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Hidden Overflow Pathway to L-Phenylalanine in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is representative of a large group of pseudomonad bacteria that possess coexisting alternative pathways to L-phenylalanine (as well as to L-tyrosine). These multiple flow routes to aromatic end products apparently account for the inordinate resistance of *P. aeruginosa* to end product analogs. Manipulation of carbon source nutrition produced a physiological state of sensitivity to *p*-fluorophenylalanine and *m*-fluorophenylalanine, each a specific antimetabolite of L-phenylalanine. Analog-resistant mutants obtained fell into two classes. One type lacked feedback sensitivity of prephenate dehydratase and was the most dramatic excretor of L-phenylalanine. The presence of L-tyrosine curbed phenylalanine excretion to one-third, a finding explained by potent early-pathway regulation of 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase-Tyr (a DAHP synthase subject to allosteric inhibition by L-tyrosine). The second class of regulatory mutants possessed a completely feedback-resistant DAHP synthase-Tyr, the major species (>90%) of two isozymes. Deregulation of DAHP synthase-Tyr resulted in the escape of most chorismate molecules produced into an unregulated overflow route consisting of chorismate mutase (monofunctional), prephenate aminotransferase, and arogenate dehydratase. In the wild type the operation of the overflow pathway is restrained by factors that restrict early-pathway flux. These factors include the highly potent feedback control of DAHP synthase isozymes by end products as well as the strikingly variable abilities of different carbon source nutrients to supply the aromatic pathway with beginning substrates. Even in the wild type, where all allosteric regulation is intact, some phenylalanine overflow was found on glucose-based medium, but not on fructose-based medium. This carbon source-dependent difference was much more exaggerated in each class of regulatory mutants.

The biochemical arrangement employed by *Pseudomonas aeruginosa* for the biosynthesis of aromatic amino acids is the most complicated gene-enzyme system of aromatic biosynthesis described to date. Two alternative flow routes exist that lead to L-phenylalanine (Fig. 1), and dual flow routes to L-tyrosine are also simultaneously present (20). This seemingly unnecessary metabolic complexity is nevertheless commonplace in nature, having been thus far described in a diverse assemblage of pseudomonad, xanthomonad, and *Alcaligenes* species (see reference 2 for review).

The complicated gene-enzyme patterns found in *P. aeruginosa* place formidable obstacles in the way of selection procedures traditionally used for isolation of both auxotrophic and regulatory mutants. A strategy of sequential mutagenesis has been devised to isolate auxotrophic mutants (21, 29). The isolation of regulatory mutants has been equally challenging since the multiple flow routes to end products apparently contribute to pool sizes sufficiently large to account for resistance of *P. aeruginosa* to the antimetabolite action of phenylalanine and tyrosine analogs. This problem of a lack of direct selective conditions to isolate regulatory mutants was resolved by the findings of Calhoun and Jensen (3, 14) that metabolite flow to aromatic amino acids is growth limiting during culture on fructose as the sole source of carbon and energy. This phenomenon is probably explained by the presence of a phosphoenolpyruvate-dependent phosphotransferase system employed by *P. aeruginosa* to transport and phosphorylate fructose (8, 22, 23). Since biosyn-

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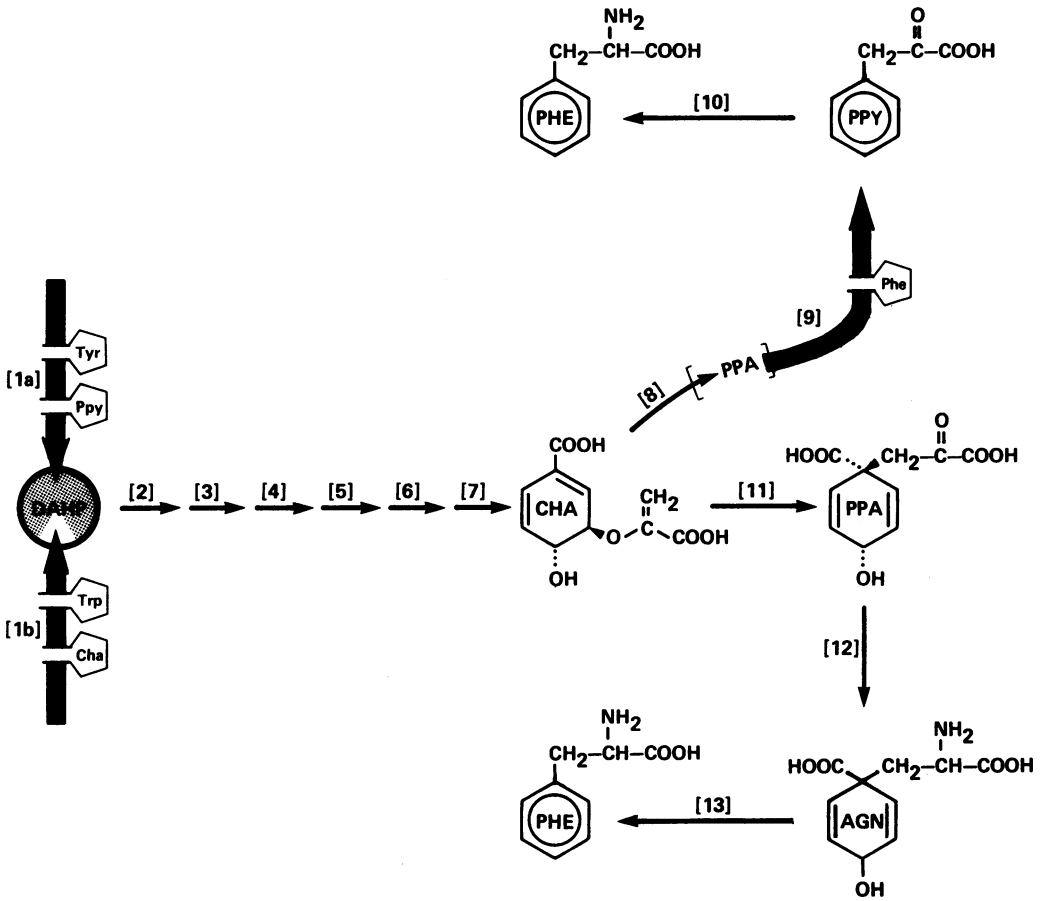

FIG. 1. Allosteric control points along two flow routes leading to L-phenylalanine in *P. aeruginosa*. The heavy arrows indicate the three control points, and the effector molecules cognate to each enzyme molecule are shown symbolically. Regulatory isozymes [1a] and [1b] contribute major and minor fractional output of DAHP as shown schematically. Enzymes [1a] and [1b] are DAHP synthase-Tyr and DAHP synthase-Trp, respectively. Enzyme activities [8] and [9] are chorismate mutase and prephenate dehydratase of the bifunctional P protein, respectively. Enzyme [11] denotes a monofunctional protein possessing a second chorismate mutase activity. Other enzymes: [10], phenylpyruvate aminotransferase; [12], prephenate aminotransferase; [13], arogenate dehydratase. Abbreviations: CHA, chorismate; PPA, prephenate; PPY, phenylpyruvate; PHE, L-phenylalanine; AGN, L-arogenate; Tyr, L-tyrosine; and Trp, L-tryptophan.

thesis of each aromatic amino acid molecule requires two molecules of phosphoenolpyruvate, aromatic pathway biosynthesis is expected to be especially sensitive to intracellular depletion of phosphoenolpyruvate. Thus, a physiological state of sensitivity to analogs of phenylalanine or of tyrosine is achieved on fructose-based medium, allowing conditions for direct selection of regulatory mutants having a phenotype of analog resistance.

The characterization of the variety of regulatory mutants possible is likely to provide a greater depth of insight into the nature and significance of the multiple flow routes in *P. aeruginosa* that contrast so sharply with the simpler gene-enzyme arrangements found in organisms such as *Escherichia coli* or *Bacillus subtilis*.

MATERIALS AND METHODS

Strains and culture conditions. *P. aeruginosa* strain 1, obtained from B. W. Holloway (13), was the parent strain of analog-resistant mutants. Cultures were grown at 37°C in a minimal salts medium (3) containing 0.5% (wt/vol) glucose or fructose and 1.5% agar (Difco Laboratories). When analog supplements were present, additions were made to give a final concentration of 50 μg/ml.

Culture turbidities were determined with a Klett-Summerson colorimeter with a green filter (no. 54). Overnight cultures were grown in minimal-glucose or minimal-fructose media and used to inoculate 15 ml of

fresh medium containing supplements as indicated. Cultures were contained in 125-ml sidearm flasks with vigorous shaking at 37°C. The initial turbidities ranged from 18 to 23 Klett units.

Extracts were prepared from cultures grown as follows. Overnight cultures of 100-ml volume contained within 250-ml flasks and incubated with shaking at 37°C were used to inoculate 16 liters of minimal-glucose medium contained within a 20-liter carboy. Incubation was carried out at 37°C with continuous aeration (sparging). Cells were harvested by centrifugation during the late-exponential phase of growth.

Isolation of analog-resistant mutants. Wild-type cultures grown overnight in minimal-fructose medium were diluted 10-fold, and 0.2-ml samples were spread on the surface of minimal-fructose agar plates. Crystals of analog were placed on the surface of the agar plates and then incubated at 37°C. After 4 days, resistant colonies appearing within the zone of growth inhibition were picked and purified by three rounds of single-colony isolation on plates containing 100 μg of analog per ml.

Cross-feeding. Analog-resistant phenotypes were initially screened for amino acid excretion by ability to cross-feed wild-type cells on minimal-fructose agar plates containing sufficient analog to inhibit the growth of the wild type. Contiguous streaks of a given analog-resistant mutant under test and the wild type were arranged on agar, cross-feeding being readily apparent after 24 h. Cross-feeding was also demonstrated by patching a small population of analog-resistant mutant on an analog-containing plate previously seeded with a confluent lawn of wild-type cells.

Identification and quantitation of excreted aromatic compounds. Quantitative assays for aromatic amino acids excreted into culture fluids were performed as follows. Cultures of 200 ml were grown in either minimal-glucose or minimal-fructose medium by shaking at 37°C. Samples of 5 ml were recovered at regular intervals to monitor culture turbidities. These samples were then centrifuged. The resulting supernatants were filtered through a 0.45-μm membrane filter (Millipore Corp.), and the filtrate was frozen until assays were performed. Phenylalanine or tyrosine was measured by the fluorometric assay of Wong et al. (30). Tryptophan was measured by the fluorescent decay assay of Guilbault and Froelich (10).

For phenylpyruvate measurements the following additional steps were carried out. A sample of filtered culture supernatant (200 μl) was acidified with 300 μl of 1 N HCl and extracted with 1 ml of ethyl acetate-toluene (4:1) for 2 min. The organic phase containing phenylpyruvate was separated and dried under nitrogen. The residue was suspended in 1 ml of 2.5 N NaOH, and phenylpyruvate absorbance was read at 320 nm.

All fluorescence measurements were performed with an Aminco-Bowman spectrophotofluorometer.

Preparation of crude extracts. Whole-cell pellets were suspended in 50 mM potassium phosphate buffer (pH 7.0) containing 1.0 mM dithiothreitol. The suspension was then disrupted at 4°C with three 30-s bursts of ultrasound at 100 mV intensity from a Lab-line Ultratip sonicator, followed by centrifugation in a Beckman ultracentrifuge at 150,000 × *g* to remove cell debris and to sediment a particulate oxidase whose presence complicated dehydrogenase assays. The extract was then passed through a 1.5 by 20-cm Sephadex G-25 column equilibrated in starting buffer to remove small molecules that might interfere with enzyme assays. The leading 80% of the protein eluate was free of small molecules.

Analytical procedures. Protein concentrations were determined by the method of Bradford (1).

3-Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase was assayed by the method of Srinivasan and Sprinson (25), as modified by Jensen and Nester (17). In crude extracts, as well as partially purified samples of each isozyme, conditions of proportionality with respect to both time and protein concentration were observed. When effector additions were made, appropriate blanks were included to detect possible interference of the chemical assay for DAHP by effector molecules (4). It is commonly found that optimal DAHP synthase activity requires the addition of divalent metal ions (26). Stock solutions of cobalt sulfate or manganese sulfate, prepared in 50 mM potassium phosphate buffer (pH 7.0), were added to give a final concentration of 1.0 mM in reaction mixtures used for assay of DAHP synthase-Tyr and DAHP synthase-Trp, respectively (28). A typical reaction mixture (200 μl) contained 50 mM potassium phosphate buffer (pH 7.0), 1.0 mM erythrose 4-phosphate, 1.0 mM phosphoenolpyruvate, 1.0 mM metal ion, enzyme, and any specified compounds under test as effector molecules. All reactions were carried out at 37°C, and the concentration of DAHP produced was calculated by using a molar extinction coefficient of 45,000 at 549 nm.

Chorismate mutase was usually assayed by the method of Patel et al. (20). The appearance of phenylpyruvate was monitored by incubating a reaction mixture (200 μl) containing 50 mM potassium phosphate (pH 7.0), 1.0 mM chorismate, and enzyme at 37°C for 20 min. Any prephenate formed was acid converted to phenylpyruvate by incubation at 37°C for 15 min after the addition of 100 μl of 1 N HCl. The absorbance of phenylpyruvate was measured at 320 nm after the addition of 700 μl of 2.5 N NaOH. The concentration of phenylpyruvate was estimated by using a molar extinction coefficient of 17,500. In a second procedure (19), the rate of chorismate disappearance was monitored continuously at 274 nm in a 1-ml cuvette with the temperature of the reaction chamber maintained at 37°C.

Prephenate dehydratase was assayed by the method of Patel et al. (20). Reaction mixtures (200 μl) contained 50 mM potassium phosphate (pH 7.0), 1.0 mM prephenate, and enzyme. After a 20-min incubation at 37°C, 800 μl of 2.5 N NaOH was added, and phenylpyruvate measured by its absorbance at 320 nm.

Aromatic aminotransferase activity was assayed by the method of Whitaker et al. (29). Reaction mixtures contained 10 mM L-phenylalanine, 2.5 mM α-ketoglutarate, 12.5 μM pyridoxal 5'-phosphate, 50 mM potassium phosphate buffer (pH 7.0), and enzyme. All assays were carried out for 20 min at 37°C. The absorbance of phenylpyruvate was measured at 320 nm after the addition of 800 μl of 2.5 N NaOH.

Arogenate dehydratase was assayed by the method of Shapiro et al. (24). Reaction mixtures (200 μl) containing 50 mM potassium phosphate (pH 7.5), 0.75 mM arogenate, and enzyme were incubated at 37°C for 45 min. Phenylalanine formation was estimated by

constructing a standard curve relating known concentrations of phenylalanine to fluorescence.

DEAE-cellulose chromatography. Approximately 440 mg of protein in crude extracts from the wild type or mutants were applied at 4°C to a 2.5- by 20-cm DEAE-cellulose (DE-52) column equilibrated in 50 mM potassium phosphate buffer (pH 7.0) containing 1.0 mM dithiothreitol. Protein was loaded onto the column, which was then washed with 300 ml of starting buffer. Bound proteins were eluted with a linear gradient of KCl, one reservoir containing 450 ml of potassium phosphate buffer and the other containing 450 ml of potassium phosphate buffer plus 0.5 M KCl. Fractions of 6.0 ml were collected. Specific activities (nanomoles per minute per milligram of protein) of wild-type enzymes recovered from DEAE-cellulose were as follows: 56, DAHP synthase-Tyr (isozyme species that is sensitive to feedback inhibition by L-tyrosine); 4.9, DAHP synthase-Trp (isozyme species that is sensitive to feedback inhibition by L-tryptophan); 653, prephenate dehydratase (P protein); 342, chorismate mutase (P protein); and 15, arogenate dehydratase.

Biochemicals. Amino acids, keto acids, amino acid analogs, erythrose 4-phosphate, phosphoenolpyruvate, pyridoxal 5'-phosphate, dithiothreitol, and Sephadex G-25 were obtained from Sigma Chemical Co. DEAE-cellulose (DE-52) was obtained from Whatman Ltd. Prephenate, prepared as the barium salt from culture supernatants of a tyrosine auxotroph of *Salmonella typhimurium* (7), was converted to the potassium salt with a twofold excess of K_2SO_4 before use. Sodium arogenate was prepared from culture supernatants of a triple auxotroph of *Neurospora crassa* ATCC 36373 (18). The purification and isolation (18) were modified by the method of Zamir et al. (31). Chorismate was isolated from the accumulation medium of the triple auxotroph, *Klebsiella pneumoniae* 62-1, and purified as the free acid (9). All chemicals were of the best grade commercially available.

RESULTS

Antimetabolite action of phenylalanine analogs. Analogs of phenylalanine (and tyrosine) do not inhibit the growth of *P. aeruginosa* on glucose-containing medium, but some analogs are effective on fructose-based medium (3). We found *p*-fluorophenylalanine and *m*-fluorophenylalanine to be the most inhibitory analogs tested. Each was a specific antimetabolite of phenylalanine on the criterion that only L-phenylalanine additions resulted in reversal of growth inhibition caused by the presence of analogs. Figure 2 illustrates that in each case growth is progressively slowed, allowing just about one mass doubling over about 7 to 8 h, followed by a nearly complete cessation of growth. *p*-Fluorophenylalanine appears to be a more straightforward analog agent than *m*-fluorophenylalanine, inhibition being completely reversed by L-phenylalanine (Fig. 2A). Figure 2B shows that 50 μg of L-phenylalanine per ml was not sufficient to reverse the inhibitor effects of an equimolar amount of *m*-fluorophenylalanine. Higher initial concentrations of L-phenylalanine in combination with 50 μg of *m*-fluorophenylalanine per ml led to a family of curves exhibiting progressively greater degrees of reversal. It appears that *m*-fluorophenylalanine triggers some enzyme activity which is able to utilize L-phenylalanine as a substrate, thus eventually depleting L-phenylalanine and restoring vulnerability to analog-mediated inhibition of growth. This conclusion was supported by the finding that further addition of L-phenylalanine (e.g., at the 10-h point under conditions shown in Fig. 2B) sustained the wild-type growth rate for a longer time. Thus, periodic additions of L-phenylalanine at 50 μg/ml were found to completely overcome *m*-fluorophenylalanine inhibition through the duration of a growth curve.

Isolation of two classes of regulatory mutants. Spontaneously arising mutants selected for resistance to either *p*-fluorophenylalanine or *m*-fluorophenylalanine were isolated and purified as described above. The most dramatic regulatory mutants were selected on the criterion of ability to cross-feed confluent lawns of wild-type cells on fructose-based medium containing inhibitory concentrations of analog. Overproduction of L-phenylalanine by regulatory mutants physiologically overcame the antimetabolite action of the analog, thereby promoting the growth of background halos of wild-type cell populations immediately surrounding mutant-cell clones patched on the lawns. None of the analog-resistant mutants isolated proved to excrete tyrosine or tryptophan.

Regulatory mutants fell into two classes, one group having a phenylalanine-insensitive prephenate dehydratase and the other having a tyrosine-insensitive DAHP synthase-Tyr isozyme. Mutants MJ-12 and MJ-13 were selected as representative of these two mutant classes for in-depth characterization. Table 1 shows that mutant MJ-13 has a DAHP synthase-Tyr isozyme that is completely insensitive to feedback inhibition by L-tyrosine. The sensitivity of DAHP synthase-Tyr to phenylpyruvate, on the other hand, was not significantly altered. In mutant MJ-13 prephenate dehydratase sensitivity to inhibition by L-phenylalanine was identical to that of wild type. The wild-type feedback sensitivities of DAHP synthase-Trp were also unaltered in mutant MJ-13. Mutant MJ-12 possesses a phenylalanine-insensitive prephenate dehydratase activity that retains, however, sensitivity to allosteric activation by L-tyrosine (Table 1). The feedback inhibition control of both regulatory isozymes of DAHP synthase was unaltered by mutation in mutant MJ-12.

The data given in Table 1 also show that the analogs, *m*-fluorophenylalanine and *p*-fluorophenylalanine, mimic L-phenylalanine as inhibi-

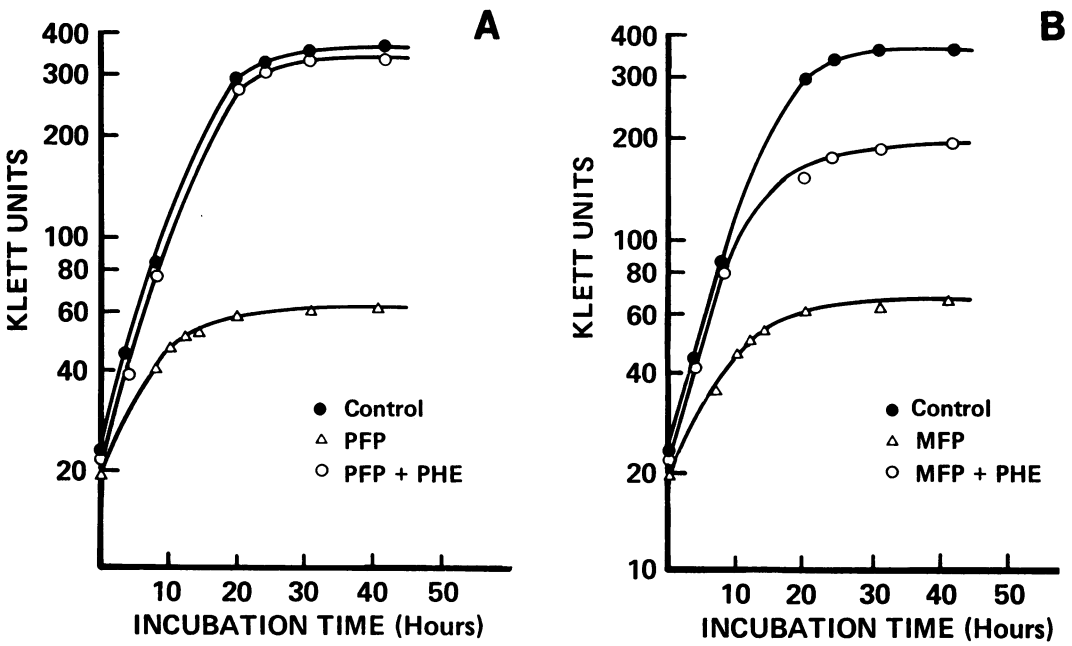

FIG. 2. Analog inhibition of growth and ability of L-phenylalanine (PHE) to reverse analog inhibition in wild-type *P. aeruginosa*. Results obtained with *p*-fluorophenylalanine (PFP) are shown in panel A, and those obtained with *m*-fluorophenylalanine (MFP) are given in panel B. The minimal salts medium contained fructose as the sole source of carbon and energy. When MFP, PFP, or PHE were present, they were initially added to a final concentration of 50 μg/ml.

tors of prephenate dehydratase in the wild type. These analogs did not affect the activities of any other regulatory enzymes involved in phenylalanine biosynthesis (i.e., isozymes of DAHP synthase). Figure 3 shows inhibition curves depicting the relative sensitivity of wild-type prephenate dehydratase to inhibition by L-phenylalanine, *m*-fluorophenylalanine, or *p*-fluorophenylalanine. D-Phenylalanine is not inhibitory. The analogs were equally effective as dehydratase inhibitors, although each was about fivefold less effective than L-phenylalanine on a molar basis (assuming that only the L-isomer in each racemic analog mixture was active as a mimic of L-phenylalanine). Chorismate mutase activity of the bifunctional "P" protein of the wild type has been shown by Calhoun et al. (4) to be subject to relatively weak feedback inhibition by L-phenylalanine. This allosteric sensitivity was reported by Calhoun et al. to be exceedingly labile; indeed, we did not find significant feedback sensitivity to be present, even in crude extracts. Thus, we have not yet determined whether the regulatory mutation in mutant MJ-12 has also desensitized chorismate mutase (P protein) to inhibition by L-phenylalanine.

Comparison of early-pathway and late-pathway deregulation. Metabolic input into aromatic biosynthesis in *P. aeruginosa* is remarkably sensitive to the source of carbon and energy during growth (3), input being maximal on glucose and minimal on fructose. Even in the wild type (Fig. 4), a measurable amount of phenylalanine was excreted into the culture medium during growth on glucose. In contrast, aromatic biosynthesis is actually growth rate limiting on fructose-based medium.

In accord with this carbon source phenomenon, *P. aeruginosa* is resistant to growth inhibition by phenylalanine analogs on glucose-based medium, but sensitive on fructose-based medium. The carbon source effect was dramatic in deregulated mutants (Fig. 4). Early-pathway deregulation (at DAHP synthase-Tyr in mutant MJ-13) resulted in less phenylalanine overproduction than did late-pathway deregulation (at prephenate dehydratase in mutant MJ-12). Figure 4 shows that regulatory mutant MJ-13 actually excreted less phenylalanine on fructose-based medium than did the wild type on glucose-based medium. The amount of phenylalanine excreted by mutant MJ-12 on fructose (Fig. 4) probably approaches the upper limit possible during growth on this carbon source.

The arrows in Fig. 4 indicate the effect of the presence of L-tyrosine during growth upon excretion of phenylalanine. Only in mutant MJ-12 grown on glucose-based medium did L-tyrosine exhibit an appreciable effect, depressing excretion by a factor of 3. This undoubtedly reflects

TABLE 1. Mutant alterations of inhibitor specificities of enzymes positioned along metabolic flow routes to L-phenylalanine

Enzyme[a]	Effector molecule[b]	% Relative activity		
		Wild type	MJ-13	MJ-12
DAHP synthase-Tyr	TYR	2	100	3
	PPY	50	69	65
	MFP	100	100	100
	PFP	100	100	100
Prephenate dehydratase (P protein)	PHE	5	9	100
	TYR	144	130	123
	PHE + TYR	29	19	118
	MFP	14	25	100
	PFP	12	16	100
DAHP synthase-Trp	TRP	26	34	30
	CHA	50	52	75
	MFP	100	100	100
	PFP	100	100	100

[a] Each enzyme was partially purified by DEAE-cellulose chromatography. Relative activities of 100 for each enzyme from top to bottom correspond to specific activities of 55, 653, and 4.9 nmol/min per mg of protein, respectively, with saturating substrate concentrations.

[b] Abbreviations: TYR, L-tyrosine; PPY, phenylpyruvate; TRP, L-tryptophan; CHA, chorismate; MFP, *m*-fluorophenylalanine; PFP, *p*-fluorophenylalanine. Effector concentrations were 0.4 mM, except for PPY (4.0 mM) and PHE (0.1 mM).

the restraining effect of L-tyrosine upon the activity of DAHP synthase-Tyr in mutant MJ-12. This is consistent with the lack of any tyrosine effect in mutant MJ-13 (Fig. 4), which possesses a feedback-resistant DAHP synthase-Tyr.

DISCUSSION

Regulation of flow route to phenylalanine via phenylpyruvate. The eventual transformation of starting substrates, phosphoenolpyruvate and erythrose 4-phosphate, into L-phenylalanine by way of phenylpyruvate is a tightly regulated train of reactions. Early-pathway control feedback inhibition is vested in two isozymes of DAHP synthase, one (DAHP synthase-Tyr) sensitive to micromolar levels of L-tyrosine and the other (DAHP synthase-Trp) sensitive to micromolar levels of L-tryptophan. Control of enzyme formation by repression or induction has not been found in *P. aeruginosa* for enzymes of aromatic biosynthesis, except for some enzymes of tryptophan biosynthesis (5). In view of the lack of direct use of phenylalanine as a molecular signal for control of DAHP synthase activity together with the great sensitivity of these isozymes to feedback control by L-tyrosine and L-tryptophan, it seems likely that chorismate flow to L-phenylalanine may be favored over chorismate flow to L-tyrosine. In support of this is the presence of the bifunctional P protein which catalyzes the overall conversion of chorismate to phenylpyruvate (i.e., chorismate mutase and prephenate dehydratase). This bifunctional protein is surprisingly ubiquitous, being present in xanthomonads, certain species of *Alcaligenes*, and a large group of mainly fluorescent pseudomonads (27) as well as in enteric bacteria (6), where the P protein denotation was first used. Such molecular "channeling" would be expected to provide prephenate dehydratase with a huge advantage over prephenate aminotransferase or prephenate dehydrogenase, all being in competition for prehenate molecules as substrate. An unregulated, monofunctional isozyme of chorismate mutase exists, thus providing prephenate molecules free for unchanneled flow to L-arogenate or to 4-hydroxyphenylpyruvate. Since prephenate dehydratase is very sensitive to feedback inhibition by L-phenylalanine, all of the elements for finely tuned and balanced regulation of phenylalanine biosynthesis via the phenylpyruvate route seem to be in place.

We previously speculated (15) that the sensitivity of DAHP synthase-Tyr to inhibition by phenylpyruvate might reflect an indirect arrangment of feedback response that is proportional to L-phenylalanine levels. Indeed, we found that phenylpyruvate levels in culture supernatants were about 20% of the L-phenylalanine levels shown (Fig. 4). It thus seems possible that the overflow pathway actually ends at phenylpyruvate. Clearly, phenylpyruvate-mediated inhibition of DAHP synthase-Tyr is too limited to offset the deregulating mutations present in MJ-12 and MJ-13. Nevertheless, it is possible that mutants MJ-12 and MJ-13 would excrete still greater amounts of L-phenylalanine (and phenylpyruvate) if a second mutation were introduced abolishing phenylpyruvate sensitivity of DAHP synthase-Tyr.

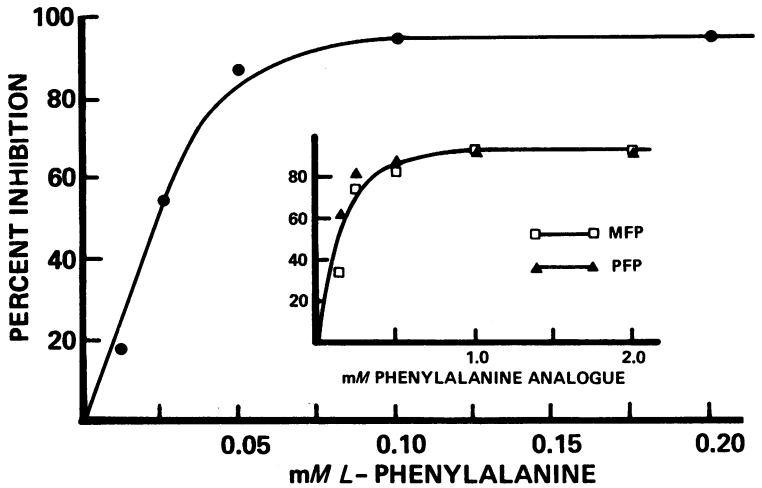

FIG. 3. Sensitivity of prephenate dehydratase (P protein) to inhibition by L-phenylalanine, *m*-fluorophenylalanine (MFP), or *p*-fluorophenylalanine (PFP). Partially purified P protein having a specific activity of 653 nmol/min per mg of protein with respect to prephenate dehydratase activity was obtained from DEAE-cellulose chromatography.

Regulation of overflow route to phenylalanine via L-arogenate. The sole point of allosteric control for the overflow route is at the level of DAHP synthase. The potential of the overflow route is largely masked in the wild type by the competitive disadvantage of the overflow route at low pathway flux, owing to the relatively poor affinities of prephenate aminotransferase and arogenate dehydratase enzymes for substrate. In fructose-based medium it is dubious whether the overflow path operates at all. In glucose-based medium the small excretion of phenylalanine found probably reflects a small fractional transit of molecules through the overflow pathway.

Comparison of results obtained with the two types of mutants shows that an unregulated phenylpyruvate pathway has more potential for phenylalanine overproduction than does an unregulated overflow pathway. Thus, regardless of the carbon source nutrition employed, mutant MJ-12 excretes about three times as much phenylalanine as mutant MJ-13 (Fig. 4). Although mutant MJ-12 still possesses a wild-type DAHP synthase-Tyr, this must be physiologically unregulated by L-tyrosine owing to preferential utilization of chorismate by the mutant-desensitized P protein. Such preferential flow to L-phenylalanine would tend to limit endogenous levels of L-tyrosine. Indeed, the finding that exogenous L-tyrosine diminished phenylalanine excretion dramatically in mutant MJ-12 on glucose-based medium supports this interpretation.

Relationship of carbon source input, L-tyrosine level, and overflow to phenylalanine. Utilization of fructose creates a metabolic drain on endogenous phosphoenolpyruvate supplies. When endogenous phosphoenolpyruvate concentration is low, sensitivity of both DAHP synthase isozymes to inhibition would be maximized since inhibition of DAHP synthase-Tyr by L-tyrosine is competitive against phosphoenolpyruvate (K_i = 23 μM) and inhibition of DAHP synthase-Trp by L-tryptophan is competitive against phosphoenolpyruvate (K_i = 40 μM) (28). It appears that L-tyrosine (and L-tryptophan) are sufficiently effective inhibitors of the DAHP synthase isozymes when phosphoenolpyruvate levels are low (e.g., on fructose) to render aromatic biosynthesis growth rate limiting. However, since the regulatory mutants are able to excrete low levels of phenylalanine on fructose medium under circumstances where at least DAHP synthase-Tyr is invulnerable (by mutation or physiologically) to feedback inhibition by L-tyrosine, it seems clear that significantly more phosphoenolpyruvate can be diverted to aromatic biosynthesis than in fact occurs in the wild type. Thus, under conditions of phosphoenolpyruvate depletion in the wild type, L-tyrosine appears to be a powerful signal favoring phosphoenolpyruvate conservation. The regulatory mutants appear to grow slightly slower in fructose-based medium than does the wild type, a result which may reflect limitation of endogenous phosphoenolpyruvate at the expense of phenylalanine overflow.

Endo-oriented nature of phenylalanine overflow? In organisms like *E. coli* or *B. subtilis* the deregulation of aromatic amino acid biosynthesis is conferred only by mutations affecting postchorismate enzymes, i.e., in the terminal

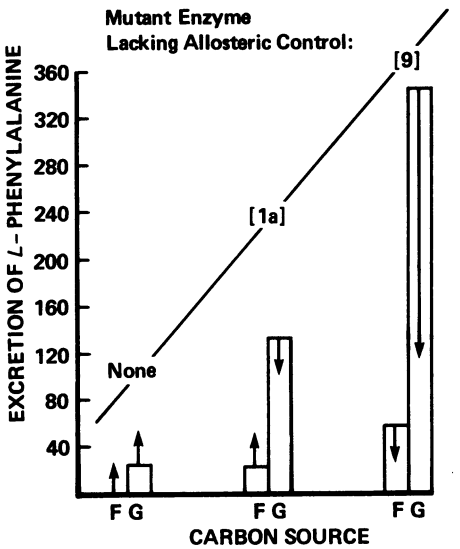

FIG. 4. Histogram showing the comparative abilities of the wild type (left bars) and mutants MJ-12 (middle bars) and MJ-13 (right bars) to excrete phenylalanine (ordinate scale) during growth at the expense of either fructose (F) or glucose (G) as the carbon source (abscissa scale). The ordinate values are expressed as micromolar concentrations of phenylalanine in culture supernatants at a culture turbitity of 300 Klett units. The arrows extending from the tops of the histogram bars indicate the effects of L-tyrosine upon L-phenylalanine excretion. L-Tyrosine additions were made at 100 μg/ml, final concentrations. Enzyme [1a] is DAHP synthase-Tyr, and enzyme [9] is prephenate dehydratase (see Fig. 1).

branchlets. Such emphasis placed upon finely tuned late-pathway regulation has been termed (16) exo-oriented regulation in contrast to that seen in endo-oriented organisms such as cyanobacteria (11, 12), where the major or sole regulation is at an early-pathway position. It is intriguing that *P. aeruginosa* possesses both of these types simultaneously. The phenylpyruvate flow route of phenylalanine biosynthesis is nearly identical to that of *E. coli* in enzymatic makeup and regulation, whereas the unregulated L-arogenate flow route is reminiscent of the lack of late-pathway regulation in some cyanobacteria. The full significance of such biochemical complexity in a single organism awaits insight hopefully to be derived from other regulatory mutants and from structural-gene mutants.

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