all carbon sources. Resistant mutants are numerous on plates containing 10 μg of 4FT per ml, and many of these overproduce tryptophan. Thus, 4FT, which had not previously been described as a growth inhibitor of *P. aeruginosa*, inhibits at low concentrations and is effective in the isolation of regulatory mutants. In another member of this genus, *P. putida*, mutants resistant to high levels (200 to 500 μg/ml) of DL-5-fluorotryptophan (5FT) or 5-fluoroindole (5FI) have recently been isolated (13). These mutants excrete anthranilic acid but not tryptophan (I. Crawford, *personal communication*), unlike the 4FT-resistant *P. aeruginosa* derivatives described here. This result implies a difference in the site of action of 4FT compared to 5FI and 5FT, or alternatively, a difference in the mode of regulation of tryptophan synthesis in these two species.

In contrast to the results with 4FT, other analogues produce significant growth inhibition in *P. aeruginosa* only with certain carbon sources. When growing at the expense of fructose, which is a poor carbon source (mass doubling time 2.3 hr versus 1 hr with glucose), the cells apparently are relatively limited in the biosynthesis of aromatic amino acids and become sensitive to the phenylalanine analogue TA and to the tyrosine analogue PAP. TA also inhibits the adaptation of cells to growth with fructose. Mutants resistant to these analogues have been isolated from fructose carbon source plates. Many of the TA-resistant regulatory mutants overproduce phenylalanine. The molecular basis for the resistance of one such mutant, strain TA2, was found to be decreased sensitivity of prephenate dehydratase to feedback inhibition by phenylalanine.

Several lines of evidence indicate that there is a relative limitation of aromatic amino acids or their precursors in cells of *P. aeruginosa* growing at the expense of fructose as the carbon source. (i) TA and PAP, analogues of phenylalanine and tyrosine, respectively, inhibit growth with fructose but not with glucose as the carbon source (Fig. 1-3). (ii) The specific growth rate of cells growing with fructose is increased when supplemented with phenylalanine, tyrosine, tryptophan, or shikimic acid (Fig. 2-4). These compounds do not stimulate the growth rate with glucose as the carbon source, and most nonaromatic amino acids do not stimulate growth in fructose. The increased growth rate in fructose medium supplemented with aromatic compounds does not reflect utilization of these compounds as carbon sources. (iii) The rate of phenylalanine excretion by strain TA2 is about ninefold greater with glucose than with fructose as the carbon source (Table 4). Cross-feeding of phenylalanine auxotrophs by strain TA2 is very weak on fructose plates.

The detailed explanation for the relative limitation of aromatic amino acids in fructose is undoubtably complex. However, it has previously been demonstrated in other microorganisms that the relative rate of aromatic amino acid biosynthesis varies with different carbon sources. Champney and Jensen (3) found that the specific tyrosine excretion of a *B. subtilis* tyrosine excretor changed dramatically with different carbon sources. Davis (5) found that, with different carbon sources, different levels of aromatic amino acid end products are required for maximal growth of mutants of *S. typhimurium* and *B. subtilis* with partial (leaky) blocks in the common part of the aromatic pathway. Thus, the ability to alter the carbon flow through the aromatic amino acid biosynthetic sequence by changing the carbon source does not seem to be limited to *P. aeruginosa*.

Regardless of the specific mechanism involved, it is clear that growth at the expense of fructose as carbon source has reversed the natural resistance of *P. aeruginosa* to one class of inhibitory compounds. Accordingly, this has

made it possible to isolate mutants with altered biosynthetic control mechanisms in this widely studied microbial species.

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